Identification of a Novel Protein with a Role in Lipoarabinomannan Biosynthesis in Mycobacteria

Received for publication, October 31, 2005, and in revised form, January 18, 2006 Published, JBC Papers in Press, February 2, 2006, DOI 10.1074/jbc.M511709200

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All species of Mycobacteria synthesize distinctive cell walls that are rich in phosphatidylinositol mannosides (PIMs), lipomannan (LM), and lipoarabinomannan (LAM). PIM glycolipids, having 2–4 mannose residues, can either be channeled into polar PIM species (with 6 Man residues) or hypermannosylated to form LM and LAM. In this study, we have identified a Mycobacterium smegmatis gene, termed lpqW, that is required for the conversion of PIMs to LAM and is highly conserved in all mycobacteria. A transposon mutant, Myco481, containing an insertion near the 3’ end of lpqW exhibited altered colony morphology on complex agar medium. This mutant was unstable and was consistently overgrown by a second mutant, represented by Myco481.1, that had normal growth and colony characteristics. Biochemical analysis and metabolic labeling studies showed that Myco481 synthesized the complete spectrum of apolar and polar PIMs but was unable to make LAM. LAM biosynthesis was restored to near wild type levels in Myco481.1. However, this mutant was unable to synthesize the major polar PIM (AcPIM6) and accumulated a smaller intermediate, AcPIM4. Targeted disruption of the lpqW gene and complementation of the initial Myco481 mutant with the wild type gene confirmed that the phenotype of this mutant was due to loss of LpqW. These studies suggest that LpqW has a role in regulating the flux of early PIM intermediates into polar PIM or LAM biosynthesis. They also suggest that AcPIM4 is the likely branch point intermediate in polar PIM and LAM biosynthesis.

Members of the genus Mycobacterium cause important diseases in humans, including tuberculosis and leprosy. Mycobacterium tuberculosis is thought to infect nearly one-third of the world population and to cause two to three million deaths each year (1). This threat to global health is growing as drug-resistant strains emerge and coinfections with human immunodeficiency virus increase the number of individuals affected. Mycobacteria are highly conserved in all mycobacteria. A transposon mutant, Myco481, containing an insertion near the 3’ end of lpqW exhibited altered colony morphology on complex agar medium. This mutant was unstable and was consistently overgrown by a second mutant, represented by Myco481.1, that had normal growth and colony characteristics. Biochemical analysis and metabolic labeling studies showed that Myco481 synthesized the complete spectrum of apolar and polar PIMs but was unable to make LAM. LAM biosynthesis was restored to near wild type levels in Myco481.1. However, this mutant was unable to synthesize the major polar PIM (AcPIM6) and accumulated a smaller intermediate, AcPIM4. Targeted disruption of the lpqW gene and complementation of the initial Myco481 mutant with the wild type gene confirmed that the phenotype of this mutant was due to loss of LpqW. These studies suggest that LpqW has a role in regulating the flux of early PIM intermediates into polar PIM or LAM biosynthesis. They also suggest that AcPIM4 is the likely branch point intermediate in polar PIM and LAM biosynthesis.

1 This work was funded by the Australian National Health and Medical Research Council.
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3 Supported by the International Human Frontier Science Program, Long-term Fellowship.
4 Supported by the Australian Research Council Centre for Structural and Functional Microbial Genomics.
5 Howard Hughes International Scholars.
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9 The abbreviations used are: PI, phosphatidylinositol; PIM, PI mannoside; AcPIM, acyl PIM; LM, lipomannan; LAM, lipoarabinomannan; DXO, double crossover mutants; MS, mass spectrometry; GC-MS, gas chromatography-Ms; HPTLC, high performance thin layer chromatography; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; PPM, poly prenyl mannoside; PPLO, pleuropneumonia-like organism medium.
A Novel Protein with a Role in LAM Biosynthesis

FIGURE 1. Pathway of PIM biosynthesis, highlighting the possible branch point between polar PIM and LM/LAM biosynthesis. Genes encoding some of the mannosyltransferases involved in PIM and LAM biosynthesis are indicated.

ations in colony morphology has resulted in the identification of several genes involved in the synthesis of outer layer glycolipids (15, 16). In this study, we describe the isolation of two M. smegmatis transposon insertion mutants that exhibit novel defects in polar PIM and LAM biosynthesis. The first of these mutants has a defect in LAM biosynthesis but expresses normal levels of polar PIMs. This mutant is unstable in complex medium, giving rise to the second mutant with normal LAM but altered polar PIM profiles. The phenotypes of these mutants were due to loss of function of the lpqW gene, which is present in the genomes of all mycobacteria sequenced to date. We present evidence that the product of this gene plays a role in regulating the conversion of AcPIM4 to more polar PIM species or LAM. This study demonstrates that LAM and polar PIM biosynthesis can be independently regulated.

MATERIALS AND METHODS

Bacterial Strains, Growth Conditions, and Recombinant DNA Methods—M. smegmatis mc²155 (17) and derivative strains were routinely grown in Middlebrook 7H9 or 7H10 media (Difco) with the addition of DC (dextrose-NaCl). Complex media used were LB (Tryptone 10 g/liter, yeast extract 5 g/liter, NaCl 10 g/liter) and PPLO (brain-heart infusion 50 g/liter, peptone 10 g/liter, NaCl 5 g/liter) in 1% w/v agar and BY (beef-yeast) media (0.3% w/v beef extract, 0.6% w/v yeast extract, 1.2% w/v peptone, 1% w/v NaCl, 1.5% w/v agar). Sauton’s minimal medium was used in labeling assays (18). Kanamycin, streptomycin, or hygromycin were added to media at 100 μg/ml as required. Transposon mutants were cultivated at 39 °C, and all other bacteria were grown at 37 °C. Cloning procedures were performed according to standard protocols (19). Electrottransformation was performed using a Bio-Rad Gene Pulser using Escherichia coli competent cells prepared for transformation according to the manufacturer’s recommendations or M. smegmatis cells prepared according to Jacobs et al. (20). Genomic DNA was extracted from mycobacteria as described previously (21). Probes for Southern blotting were labeled with digoxigenin-labeled dNTPs using a digoxigenin labeling kit (Roche Applied Science), and membranes were developed according to the manufacturer’s instructions. Restriction endonucleases and DNA modification enzymes were obtained from Amersham Biosciences. All PCR amplifications and sequencings were carried out using a PTC-200 Peltier Thermal Cycler (Bresatech Pty. Ltd.). Sequencing products were analyzed on an ABI 373A automated fluorescent sequencing apparatus (Applied Biosystems Inc.). Sequencher™ Version 3.0 (Gene Codes Corp.) and BLAST algorithms (Basic Local Alignment Search Tool) were used to analyze DNA sequences.

Isolation of lpqW::Tn611 Mutants—A library of Tn611 mutants of M. smegmatis was prepared as described previously (15) except that the mutant library was plated on beef-yeast agar. A mutant strain that formed small colonies (0.5–1 mm diameter) was isolated. This mutant, Myco481, rapidly reverted to the normal sized (2–3 mm diameter) colony upon subculture, and one of the normal sized variants was isolated and named Myco481.1. Both Myco481 and the variant (Myco481.1) formed normal sized colonies on Middlebrook 7H10 agar and only differed from each other on complex media such as beef-yeast, LB, or PPLO; therefore, Middlebrook media were used for all subsequent analyses.

Construction of an M. smegmatis lpqW::aphA3 Mutant by Gene Replacement—A new lpqW mutator was created by directed gene replacement to confirm that the phenotype was related to the Tn disruption of lpqW in Myco481. The procedure for gene replacement used an allelic exchange cassette comprised of a 2.5-kb fragment of M. smegmatis genomic DNA with the lpqW gene centrally located. The lpqW gene was disrupted at an internal HindIII site with a kanamycin resistance marker, aphA3. The vector carrying the allelic exchange cassette carried sacB and a streptomycin resistance marker and cannot replicate in mycobacteria (21). The plasmid, pHBJ508, was introduced into M. smegmatis mc²155 by electroporation, and transformants were recovered on Middlebrook 7H10 agar containing kanamycin and streptomycin at 37 °C. One transformant was grown without antibiotics for 3 days (Middlebrook 7H9 broth, 37 °C) and then plated on medium with kanamycin and 10% w/v sucrose. Putative double crossover mutants (DXO; kanamycin-resistant, streptomycin-sensitive, sucrose-resistant) were streaked out to purity and then grown in Middlebrook 7H9 broth for 3 days. Aliquots of the broth cultures were stored at −70 °C and used as the inocula for subsequent experiments. Disruption of lpqW in the DXO mutant was confirmed by Southern blotting. The DXO mutant was named Myco721. This mutant produced small colonies on complex medium and rapidly converted to a normal sized colony variant. A variant was isolated for further analysis and was named Myco721.1. A plasmid was constructed to complement the lpqW mutation. The plasmid contained the entire lpqW open reading frame under the control of the GroEL promoter on pVV16 (gift of T. Eckstein). The complementation plasmid, pHBJ212, was transferred to Myco721 by transformation.

Extraction and Analysis of PIMs and LAM—PIMs were extracted from mycobacterial cell pellets in chloroform:methanol (2:1, v/v) and chloroform:methanol:water (1:2:0.8 v/v), and dried extracts were resolved by high performance thin layer chromatography (HPTLC) as described previously (15). Individual PIM species were purified by scraping silica from preparative HPTLC sheets and extracted in chloroform:methanol:water (10:10:3, v/v). HPTLC-purified PIMs were analyzed by electrospray ionization-MS and MALDI-TOF-MS as described previously (12). LAM was extracted from delipidated pellets by reflux (3×) in 50% (v/v) ethanol (100 °C, 2 h), and insoluble material was removed by centrifugation (10,000 × g, 10 min). The combined extracts
were dried under N₂, and the LAM/LM was purified by octyl-Sepharose (Amersham Biosciences) chromatography. Samples in 0.1 M NH₄O(OH)CH₃, 5% (v/v) 1-propanol were loaded onto a small column (500 µl) of octyl-Sepharose equilibrated in the same buffer. The column was washed with 0.1 M NH₄O(OH)CO(CH₃)₂ containing 5% 1-propanol, and the bound LAM/LM was eluted in 30, 40, and 50% (w/v) 1-propanol. Extracts from 150 mg (wet weight) of cells were run on SDS-PAGE gels and silver-periödacid-stained. The monosaccharide compositions of purified LAM/LM fractions were determined after hydrolysis in 2 M trifluoroacetic acid (2 h, 100 °C), and conversion of the released monosaccharides to their alditol acetate derivatives by reduction in NaBD₄ and acetylation in acetic anhydride using 1-methyl-imidazole as a catalyst (22) was determined. Gas chromatography-mass spectrometry (GC-MS) was performed using an Agilent 6890-5973 GC-MS system and an HP-1 column. Samples (1 µl) were injected in splitless mode, and the column was held at 80 °C for 1 min, ramped at 30 °C min⁻¹ to 140 °C, and then at 5 °C min⁻¹ to 250 °C, and held at 250 °C for 20 min.

Metabolic Labeling—Mid-exponential phase cultures of wild type and mutant M. smegmatis (25 ml) were harvested by centrifugation, washed once in Sauton’s medium, and resuspended in 1 ml of prewarmed Sauton’s medium (39 °C, 15 min) with shaking. Cells were pulse-labeled with [³H]mannose (50 µCi; 17 Ci/mmol of specific activity, PerkinElmer Life Sciences) for 10 min and then diluted with 5 ml of prewarmed Middlebrook 7H9 medium (Difco). Samples (1 ml) were removed at indicated times during this “chase” period, and PIMs were extracted as described above. Labeled lipids were resolved by HPTLC and detected either with an TLC linear analyzer (Berthold) or by fluorography after coating HPTLC sheets with EA scintillant wax (EA Biosciences) and exposed to Biomax MR film (Eastman Kodak Co.) at −70 °C. LAM was extracted from the delipidated pellets as described above. Radioactivity in the combined 30–40% 1-propanol fractions was measured by liquid scintillation counting. Incorporation of [³H]Man into LAM was normalized to cell weight, and the results shown are representative of three independent experiments.

RESULTS

Phenotype of a lpqW Mutant—The M. smegmatis transposon mutant, Myco481, was isolated from a library of Tn611 mutants on the basis that it formed unusual, small (0.5–1 mm diameter) colonies on complex agar medium, such as beef-yeast and PPLO agar. Myco481 formed normal sized colonies on Middlebrook 7H10 and PPLO agar (Fig. 2 A). This mutant was consistently found to be unstable when cultured on complex media, and colonies of the same size as wild type M. smegmatis mc²¹⁵⁵ (2–3 mm diameter) appeared after subculturing on complex medium (Fig. 2 A). One of the normal sized variants was isolated and named Myco481.1. The colony size was dependent on medium composition, and both Myco481 and Myco481.1 formed normal sized colonies on Middlebrook 7H10 agar (Fig. 2 A) but could be differentiated on PPLO agar. Myco481 cultured on 7H10 agar consistently formed small colonies when subcultured on PPLO agar, suggesting that the rate of reversion was slower or happened much less frequently in Middlebrook medium than complex medium. Southern blotting of digested genomic DNA of both Myco481 and Myco481.1 using an IS6100 probe showed the three bands typical of a single copy of Tn611, indicating that the Tn was inserted in the same place in each strain (Fig. 2 A). The site of Tn611 insertion in each strain was also determined using ligation-mediated PCR (24) and marker rescue (16). The data showed that both Myco481 and the variant, Myco481.1, had a single copy of Tn611 inserted in identical positions in an open reading frame that is similar to Rv1166 of M. tuberculosis that has been designated as lpqW because it is predicted to encode a lipoprotein (26). Disruption of the gene downstream of lpqW did not affect PIM/LAM synthesis, showing that the lpqW mutation did not cause polar genetic effects (27). The lpqW gene is highly conserved (>80% similarity) in M. tuberculosis, Mycobacterium leprae, Mycobacterium avium paratuberculosis, and M. smegmatis. The M. tuberculosis and Mycobacterium bovis alleles are identical to each other. The LpqW polypeptide sequence has a putative secretion signal sequence and signal peptidase II cleavage site, suggesting that the mature protein is a secreted lipoprotein.

Cell Wall Composition of Mutants and Variants—The cell wall composition of the Myco481 and Myco481.1 mutants was analyzed and compared with the parental strain, M. smegmatis mc²¹⁵⁵. No differences were observed in core cell wall components such as mycolic acids and arabinogalactan or in the outer layer glycopeptidolipids (data not shown). However, analysis of fractions containing the PIMs and LAM/LM revealed marked differences between the wild type M. smegmatis and the two mutants. Samples of LAM/LM extracts were applied to SDS-PAGE and silver-periodic acid-stained to reveal LAM and LM (Fig. 3 A). Myco481 contained very little LAM; however, Myco481.1 had LAM levels similar to the wild type. LM was evident in both strains. GC-MS compositional analysis of the mannose of LAM/LM was performed to quantitate the LAM/LM. This revealed that Myco481 had greatly reduced levels of mannose (20% of wild type) (Fig. 3 B), which can be accounted for substantially by the loss of LAM. Wild type levels of LAM expression were restored in Myco481.1 (Fig. 3 B). The LAM/LM fractions of wild type and Myco481.1 contained mannose, arabinose, and myo-inositol in similar ratios, indicating that the pool of Myco481.1 LAM is capped with a mature arabian component (data not shown). The PIM profile of Myco481 was indistinguishable from that of wild type cells, whereas Myco481.1 had a clearly altered PIM profile (Fig. 3 B). The PIMs of Myco481 and Myco481.1 accumulated four major PIM species, previously characterized as AcPIM2, Ac₅PIM2, AcPIM6, and Ac₆PIM6 (12). Although Myco481.1 expressed similar levels of AcPIM2 and Ac₅PIM2, this mutant lacked detectable levels of the polar PIM species, AcPIM6 and Ac₅PIM6, and it accumulated a new glycolipid species of intermediate polarity (Fig. 3 B). GC-MS compositional analysis of this glycolipid indicated the presence of Man, myo-inositol, palmitic acid, and 10-methyl octadecanoic acid (C₁₉:0) in the molar ratio 4:1:1:1, whereas MALDI-TOF analysis of the intact glycolipid gave a [M-H]⁻ molecular ion of 1733, the expected molecular weight of AcPIM4. These data suggest that Myco481.1 is unable to extend AcPIM4 with α₁–2-linked Man residues to form AcPIM6, although this species can presumably be extended with long α₁–6-Man chains to form LAM. As the α₁–6-Man backbone of mature LM/LAM is also modified with α₁–2-linked Man side chains, we subjected the mature LAM from wild type cells and the residual LAM pool from Myco481 to methylation linkage.
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Care was taken to minimize the sub culturing of the mutants created. A mutant, named Myco721, was isolated from a plate selecting for DXO mutants and streaked to purity, and a colony was subcultured once in Middlebrook 7H9 broth, which was then stored in aliquots at −70 °C. One aliquot was used to inoculate LB broth, which was grown at 37 °C and sub cultured into fresh LB broth every 3 days. At each subculture, a sample was plated on PPLO agar to determine colony morphology. Fig. 4C shows how at first, all colonies were small (a), but after five subcultures, some large colonies appeared (b), and after 10 subcultures, virtually all of the colonies were large (c). One large colony (Fig. 4C, indicated with arrow in b) was picked and named Myco721.1 and was used for further analysis. Disruption of \(lpqW\) was confirmed by Southern blot analysis (Fig. 4B).

Cell wall analysis of Myco721 showed that it had little LAM or LM but had a normal PIM profile (Fig. 3). The variant, Myco721.1, regained full LAM/LM synthesis but had a PIM profile altered by accumulation of AcPIM4 and reduced levels of AcPIM6 (Fig. 3). The amount of LM proportional to LAM appears to be elevated in this mutant (Fig. 3A), which accounts for the higher levels of mannose determined by compositional analysis (Fig. 3B).

Myco721 was purified and stored immediately after its creation. Frozen aliquots were used as inocula for all subsequent experiments to avoid sub culturing. In comparison, Myco481 underwent extensive sub culturing on complex media before storage and may have accumulated mutations; therefore, Myco721 is the best genetic representative of a \(lpqW\) mutant. Variants derived from Myco481 and Myco721 (Myco481.1 and Myco721.1) were stable in colony phenotype and chemical composition even after extensive sub culturing. These results show that disruption of \(lpqW\) results in a defect in PIM/LAM synthesis and the mutants are not stable.

Complementation of the defect in Myco721 was achieved by introducing an intact copy of \(lpqW\) on a plasmid. Myco721 was transformed with the \(lpqW\) complementation plasmid pHBJ212 and with plasmid controls. Transformants containing the control plasmid formed small colonies, whereas transformants containing pHBJ212 (Myco721C) grew as normal sized colonies on complex media. The PIM profile of Myco721C contained the four PIMs seen in wild type and Myco721, but importantly, the LAM/LM was restored by complementation (Fig. 3). These results indicate that disruption of \(lpqW\) was responsible for the small colony phenotype and the LAM defect observed in Myco721. Similar results were obtained by complementation of Myco481 (data not shown). The parental strain, Myco481, and Myco721 were readily transformed with pHBJ212; however, complementation of Myco481.1 and Myco721.1 with pHBJ212 was unsuccessful. Either repeated attempts to transform those strains with pHBJ212 failed or the few surviving transformants contained plasmids with large deletions in the \(lpqW\) gene. All strains could be transformed with the empty vector or vector containing unrelated genes, indicating that the strains were competent. The wild type could tolerate pHBJ212 and had a normal cell wall composition (data not shown). These results suggest that expression of functional LpqW in the variant strains is deleterious.

\[^{3}H\]Mannose Labeling of Growing Cells—AcPIM4 is thought to represent a branch point intermediate in polar PIM and LAM biosynthesis in M. smegmatis (10, 12). The absence of PIM6 could occur through lack of synthesis, catabolism, or redirection of its precursor to another pathway. To investigate the metabolic fate of AcPIM4 in the mutants, actively growing cells were metabolically labeled with \[^{3}H\]Man. Label was initially incorporated into PPM and the expected spectrum of PIM intermediates in wild type cells (Fig. 5A) (12). During the chase period, label accumulated in the end product mono- and diacylated PIM2 and

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**FIGURE 3. Analysis of LAM and PIM content of wild type M. smegmatis and \(lpqW\) mutants.** A, LAM/LM extracted from 150 mg (wet weight) of cells was resolved on SDS-PAGE and silver-periodic acid-stained. Migration of molecular mass standards is shown on the left in kDa. B, the amount of mannose contained in LAM/LM extracts from wild type (mc155), mutant, and complemented cell lines. Mycobacteria were harvested from duplicate cultures in mid-exponential phase, and levels mannose of purified LAM/LM were determined by GC-MS. Myco481 and Myco481.1 are representative of the initial unstable transposon mutant and the second stable mutant, respectively. Myco721 and Myco721.1 represent the equivalent mutants generated after targeted disruption of the \(lpqW\) gene. Myco721 C is the complemented Myco721. The error bars represent the maximum and minimum values. C, PIM profiles. PIMs were extracted from mid-exponential phase cells and analyzed by HPTLC developed in chloroform, methanol, 1M ammonium acetate, 13M NH3, water (180:140:9:9:23 by volume). PIMs were visualized with orcinol-H2SO4 staining.

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analysis. No difference was observed in the degree of substitution and α1–2 branching in the LAM from Myco481, indicating that the mutant does not have a defect in the side chain α1–2-mannosyltransferase (data not shown).

**Genetic Analysis**—As Myco481 contained a Tn611 insertion just 157 bp before the end of the \(lpqW\) open reading frame (Fig. 4A), it is possible that the mutant may express a truncated form of the protein that might retain some function. To confirm the phenotype of a mutant lacking functional LpqW protein, a \(lpqW\) mutant was created by placing a kanamycin resistance gene, aphA3, in the middle of \(lpqW\) (Fig. 4A).
PIM6 species (Fig. 5A). A similar labeling pattern was observed in the Myco481 mutant that contains an identical PIM profile to that seen in wild type cells. In contrast, $^{[3}H]MNSM$ in Myco481.1 was initially incorporated into PPM and the PIM species, AcPIM1, AcPIM2, AcPIM3, and AcPIM4. The polar PIM species AcPIM5 and AcPIM6 were not labeled. During the chase period, label in PPM, AcPIM1, and AcPIM3 was depleted with a corresponding increase in label in mono-/diacylated PIM2 and PIM4 species. These data show that AcPIM4 is a transient intermediate in wild type mycobacteria and Myco481 but that it is a relatively stable metabolic end product in Myco481.1, accounting for its accumulation in this mutant.

As expected from the chemical analysis, $^{[3}H]MNSM$ was actively incorporated into LAM during the chase period in both wild type and Myco481.1 (Fig. 5B). In contrast, very little label was incorporated into LAM in Myco481. These data strongly suggest that disruption of $lpqW$ dramatically reduces the flux of PIM intermediates into LAM, without affecting the rate of synthesis of polar PIMs (i.e. AcPIM6). In subsequently derived mutants, represented by Myco481.1, restoration of LAM biosynthesis occurs at the expense of polar PIM biosynthesis, although less polar PIMs (i.e. AcPIM4) still accumulate to high levels. Despite the differences in cell envelopes, all strains grew equally well in Middlebrook broth (Fig. 5C).

**DISCUSSION**

PIMs and LAM are major plasma membrane and cell wall components of all mycobacteria and appear to be important virulence factors in pathogenic species. Although it is clear that PIMs are both biosynthetic precursors to LAM and metabolic end products in their own right, very little is known about the regulation of these pathways. In this study, we show that disruption of the $lpqW$ gene has a dramatic affect on the ability of *M. smegmatis* to grow on complex medium and influences the flux of PIM intermediates into LAM. Mutants lacking functional $lpqW$ are unstable but give rise to stable variants in which LAM biosynthesis is restored at the expense of polar PIM biosynthesis. LpqW thus appears to have dual, and possibly related, functions in regulating LM/LAM biosynthesis and the adaptation of mycobacteria to different nutritional environments.

The biosynthesis of polar PIMs and LM/LAM likely involves the same early steps, with a bifurcation after the synthesis of AcPIM4 (Fig. 1) (10, 12, 25). This conclusion is strongly supported by the finding that AcPIM4 accumulated in Myco481.1 and Myco721.1 mutant lines that had no or reduced levels of AcPIM6 biosynthesis, respectively. Myco721.1 differed from Myco481.1 in that it retained some AcPIM6 and had slightly more LM even than WT. These differences suggest that the two variants are not genetically identical; however, the genetic basis of the variation has not been elucidated. The commitment of AcPIM4 to polar PIM or LM/LAM biosynthesis requires the activity of at least two enzymes: a putative $\alpha_1$–$\alpha_6$-mannosyltransferase and an $\alpha_1$–$\alpha_6$-mannosyltransferase, respectively (Fig. 1). Essentially nothing is known about these enzymes. Although LpqW is clearly not essential for the biosynthesis of either polar PIMs or LM/LAM, as mutants lacking this protein gene is 2.0 kb, and a vector fragment containing part of $lpqW$ in the single cross-over is 1.6 kb. The probe is shown in gray in panel A, C, the appearance of variants. Myco721 was isolated from a plate selecting for DOX mutants and streaked to purity, and a colony was subcultured once in Middlebrook 7H9 broth, which was then stored in aliquots at $-70^\circ$C. One aliquot was used to inoculate LB broth, which was grown at 37°C and subcultured into fresh LB broth every 3 days. At each subculture, a sample was plated on PPLO agar to determine colony morphology. All colonies were small for the first two subcultures (a), but after five subcultures, some large colonies appeared (b). After 10 subcultures, virtually all of the colonies were large (c). One large colony (b, arrow) was picked and named Myco721.1 and was used for further analysis.

**FIGURE 4.** Targeted disruption of $lpqW$ and overgrowth by a variant. **A**, gene map of $lpqW$ showing sites of insertion by Tn611 in Myco481 and 481.1 (bp 1727) and by aphA3 in Myco721 and Myco721.1 (bp 952). BalI (B) and HindIII (H) restriction endonuclease sites are indicated. **B**, Southern blot analysis of BalI-digested genomic DNA of wild type WT, SXO, and 721 showing sites of insertion by Tn611, a single cross-over (SXO) mutant, and Myco721 (721) showing that Myco721 has a $lpqW$ disruption. The size of the normal gene is 1.12 kb, the disrupted...
can make both classes of glycolipids, our data suggest that LpqW and/or interacting proteins plays a key role in regulating the activity of the α1–2/α1–6 mannosyltransferases or access of AcPIM4 to these transferases. Specifically, loss of lpqW was initially associated with a dramatic reduction in the synthesis of LAM, whereas the modification of AcPIM4 to form AcPIM6 was unaffected. Following conversion of Myco481 to the stable mutant, Myco481.1, LAM biosynthesis was restored at the expense of AcPIM6 biosynthesis. Overall, PIM biosynthesis in the two mutant cell lines was similar to that of wild type cells.

These results provide compelling evidence that AcPIM4 is indeed a branch point intermediate and represent the first demonstration that the synthesis of polar PIMs and LAM can be independently regulated. The capacity to differentially regulate these pathways may be important in ensuring that mycobacteria retain a sizable pool of PIM species (see below) and in regulating the properties of either the plasma membrane, which is rich in PIMs, or the cell wall, which is rich in LAM.

There are LpqW homologues in the genomes of all mycobacteria analyzed to date. Although its exact role has not been identified, LpqW appears to be essential for growth of M. tuberculosis as it cannot be disrupted (29). LpqW shares no homology with known classes of glycosyltransferases, and its role in PIM and LM/LAM biosynthesis is currently undefined. All mycobacterial LpqW homologues are predicted to be lipoproteins with a secretion signal sequence and signal peptidase II cleavage site, suggesting that they are associated with the external face of plasma membrane. Similarly, we have shown that AcPIM4 is synthesized by a PPM-dependent mannosyltransferase that is spatially separated from the cytoplasmically orientated GDP-Man-dependent transferases that catalyze early steps in PIM biosynthesis (10, 12). As polyprenyl-phosphosugar-dependent transferases are thought to catalyze reactions on the external face of the plasma membrane, most of the AcPIM4 is probably located in the external leaflet of the plasma membrane and/or cell wall. It is possible that LpqW could directly regulate the activity of the α1–2/α1–6-mannosyltransferases or access of AcPIM4 to these transferases. Alternatively, LpqW may have a more general signaling function that indirectly leads to changes in PIM/LAM biosynthesis. LpqW resembles solute-binding proteins that bind a wide variety of ligands, which are subsequently transported across the plasma membrane by ATP-binding cassette transport proteins. LpqW is similar to solute-binding proteins whose roles include internalization of peptides for nutrition and sensing the external medium for specific and nonspecific peptides (30). A potential role for LpqW in nutrient sensing is supported by the finding that Myco481 and Myco721 exhibited markedly different growth phenotypes and colony morphologies on different medium. Identifying the connection between LpqW and PIM biosynthesis may shed new insights into signaling mechanisms that regulate cell wall assembly.

The finding that the biosynthesis of polar PIMs and LAM can be differentially regulated raises the question as to whether each of these glycolipids have different functions. Recent gene deletion studies in M. smegmatis have provided some insights into the requirement for different PI lipids for mycobacteria growth. PimA and PimB are mannosyltransferases that catalyze the first two mannosylation steps in PIM biosynthesis. Deletion of either of the genes encoding these enzymes is lethal, suggesting that the PIM pathways are essential for growth (9, 13). In contrast, mutants lacking mature LAM remain viable (25, 31). The selective depletion of all inositol lipids was achieved following inositol starvation of a M. smegmatis inositol auxotroph (10). These analyses indicated that the large pools of PI and apolar PIMs (Ac/Ac2PIM2) are
not essential for viability, whereas loss of polar PIMs was associated with cell death (10). Although the stable Myco481.1 mutant lacked a large pool of AcPIM6, this mutant accumulated a pool of AcPIM4 that likely substitutes for AcPIM6. Collectively, these data suggest that the accumulation of a steady-state pool of PIMs is essential for mycobacteria viability but that the precise structure of the glycan head group may not be critical. In this respect, the capping of AcPIM4 with two α1–2-linked Man residues may constitute a mechanism for preventing the depletion of the PIM pool for LM/LAM biosynthesis under nutrient-limiting conditions.

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