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Mutations in *pimE* Restore Lipoarabinomannan Synthesis and Growth in a *Mycobacterium smegmatis lpqW* Mutant▽‡

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Lipoarabinomannans (LAMs) and phosphatidylinositol mannosides (PIMs) are abundant glycolipids in the cell walls of all corynebacteria and mycobacteria, including the devastating human pathogen *Mycobacterium tuberculosis*. We have recently shown that *M. smegmatis* mutants of the lipoprotein-encoding *lpqW* gene have a profound defect in LAM biosynthesis. When these mutants are cultured in complex medium, spontaneous bypass mutants consistently evolve in which LAM biosynthesis is restored at the expense of polar PIM synthesis. Here we show that restoration of LAM biosynthesis in the *lpqW* mutant results from secondary mutations in the *pimE* gene. PimE is a mannosyltransferase involved in converting AcPIM4, a proposed branch point intermediate in the PIM and LAM biosynthetic pathways, to more polar PIMs. Mutations in *pimE* arose due to insertion of the mobile genetic element ISMsm1 and independent point mutations that were clustered in predicted extracytoplasmic loops of this polytopic membrane protein. Our findings provide the first strong evidence that LpqW is required to channel intermediates such as AcPIM4 into LAM synthesis and that loss of PimE function results in the accumulation of AcPIM4, bypassing the need for LpqW. These data highlight new mechanisms regulating the biosynthetic pathways of these essential cell wall components.

Mycobacteria are the etiological agents of devastating diseases such as tuberculosis (*Mycobacterium tuberculosis*), leprosy (*Mycobacterium leprae*), and opportunistic infections of human immunodeficiency virus-infected individuals (*Mycobacterium avium*). *M. tuberculosis* infects around one-third of the entire human population and kills two to three million people each year, mostly in developing countries (22). Once thought to have been controlled in the developed world, tuberculosis has reemerged in recent years primarily due to the human immunodeficiency virus epidemic and the appearance of drug-resistant strains of *M. tuberculosis* (2). The emergence of such strains is particularly serious since mycobacteria are intrinsically resistant to most antibiotics and disinfectants. The key factor in this generalized resistance and the success of *M. tuberculosis* as a human pathogen is its unusual, waxy cell wall, which provides an effective permeability barrier. This structure is of particular interest since the biochemical processes involved in its synthesis are the targets of existing antimycobacterial drugs and are potential targets for the design of new generations of antimycobacterial compounds.

The mycobacterial cell wall comprises a layer of peptidoglycan to which is attached arabinogalactan and long-chain mycolic acids (3). The mycolic acids form the inner leaflet of an asymmetric lipid bilayer with the outer leaflet comprising a range of noncovalently linked (glyco)lipids. Although the composition of the noncovalently linked (glyco)lipids varies in different species, some are highly conserved in all mycobacteria. The most important of these are the phosphatidylinositol mannosides (PIMs) and their hyperglycosylated derivatives, lipomannan (LM) and lipoarabinomannan (LAM). It is proposed that these mannolipids are important virulence factors in pathogenic mycobacteria, acting as ligands for host cell receptors and having potent immunomodulatory properties (reviewed in reference 12). Considerable progress has been made in delineating the early steps in PIM biosynthesis and the assembly of the mannan and arabinan components of LM and LAM (reviewed in reference 1) (Fig. 1). PIM biosynthesis is initiated by the transfer of two mannose residues and a fatty acyl chain to phosphatidylinositol (11). The first mannose is transferred from GDP-Man by the enzyme PimA (10), while the second is added by a recently identified mannosyltransferase Rv2188c, now known as PimB′ (14). AcPIM2 and its acylated variant Ac_2PIM2 are the most abundant apolar PIMs of the mycobacterial cell wall. A subpopulation of AcPIM2 is elongated with two more mannose residues to form AcPIM4, the proposed branch point intermediate of polar PIM and LM/LAM biosynthesis (13, 19). AcPIM4 can be processed by the PimE $\alpha(1\rightarrow2)$ mannosyltransferase (20) and another uncharacterized enzyme to form the abundant polar PIMs AcPIM6 and Ac_2PIM6. Alternatively, AcPIM4 can be elongated with long chains of $\alpha(1\rightarrow6)$-linked mannoses with

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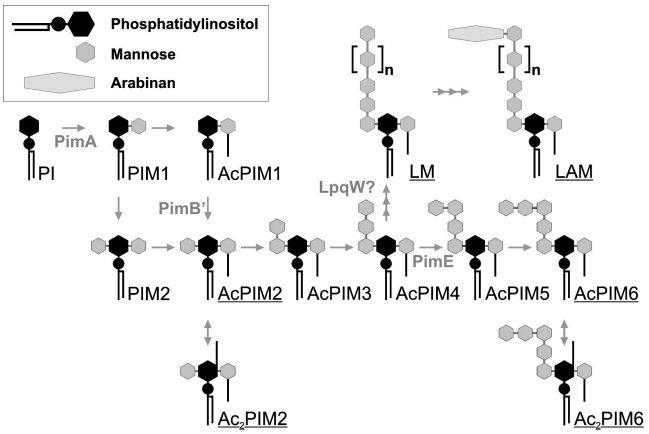

FIG. 1. PIM/LAM biosynthetic pathway. Phosphatidylinositol (PI) is converted to PIMs via substitution with up to six mannose units by mannosyltransferases, some of which have been identified. The sequence of reactions converting PIM1 to AcPIM2 may be flexible. PIM2, PIM4, and PIM6 can be substituted with one or two additional acyl groups. The underlined species accumulate as metabolic end products in wild-type *M. smegmatis*. AcPIM4 is modified by the α(1→2) mannosyltransferase PimE to form the higher PIMs and is thought to be hyperextended by α(1→6) mannosyltransferases to form LM, which is further extended with branched arabinans to form LAM. Thus, AcPIM4 is the likely bifurcation point of LM/LAM and polar PIM synthesis. The role of LpqW is unclear, but its loss leads to a decrease in LM/LAM biosynthesis.

α(1→2)-linked side chains to form LM (8, 9, 18). LM is subsequently modified with single arabinose or more complex arabinan oligosaccharides, depending on the species, to form the mature LAM (26).

Although the essential features of this pathway are established, less is known about the topology of these reactions or mechanisms that regulate flux of intermediates into polar PIM, LM, or LAM biosynthesis and hence the steady-state levels of these mannolipids. Based on the predicted topologies and sugar donors utilized by the respective glycosyltransferases, it is thought that the early PIM species are synthesized on the cytoplasmic leaflet of the plasma membrane, whereas polar PIM species, LMs, and LAMs are assembled by plasma membrane or cell wall-localized glycosyltransferases with extracytoplasmic catalytic sites. We have recently identified a putative lipoprotein, termed LpqW, that is likely to have an extracytoplasmic location and a role in regulating LAM biosynthesis in *M. smegmatis* (15, 16). The gene encoding LpqW was initially isolated when a *M. smegmatis* transposon library was screened for mutants with defects in cell wall synthesis (13). The transposon mutant, designated Myco481, formed small colonies on complex media and synthesized structurally normal LAMs, but at significantly reduced levels relative to the wild type (13). When grown in complex liquid media such as Luria-Bertani (LB) or pleuropneumonia-like organism media (PPLO), Myco481 was observed to give rise to variants that had normal colony morphology and restored LAM biosynthesis. Analysis of one such variant, designated Myco481.1, indicated that the restoration of LAM synthesis was associated with the loss of polar PIM biosynthesis and the accumulation of the branch point intermediate, AcPIM4. Targeted disruption of *lpqW* with a drug resistance cassette also generated a LAM-deficient mutant (Myco721) from which a LAM-restored strain could be derived (Myco721.1). Theses analyses indicate that loss of LpqW function and LAM synthesis has a profound impact on the growth of *M. smegmatis* but that this defect can be largely bypassed through inhibition of polar PIM biosynthesis and accumulation of AcPIM4. In the present study we show that this bypass results from the introduction of secondary mutations into *pimE*, encoding the mannosyltransferase involved in converting AcPIM4 to polar PIMs (20). Analysis of independently derived *lpqW pimE* mutants has revealed functionally important domains in the polytopic PimE protein. Our data suggest that LpqW might function in chaperoning low-abundance PIM intermediates into the LM/LAM pathway in *M. smegmatis*.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and recombinant DNA methods. The wild-type *M. smegmatis* mc^2155 (24), *lpqW* mutants Myco481 and Myco721, and their derivative strains (13; the present study) were cultivated at 37°C. Cultures were grown in Middlebrook 7H9 broth or 7H10 solid medium (Difco) with the addition of a modified DC supplement (0.2% [wt/vol] dextrose, 0.085% [wt/vol] NaCl), LB complex medium (tryptone, 10 g/liter; yeast extract, 5 g/liter; NaCl, 10 g/liter) or PPLO complex medium (brain heart infusion, 50 g/liter; peptone, 10 g/liter; NaCl, 5 g/liter). For LB and PPLO, 1% (wt/vol) agar was included where required. Kanamycin and streptomycin were added to media to 20 μg/ml, as required. PCR amplifications and sequencing cycles were carried out by using a PTC-200 Peltier thermal cycler (Bresatech Pty., Ltd.) and Proofstart DNA polymerase (Qiagen) according to the manufacturer's instructions. The genomic sequence surrounding *pimE* (MSMEG_5149) was amplified and sequenced by using the primers *pimE*-L (5′-CGCGGGCCTGTACTTCCTCG-3′), *pimE*-R (5′-CCATCATCCGGATATCGAC-3′), *pimE*-L2 (5′-GCGCCGCTCAGGGCCAC CG-3′), and *pimE*-R2 (5′-CGAGGAAGTACAGGCCCGCG-3′). Sequencing products were analyzed on an ABI 373A automated fluorescent sequencing apparatus (Applied Biosystems, Inc.). Sequencher version 3.0 (Gene Codes Corp.) and BLAST (for Basic Local Alignment Search Tool) algorithms were used to analyze DNA sequences. Genomic DNA was extracted from mycobacteria as described previously (7). Expression vector for the C-terminally hemagglutinin (HA)-tagged PimE, pYAB53, was generated from a PimE expression vector, pYAB34 (20). A C-terminal HA epitope tag was introduced by amplifying a fragment of the MSMEG_5149 gene using the primers 5′-TGTCGTGGCTC GTGGTGGGT and 5′-TTCGAATTC**ACGCGTAGTCCGGGACGTCGTACGG GTA**AATATTACCGGATCCTTTCCGACTGA (the EcoRI and SspI restriction enzyme sites are underlined; the sequence encoding the HA tag is indicated in boldface) and ligating the PCR fragment using EcoRI and ApaI sites. Electrocompetent *M. smegmatis* cells were prepared and transformed according to the method of Jacobs et al. (6).

Isolation of *lpqW pimE* double mutants. The stability of *lpqW* mutants at a population level was examined by serially subculturing wild-type, Myco481, and Myco481.1 strains in LB, PPLO, and Middlebrook media. Single colonies of each strain were resuspended and subsequently inoculated into 30 ml of broth of each of the three media. After incubation for 3 days, strains were subcultured 1 in 100 into fresh medium, and incubation was continued. A total of five subculture cycles were carried out for analysis. At the end of each cycle, cells were collected by centrifugation (3,000 × *g*, 10 min), and the PIMs were extracted (see below). Cells were also plated onto PPLO agar to determine the colony morphology of the population at each subculture. Individual Myco481 variants were isolated in a similar fashion using only LB media. After two rounds of subculture, cells were plated on LB agar and showed a mixed population of small and normal-sized colonies. Thirty normal colonies were chosen for further analysis, with two small colonies selected as controls. The selected variants were grown on Middlebrook medium for all subsequent genetic and biochemical analyses.

Extraction and analysis of PIMs and LAM. PIMs and LM/LAMs were sequentially extracted in chloroform-methanol-water (1:2:0.8 [vol/vol/vol]) and refluxed in 50% ethanol, respectively, as previously described (13). PIMs were analyzed on aluminum-backed silica high-performance thin-layer chromatography (HPTLC) sheets developed in chloroform-methanol-ammonium acetate-ammonium-water (180:140:9:9:23 [vol/vol/vol/vol/vol]). Glycolipids were detected with orcinol-H_2SO_4 spray. LM/LAM levels were determined by gas chromatography-mass spectrometry (GC-MS) monosaccharide composition

analysis, following methanolysis (0.5 M methanolic HCl, 80°C, 16 h) and conversion of the released methylglycosides to their corresponding trimethylsilyl derivatives (13). For quantitative analysis of LM/LAM content, culture conditions corresponding to mid-exponential growth were determined by using the biomass quantitation method described by Meyers et al. (17) in Middlebrook 7H9 media. Growth curves and subsequent cultures for LAM extraction were produced by using seeder stocks prepared by growing all strains to saturation, adjusting the protein concentration to 200 μg/ml, and storing the samples frozen as single-use aliquots in Middlebrook 7H9 medium containing 50% (vol/vol) glycerol.

RESULTS

***M. smegmatis lpqW* mutants evolve in complex media but are stable in a defined medium.** When propagated in complex media, Myco481 grows poorly and forms small colonies compared to its derivative strain Myco481.1 or the wild type. In contrast, Myco481 grows normally in Middlebrook 7H9 broth and forms normal colonies on Middlebrook 7H10 agar (13). To gain an understanding of the mechanisms by which variants arise from the *lpqW* mutants, we devised a pilot experiment to more closely observe the phenomenon on a population of cells grown in these media. Wild-type *M. smegmatis*, the *lpqW* transposon mutant Myco481, and its derivative Myco481.1 were serially subcultured through either complex (LB and PPLO) or defined (Middlebrook 7H9) broth media. After each subculture, bacteria were harvested for analysis of their PIM content and transposon stability and plated for assessment of the colony morphology on PPLO agar.

When Myco481 was cultured in complex media, pronounced changes in phenotype were observed after two to three rounds of broth subculture. First, the growth of these bacteria in liquid culture and the colony morphology on agar plates were restored to those of wild-type *M. smegmatis*. The shift from a poorly growing Myco481 culture population dominated by small-colony-forming cells to a culture showing normal growth characteristics corresponded to a change in PIM profile (Fig. 2). The emergent populations derived from the Myco481 parent culture had markedly reduced levels of polar PIMs (AcPIM6, Ac_2PIM6) and progressively accumulated AcPIM4, a minor species in wild-type bacteria (Fig. 2). In marked contrast, such changes in population growth, colony morphology, or PIM profiles were not observed when Myco481 was continuously cultured in defined medium. The observations were reproducible in Myco721, the targeted *lpqW* knockout strain (13), confirming that independent genetic disruption events of *lpqW* result in phenotypic instability in complex media (data not shown). Detailed LAM analysis was not performed on the subcultured strains due to the apparent heterogeneity of the culture populations, which is described in detail below.

Myco481.1 showed no significant variation through identical culture conditions in each medium tested, suggesting that the change from its parental strain Myco481 is stable and irreversible. Myco481.1 grew comparably to the wild-type strain, which also exhibited no change during serial subculturing. No change in transposon orientation or location within *lpqW* as determined by Southern blot analysis following the first and fifth subcultures in either complex or defined media for either Myco481 or Myco481.1 (data not shown) and no change in the *lpqW* sequence itself (13) suggest that factors other than *lpqW* disruption were contributing to the change in phenotype. Taken together, these results suggest that the *lpqW* mutant

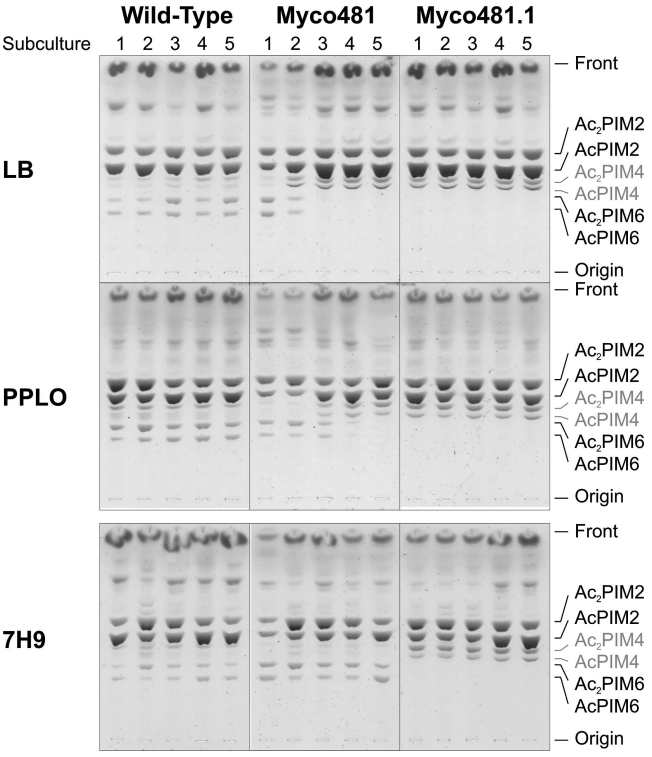

FIG. 2. Evolution of *lpqW* mutant Myco481 is observed in complex but not defined media. The *lpqW* mutant Myco481 carries a transposon insertion near the 3′ end of the gene and was previously characterized as being deficient in LAM. The derivative strain Myco481.1 has lost the ability to produce polar PIMs at the expense of restoration of LAM levels (13). PIM extracts from wild-type *M. smegmatis* mc^2155, Myco481, and Myco481.1 following five successive subcultures (1–5) in complex (LB, PPLO) and defined (Middlebrook 7H9) media were resolved by HPTLC and visualized by orcinol staining. The identities of the major PIM species (19) are indicated in relation to the sample origin and solvent front.

Myco481 readily evolves when grown in complex media to form a stable derivative that grows normally, is deficient in polar PIMs, and accumulates PIM4. This instability is not observed in defined media, since no change in colony morphology or PIMs was observed. Importantly, the lack of phenotypic change in defined media offers a means to reliably analyze the *lpqW* mutant phenotype following culture and subsequent change in complex media.

Evolution of Myco481 to Myco481.1 is caused by insertion of a transposon into *pimE*. A recent study identified PimE as the α(1→2) mannosyltransferase responsible for converting AcPIM4 to a PIM-specific isomer of AcPIM5 in the PIM/LAM biosynthetic pathway (Fig. 1) (20). That study showed that a *pimE* knockout strain of *M. smegmatis* failed to synthesize AcPIM5 and AcPIM6, instead accumulating AcPIM4. Since the evolution of Myco481 and Myco721 also manifested as an accumulation of AcPIM4 at the expense of polar PIM species, and since this transformation appeared to be permanent, we speculated that mutation of *pimE* might be responsible for this phenotypic change. Initial studies focused on the two previously characterized *lpqW* mutant variants, Myco481.1 and Myco721.1 (13).

Intriguingly, the *pimE* gene (Rv1159) was found to be lo-

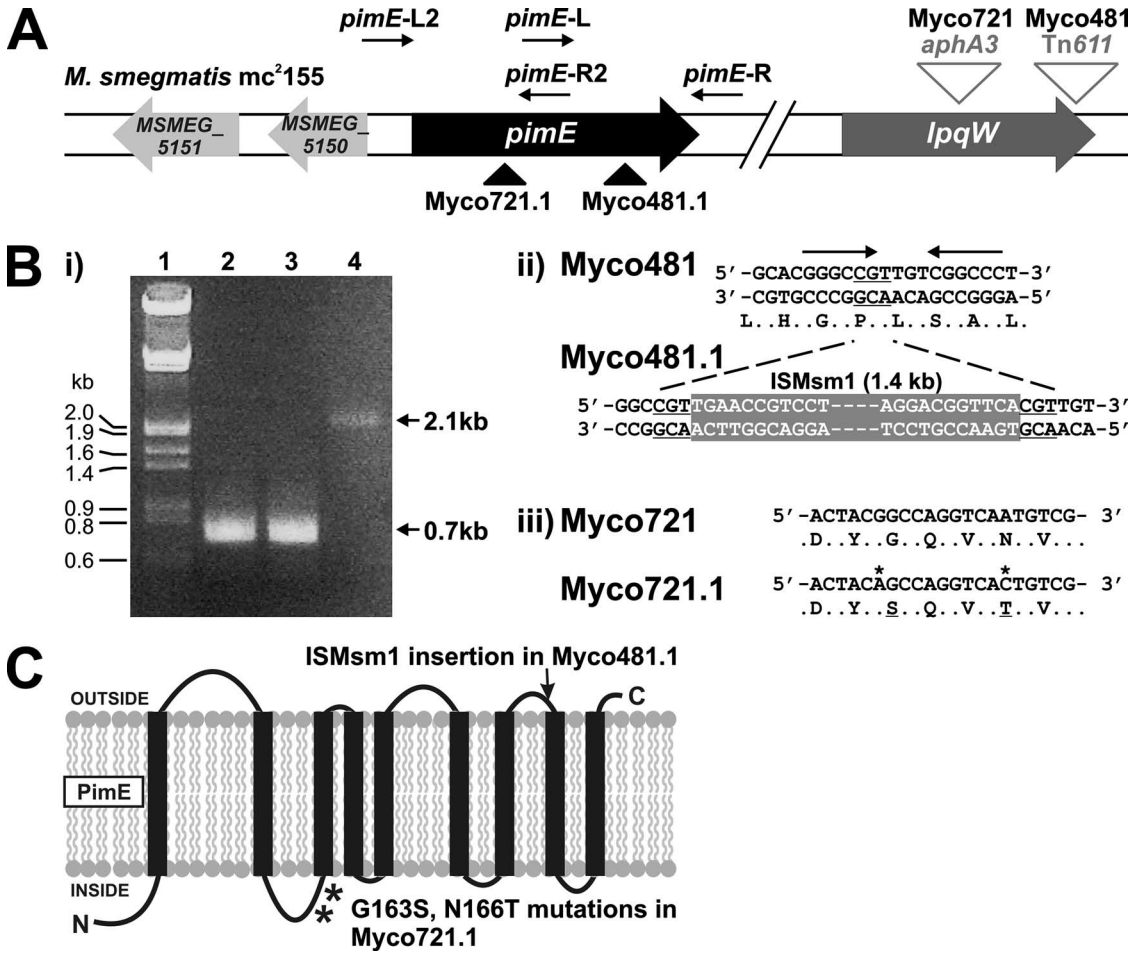

FIG. 3. *lpqW* mutants evolve via mutations in the *pimE* gene. (A) Gene map of the *pimE-lpqW* genomic region in *M. smegmatis*. The *lpqW* and *pimE* genes are separated by 22 kb. The disruption sites within *lpqW* in Myco481 (Tn*611* insertion) and Myco721 (*aphA3* insertion) are indicated by open triangles. Small horizontal arrows indicate binding sites for primers used to amplify and sequence the 5′ and 3′ ends of *pimE*. Sequence analysis showed that the LAM-restored/polar PIM-deficient derivative strains Myco481.1 and Myco721.1 both contain mutations in differing locations in *pimE* (indicated by filled triangles). (B) Comparative genetic analysis of Myco481/Myco481.1 and Myco721/Myco721.1. (i) Detection of ISMsm1 insertion by PCR using the primers *pimE*-L and *pimE*-R. Lane 1, λHindIII/EcoRI DNA size markers indicated in kilobases; lane 2, *M. smegmatis* mc²155; lane 3, Myco481; lane 4, Myco481.1. (ii) *pimE* sequence from Myco481 showing the 3-bp target sequence for ISMsm1 (underlined) and the corresponding amino acids of PimE. Arrows indicate a small inverted repeat surrounding the target site. Sequence of the equivalent region in Myco481.1 shows the inserted IS element (reverse shaded) and the 3-bp target site duplication (underlined). (iii) *pimE* sequence from Myco721 and Myco721.1 show that two nonconservative point mutations (*) have been created in the derivative line. The affected amino acid residues are underlined. (C) Proposed membrane topology of PimE. Topology was predicted by using the TMHMM server version. 2.0 (Center of Biological Sequence Analysis, Technical University of Denmark), which predicted nine putative transmembrane helices. The position of insertion of the ISMsm1 element within *pimE* in Myco481.1 is indicated, as are the two mutated residues in Myco721.1 (*).

cated just 12 kb upstream of *lpqW* (Rv1166) in the genome of *M. tuberculosis* $H_{37}R_v$ (4). We examined the equivalent region of the *M. smegmatis* chromosome and found the homologs of *lpqW* (MSMEG_5130) and *pimE* (MSMEG_5149) to be arranged similarly but separated by 22 kb and 18 genes (Fig. 3A). These include genes encode conserved hypothetical proteins, a set of putative nitrate reductase components, and permease and membrane transport components of a putative sugar ABC transporter. To investigate our hypothesis of a genetic change within this genomic region in Myco481.1, we examined the DNA upstream of *lpqW* in an initial set of Southern hybridization experiments using an *lpqW*-specific probe. We found that *lpqW* and 17 kb of DNA upstream was unaltered in Myco481.1 compared to Myco481 and the wild-type strain (data not shown), indicating that gross changes within *lpqW* itself, or in nearby upstream genes, were not responsible for the evolution of Myco481 to Myco481.1.

To specifically examine the *pimE* region of the genome in the *lpqW* mutants, two primer pairs were used in PCR amplifications of genomic DNA templates (Fig. 3A). Reactions using *pimE*-L2 and *pimE*-R2, which were designed to amplify the 5′ half of *pimE* and 100 bp upstream, gave identical products of the expected size from wild-type *M. smegmatis*, Myco481 and Myco481.1 genomic DNA. Further analysis showed that the nucleotide sequences from each amplification product were identical. However, when *pimE*-L (targeting the middle of *pimE*) and *pimE*-R (targeting 104 bp beyond the 3′ end of *pimE*) were used in PCRs, both wild-type and Myco481 gave

the expected product of 732 bp, whereas Myco481.1 gave a product that was approximately 1.4 kb larger (Fig.3Bi). This result suggested that, rather than a deletion or rearrangement, an insertion of DNA had occurred in the 3′ half of *pimE* in Myco481.1. Sequence analysis of the 2.1-kb PCR product revealed an insertion 209 bp from the 3′ end of *pimE* and a concomitant duplication of the sequence CGT, a finding consistent with the incorporation of a transposable element (Fig.3Bii). The inserted sequence was found to correspond exactly to the insertion sequence (IS) element ISMsm1 identified during the sequencing of the *M. smegmatis* genome at the J. Craig Venter Institute (JCVI; http://www.jcvi.org).

Examination of the 1.4-kb ISMsm1 element showed it to be a member of the IS*3*/IS*911* family and flanked by 27-bp imperfect inverted repeats containing 5-bp mismatches in the central region. The element terminated with 5′-TG-3′, as is normal for elements of this class and carried two genes, designated *orfA* and *orfB*, encoding transposition functions. Inspection of the *M. smegmatis* genome revealed three copies of ISMsm1, with the two transposition genes annotated as MSMEG_1728/1730, MSMEG_2818/2819, and MSMEG_5095/5096. MSMEG_5095 is a truncated *orfB*, suggesting that this copy of ISMsm1 may be defective. Searching of the completed genomes available at the JCVI showed that ISMsm1 is restricted to *M. smegmatis*, *Corynebacterium jeikeium* strain K411, and two strains of *Mycoplasma hyopneumoniae*.

When mapped onto a predicted membrane topology model for PimE, the site of insertion of ISMsm1 in *pimE* corresponds to the second last extracytoplasmic loop of the translated protein (Fig. 3C). Translation of the fused *pimE*-ISMsm1 sequence in silico revealed that Myco481.1 should produce a truncated PimE in which Leu-343 is followed by 10 residues (NRPGLDGDLG) encoded by the IS element. Thus, we predict that the IS insertion resulted in the loss of transmembrane domains 8 and 9, along with the last cytoplasmic loop and final extracytoplasmic loop of PimE. This genetic event is the likely cause of the defect in AcPIM4 processing observed in Myco481.1 (Fig. 2) (13).

Evolution of Myco721 to Myco721.1 results from two point mutations in the *pimE* gene. Myco721, the *lpqW* mutant created by targeted insertion of a kanamycin resistance cassette (Fig. 3A) has an unstable phenotype similar to that observed for Myco481, giving rise to a derivative, Myco721.1, in which the LAM biosynthetic defect is reversed and PIM4 accumulates at the expense of higher PIM synthesis during subculture in complex medium (13). However, unlike Myco481.1 in which higher PIMs are absent, AcPIM6 and Ac_2PIM6 still accumulate in Myco721.1 but at dramatically reduced levels, suggesting a different kind of *pimE* defect in this strain (13).

To identify the putative *pimE* lesion, PCRs were performed with the same primer pairs described above to amplify the 5′ and 3′ halves of *pimE* (Fig. 3A). Genomic DNA templates from the wild-type, Myco721, and Myco721.1 strains were found to amplify PCR products of the same sizes using these primers, demonstrating the absence of IS elements, large deletions, and rearrangements. The sequence of *pimE* from Myco721 was found to be identical to that of the wild-type gene. However, Myco721.1 contained two nucleotide changes separated by just 9 bp (Fig.3Biii): one modified the codon GGC, coding for glycine, to AGC, coding for serine (G163S), while the other converted AAT to ACT (N166T). These residues are located at the interface between the proposed second cytoplasmic loop and third transmembrane domain of the *M. smegmatis* homolog of PimE (Fig. 3C).

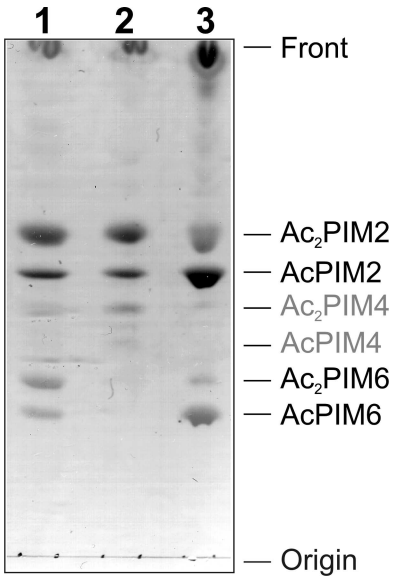

FIG. 4. PIM analysis after complementation of Myco721.1 with an extrachromosomal *pimE* gene. Plasmid pYAB53 (Y. Morita, unpublished data) enables *pimE* expression under the control of the *Mycobacterium bovis* BCG *hsp60* promoter (25). PIMs were extracted from cells using organic solvents, purified, resolved by HPTLC, and visualized by orcinol staining. Lane 1, *M. smegmatis* mc^2155; lane 2, Myco721.1; lane 3, Myco721.1(pYAB53). Major PIM species are indicated in relation to the sample origin and solvent front.

Introduction of a plasmid expressing *pimE* normalizes the PIM profile of Myco721.1. To test our hypothesis that disruption of *pimE* was responsible for the evolution of the *lpqW* mutants, a plasmid-encoded *pimE* gene (pYAB53, expressing a functional PimE-HA [influenza HA epitope] fusion protein [Y. Morita, unpublished data]) was electroporated into Myco481.1 and Myco721.1. Transformation of Myco481.1 with pYAB53 failed to yield any colonies, suggesting that the *pimE* gene was lethal in this background. However, transformation of Myco721.1 was successful. Introduction of pYAB53 resulted in an enfeebled strain that could be cultured on solid complex and defined media but not in liquid broth, limiting our ability to reliably analyze components of the PIM/LAM biosynthetic pathway. This strain, designated Myco721.1(pYAB53) was cultured on agar plates alongside wild-type and Myco721.1 control strains and cells scraped from the plate surface were analyzed for PIM content. As shown in Fig. 4, the wild-type strain displayed a normal complement of PIM species dominated by acylation variants of PIM2 and PIM6, whereas Myco721.1 synthesized dramatically reduced levels of polar PIMs, particularly AcPIM6 and Ac_2PIM6, and accumulated AcPIM4 as previously described (13). Introduction of pYAB53 into Myco721.1 restored the wild-type PIM profile to this strain, with PIM6 acylation variants clearly evident (Fig. 4, lane 3), demonstrating that the absence of a functional *pimE* was responsible for the PIM biosynthetic defect in Myco721.1.

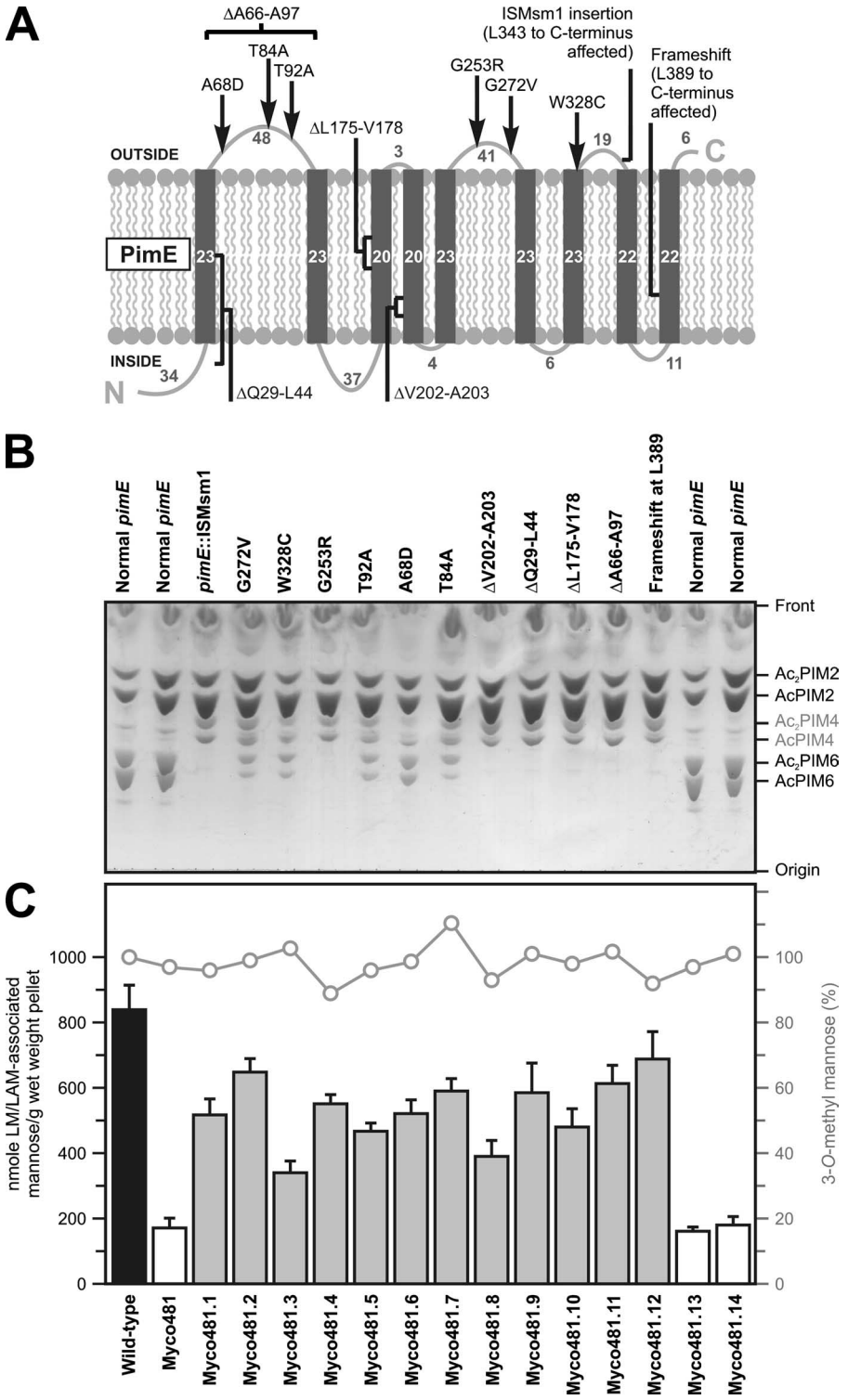

FIG. 5. Phenotypic analysis of Myco481-derived *pimE* mutants. The *lpqW* mutant Myco481 was serially subcultured through LB broth to generate a number of derivative strains with normal colony morphology on LB agar. (A) *pimE* was sequenced from each strain to identify mutations, and these were mapped to the proposed membrane topology model of PimE, predicted by using the TMHMM server version 2.0. The number of amino acid residues in each proposed transmembrane domain or loop are indicated. (B) PIMs were extracted from the *pimE* mutants in organic solvents, purified, resolved by HPTLC, and visualized by orcinol staining. Major PIM species are indicated in relation to the sample origin and solvent front. The status of each *pimE* gene is shown above each lane, and the strains correspond to the lanes underneath that form panel C. (C) LM/LAMs were extracted from the *pimE* mutants and quantified by GC-MS. The amount of LM/LAM-associated mannolipids in each variant strain (▨) is shown alongside wild type (■) and *lpqW* mutants with normal *pimE* genes (□). Sample loading was controlled by assessing the percentage of 3-*O*-methylmannose relative to the wild-type sample, as shown on the right-hand axis. Strain numbers are shown on the *x* axis, and the strains correspond to the lanes above that form panel B.

TABLE 1. Characteristics of *M. smegmatis* strains used in this study

Strain	Characteristics[a]	Genomic *pimE* mutation	PimE mutation	PIM profile	Source or reference
Wild-type	*M. smegmatis* mc^{2}155	None	None	Normal	24
Myco481	*lpqW* transposon mutant, small colonies on complex media, LAM defect, Sm^r Km^r	None	None	Normal	13
Myco481.1	Normal colony variant of Myco481, Sm^r Km^r	ISMsm1 insertion at bp 1025	Truncated at P342	Accumulates AcPIM4, no higher PIMs	13
Myco481.2	Normal colony variant of Myco481, Sm^r Km^r	G to T at bp 815	G272V	Accumulates AcPIM4, Ac/Ac$_2$PIM6 reduced	This study
Myco481.3	Normal colony variant of Myco481, Sm^r Km^r	G to T at bp 984	W328C	Accumulates AcPIM4, Ac/Ac$_2$PIM6 reduced	This study
Myco481.4	Normal colony variant of Myco481, Sm^r Km^r	G to C at bp 757	G253R	Accumulates AcPIM4, no Ac/Ac$_2$PIM6	This study
Myco481.5	Normal colony variant of Myco481, Sm^r Km^r	A to G at bp 274	T92A	Accumulates AcPIM4, Ac/Ac$_2$PIM6 reduced	This study
Myco481.6	Normal colony variant of Myco481, Sm^r Km^r	C to A at bp 203	A68D	Accumulates AcPIM4, Ac/Ac$_2$PIM6 reduced	This study
Myco481.7	Normal colony variant of Myco481, Sm^r Km^r	A to G at bp 250	T84A	Accumulates AcPIM4, Ac/Ac$_2$PIM6 reduced	This study
Myco481.8	Normal colony variant of Myco481, Sm^r Km^r	Deletion of bp 604 to 609	Deletion of V202 to A203	Accumulates AcPIM4, no higher PIMs	This study
Myco481.9	Normal colony variant of Myco481, Sm^r Km^r	Deletion of bp 85 to 132	Deletion of Q29 to L44	Accumulates AcPIM4, no higher PIMs	This study
Myco481.10	Normal colony variant of Myco481, Sm^r Km^r	Deletion of bp 523 to 534	Deletion of L175 to V178	Accumulates AcPIM4, no higher PIMs	This study
Myco481.11	Normal colony variant of Myco481, Sm^r Km^r	Deletion of bp 195 to 294	Deletion of A66 to A97	Accumulates AcPIM4, no higher PIMs	This study
Myco481.12	Normal colony variant of Myco481, Sm^r Km^r	Additional G at bp 1157	Frameshift disrupting L389 to C terminus	Accumulates AcPIM4, no higher PIMs	This study
Myco481.13	Small colony variant of Myco481, Sm^r Km^r	None	None	Normal	This study
Myco481.14	Small colony variant of Myco481, Sm^r Km^r	None	None	Normal	This study
Myco721	*lpqW*::*aphA3* mutant, small colonies on complex media, LAM defect, Km^r	None	None	Normal	13
Myco721.1	Normal colony variant of Myco721, Km^r	G to A at bp 490, A to C at bp 500	G164S, N167T	Accumulates AcPIM4, Ac/Ac$_2$PIM6 reduced	13
Myco721.1 (pYAB53)	Myco721.1 complemented with *pimE*-expressing plasmid, Sm^r Km^r	G to A at bp 490, A to C at bp 500	G164S, N167T	Normal	This study

[a] Km^r, kanamycin resistance; Sm^r, streptomycin resistance.

Functional analysis of PimE reveals an extracytoplasmic activity. Having shown that the evolution of the *lpqW* mutants occurs via the accumulation of mutations within *pimE*, we decided to exploit this phenomenon as a means to produce a panel of defective mutants and thus identify functionally important residues within PimE. New variants of Myco481 were generated by using a simplified serial subculturing method aimed at producing a heterogeneous population of Myco481 variants using LB media, similar to the population of cells seen in previous early subcultures (Fig. 2). These variants exhibited normal colony morphology on LB agar. Since the second subculture was the earliest stage in which normal colonies appeared, we collected the variants at this point in order to limit the isolation of siblings. Thirty normal-colony variants were chosen, along with two small-colony variants and a wild-type strain serving as controls. Strains were then further propagated using Middlebrook medium to ensure phenotypic stability as demonstrated previously (Fig. 2) and tested for *pimE* mutation, PIM profile, and LAM content (Fig. 5). PCR amplification and sequencing of *pimE* genes using the primers *pimE*-L2 and *pimE*-R (see Fig. 3A) showed that all strains gave a *pimE* PCR product of 1.5 kb, suggesting a lack of large IS elements, deletions, or insertions. As expected, sequence analysis of all 30 of the normal-colony variants of Myco481 revealed mutations within *pimE*, while the sequences of products derived from the small colonies and wild-type control revealed unaltered *pimE* genes. To exclude the possibility of PCR errors, all mutations were confirmed by sequencing a second, independently derived PCR product. Eleven distinct *pimE* mutations were identified from the 30 Myco481 derivative strains: six clones contained single amino acid substitutions, and four contained *pimE* deletions, while one contained a single base pair insertion resulting in a frameshift near the 3′ end of *pimE* (for details, see Table 1 and Fig. S1 in the supplemental material).

When the mutations were mapped onto the proposed membrane topology model of PimE (Fig. 5A), all amino acid substitutions mapped to the extracytoplasmic loops of the protein, while three of the four deletions were located within proposed transmembrane domains. The remaining deletion removed the majority of the first extracytoplasmic loop, whereas the frameshift was predicted to affect all residues after and including L389, located within the last transmembrane domain.

An analysis of PIM and LAM content was carried out to assess the functional importance of the mutated PimE residues. As expected, the wild-type strain, the Myco481 strain, and two representative small colonies had normal PIM profiles (Fig. 5B). In contrast, all of the *pimE* mutants accumulated PIM species previously identified as Ac_2PIM4 and AcPIM4 (Fig. 5B), the substrate of PimE (20). A spectrum of accumulation levels for AcPIM6 and Ac_2PIM6 was observed, ranging from reduced levels compared to the *pimE*-normal controls (e.g., Myco481.6) to undetectable levels of AcPIM6 and Ac_2PIM6 (e.g., Myco481.8). Several of the single-amino-acid-substitution mutants resulted in reduced but detectable polar PIMs, with the G253R strain Myco481.4 being the only example that failed to accumulate any detectable AcPIM6 and Ac_2PIM6 (Fig. 5B). The lack of metabolic products downstream of the PimE substrate in this mutant suggests that G253R is a particularly damaging mutation. As for the G253R mutation, *pimE* mutations caused by the frameshift and the four deletions all resulted in a complete loss of AcPIM6 and Ac_2PIM6 and accumulation of AcPIM4. These PIM profiles are identical to that of Myco481.1 (Fig. 5B, lane 3), in which PimE activity is apparently abolished due to disruption of *pimE* by the transposable element ISMsm1 (see above).

To determine the effects of the mutations on LM/LAM biosynthesis, the *pimE* mutants described above were analyzed for LM/LAM content. LM/LAMs were extracted from delipidated pellets, and the levels were quantified by GC-MS (Fig. 5C). The wild-type strain, Myco481, its previously characterized derivative Myco481.1 (13), and the small colony derivatives Myco481.13 and Myco481.14 with normal *pimE* genes served as controls. Myco481 contained significantly reduced (ca. 25%) LM/LAM compared to the wild type, as noted previously (13), as did the Myco481.13 and Myco481.14 controls. In contrast, all of the *pimE* mutants had significantly higher LM/LAM levels than their parent (up to 75%), although LM/LAM biosynthesis was never restored fully to wild-type levels. Interestingly, the W328C mutant Myco481.3 had significantly less LM/LAM than the other derivatives but still produced greater amounts than its parent Myco481. This amino acid substitution mapped to the interface between the seventh transmembrane domain and the fourth extracytoplasmic loop and resulted in a mild PIM biosynthetic defect (Fig. 5B). Levels of other mannoconjugates, such as polymethylmannose (20), that are unrelated to PIM, LM, and/or LAM were present at similar levels in all strains (Fig. 5C).

DISCUSSION

This study provides new insights into the function and biosynthesis of LAM, the major cell wall lipoglycoconjugate of mycobacteria. Specifically, we provide evidence that, in *M. smegmatis*, two proteins—LpqW and PimE—can regulate the flux of PIM intermediates into LAM. The functional domains of these proteins are predicted to have an extracytoplasmic orientation, indicating that key pathway branch points in PIM/LM/LAM biosynthesis occur in this compartment. Finally, analysis of *lpqW* mutants supports the notion that LM/LAM is required for normal growth of *M. smegmatis* under certain culture conditions.

Homologues of *lpqW* have been identified in all species of mycobacteria, although the precise function of the encoded protein is unknown. We showed previously that disruption of *lpqW* in *M. smegmatis* results in the selective loss of LM/LAM without affecting PIM biosynthesis. This phenotype is stable while *lpqW* mutants are grown in minimal defined medium but is unstable when the same mutants are cultured in complex medium. Specifically, growth in complex medium results in the selection of faster-growing bypass mutants that express LM/LAM at levels intermediate between the original mutant and wild type and have altered PIM profiles. Remarkably, we show that in all of the bypass mutants examined to date, the *pimE* gene is disrupted by transposon insertion, frameshift, deletion, or point mutation, resulting in partially or completely inactivated PimE, the mannosyltransferase that converts AcPIM4 to AcPIM5 (20). We propose that LpqW and PimE are therefore likely to be involved in closely related metabolic processes (Fig. 6), providing the strongest evidence to date that LpqW is directly involved in LM/LAM biosynthesis.

How might LpqW and PimE regulate LAM biosynthesis in wild-type cells? LpqW and the functional domains of PimE are predicted to have an extracytoplasmic location (16, 20; see also below) and are involved indirectly or directly in determining the fate of the branch point intermediate AcPIM4. The capping of AcPIM4 with $\alpha(1\rightarrow2)$-linked mannose residues by PimE diverts this intermediate from LM/LAM biosynthesis. Specifically, we have shown that AcPIM5/6 are not utilized as LM precursors, even when the flux through this pathway is reduced and apolar PIM species are depleted (5). In contrast, LpqW appears to be required for incorporation of AcPIM4 into the LM/LAM pathway when PimE is active. We have recently shown that the reactions in the PIM biosynthetic pathway are compartmentalized in the plasma membrane, with the early steps occurring in domains that are not associated with the cell wall, whereas later steps occur in a tightly bound plasma membrane-cell wall fraction (21). Both PimE and the $\alpha(1\rightarrow6)$ mannosyltransferases involved in assembling the mannan backbone of LM are located in the latter fraction (20, 21). It is possible that LpqW is involved in chaperoning early PIM intermediates between these domains or to the $\alpha(1\rightarrow6)$ mannosyltransferases within the plasma membrane-cell wall domain. Consistent with this hypothesis, we have recently determined the crystal structure of LpqW and shown that it contains a potential ligand-binding domain that theoretically accommodates the trimannose structure representing the AcPIM4 head group (16). Alternatively, LpqW could be involved in excluding PimE from this domain or otherwise regulating the activity of this mannosyltransferase. In the absence of LpqW, loss-of-function mutations in PimE result in the accumulation of uncapped AcPIM4, which appears to bypass the need for LpqW. Some correlation was observed between the levels of AcPIM4 in the different *lpqW* bypass mutants and the levels of LM/LAM, supporting the notion that in the absence of LpqW,

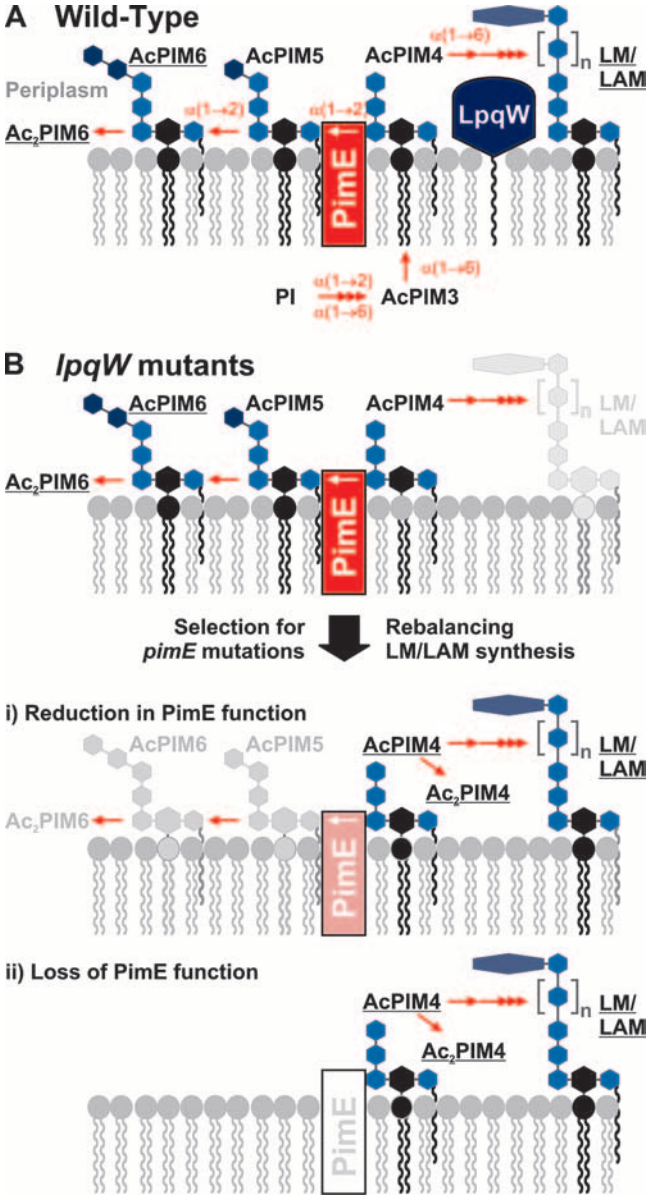

FIG. 6. Altered fluxes in the PIM and LM/LAM biosynthetic pathways in the *lpqW* mutant and derived bypass mutants. The major accumulating PIM and LAM species in wild-type and mutant strains are underlined. (A) Proposed role of LpqW and PimE in the wild type. PimE converts AcPIM4 into AcPIM5 on the extracytoplasmic face of the cytoplasmic membrane. LpqW, on the other hand, is involved in channeling AcPIM4 into the LM/LAM biosynthetic pathway (see Discussion). (B) Mutants of *lpqW* lose the ability to produce normal quantities of LAM (top panel). This is overcome through evolution in complex media, resulting from the selection of spontaneous *pimE* mutants. These mutants accumulate AcPIM4, rebalancing the biosynthetic pathway to restore LAM production. The *pimE* mutants either show partial PimE activity, where AcPIM5 production is reduced and AcPIM4 accumulates, or a complete loss of PimE function, where AcPIM5 synthesis is absent.

LM/LAM biosynthesis is dependent on high steady-state levels of AcPIM4 (Fig. 5). Similarly, expression of functional PimE in the Myco721.1 bypass mutant depleted this new pool of AcPIM4 and reversed the fast-growth phenotype of the bypass mutant. AcPIM4 accumulates to high levels in the *pimE* mutant, although no increase in LM/LAM over wild-type levels was observed in this strain (20). It is possible that in the presence of LpqW, LM/LAM synthesis is regulated by the activity of the elongating α(1→6) mannosyltransferases. Alternatively, LpqW may both promote and restrict the entry of AcPIM4 into this pathway.

Genes involved in initiating PIM biosynthesis are essential for the viability of mycobacteria, indicating that the PIMs, LMs, and/or LAMs are essential for viability and growth (1, 10, 14). However, the function of each of these components and the extent to which the roles of one can compensate for the loss of an other has not been clearly defined. Our analyses of *lpqW* mutants shows that LM/LAM is required for optimal growth in nutritionally complex medium. This was indicated by the altered colony morphology of the initial mutant and the reproducibility with which this mutant was overgrown by the *pimE* bypass mutants. The fact that all of the bypass mutants contained mutated *pimE* genes indicates that the growth phenotype of the *lpqW* mutant was directly related to its defect in LM/LAM synthesis. In contrast, colony morphology of the *lpqW* mutant grown on defined medium was similar to wild-type bacteria during continuous subculture. These data indicate that LM/LAM is not essential for optimal growth in this minimal defined medium. The appearance of bypass mutants occurs within a few subcultures, indicating a strong selective advantage in having restored LM/LAM synthesis. A high rate of LM/LAM synthesis may be required to stabilize the mycobacterial cell wall under the specific (i.e., osmotic) conditions encountered in the complex medium. Regardless, these studies highlight the possibility that LM/LAMs (and other cell wall components) are functionally important under some, but not all, growth conditions. The importance of LM/LAM appears to vary in different members of the mycobacteria and corynebacteria. For example, the *M. tuberculosis lpqW* homologue (Rv1166) is essential under standard culture conditions (23). On the other hand, we recently generated a *Corynebacterium glutamicum* mutant that is unable to synthesize AcPIM2 and consequently polar PIMs, LMs, and LAMs, by targeted disruption of the newly identified PimB′ gene (14). However, this bacterium synthesizes a second family of LM-like molecules that are assembled on a GlcA-diacylglycerol rather than a PI-lipid anchor, and this LM may compensate for the loss of PIM and PI-anchored LM/LAM in this mutant. Interestingly, the genes required for the synthesis of GlcA-diacylglycerol anchor are present in *M. smegmatis*, but we found no evidence that this pathway is upregulated in either Myco481 or Myco721 or the corresponding bypass mutants.

The *lpqW* mutant provided a unique experimental system for generating *pimE* mutants with reduced or abolished mannosyltransferase function. These analyses provided the first evidence that ISMsm1, a putative mobile element identified during the sequencing of the *M. smegmatis* genome, is an active transposon capable of driving the evolution of *M. smegmatis*. Analysis of the *M. smegmatis* genome has revealed several potential new elements, but it is not known whether these are functional for transposition or remnants of ancient elements. Thus, ISMsm1 could be modified and exploited for future mutagenesis studies on *M. smegmatis* or related species. These analyses also indicated domains within PimE that are essential for mannosyltransferase

function. All of the single-amino-acid mutations in PimE were mapped to hydrophilic loops that are predicted to be extracytoplasmic. Such an orientation for the catalytic residues of PimE is consistent with the fact that this enzyme utilizes the lipid-linked sugar donor, polyprenyl-phospho-mannose (20), a characteristic feature of glycosyltransferases in the exoplasmic leaflet of the prokaryote plasma membrane or the inner leaflet of the eukaryotic endoplasmic reticulum or Golgi membrane. Intriguingly, none of the identified mutations in *pimE* are predicted to result in the complete loss of the protein. Hypothetical translation of the mutated genes shows that all of the deletions are in-frame and should allow most of PimE to be translated normally. Even the two most disruptive mutations, the frameshift affecting all residues from L343 onward (Myco481.12) and the ISMsm1 insertion (Myco481.1), are near the 3′ end of *pimE* and should allow most of the gene to be translated. If complete loss of PimE function provided a competitive advantage to the *lpqW* mutant, we would anticipate finding frameshifts and/or transposon insertions near the 5′ end of the gene. The absence of such events suggests that mutations that abolish or limit the enzymatic activity of PimE, while still allowing expression of the N-terminal domain of the protein, are being selected. The continued translation of PimE may be required to maintain an enzyme complex in the biosynthetically active plasma membrane-cell wall domain (20, 21). Alternatively, PimE might have other functions unrelated to PIM biosynthesis that are important for normal growth.

In summary, our analyses of the *lpqW* bypass mutants suggest that both LpqW and PimE are involved in regulating LM/LAM biosynthesis in *M. smegmatis*. Whether this is also true for pathogenic species such as *M. tuberculosis* remains to be determined. Both proteins may indirectly or directly regulate the accessibility of the key branch point intermediate, AcPIM4, to elongating $\alpha(1\rightarrow6)$ mannosyltransferases. These studies are the first to identify proteins involved in regulating the flux of early PIM intermediates into polar PIMs or LM/LAMs and in balanced cell wall assembly under different growth conditions. Given that LM/LAM synthesis is essential for the infectivity of pathogenic species of mycobacteria, the dissection of these steps may facilitate the identification of new drug targets.

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