MmpL11 Protein Transports Mycolic Acid-containing Lipids to the Mycobacterial Cell Wall and Contributes to Biofilm Formation in Mycobacterium smegmatis

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Background: The role of the MmpL11 transporter in mycobacteria is not understood.

Results: Mycobacterium smegmatis mmpL11 mutants accumulate mycolic acid precursors and fail to transport monomeromycolyl diacylglycerol and mycolate ester wax to the bacterial surface.

Conclusion: MmpL11 contributes to mycobacterial cell wall biosynthesis.

Significance: MmpL11 plays a conserved role in mycobacterial cell wall biogenesis that is important for M. tuberculosis virulence.

A growing body of evidence indicates that MmpL (mycobacterial membrane protein large) transporters are dedicated to cell wall biosynthesis and transport mycobacterial lipids. How MmpL transporters function and the identities of their substrates have not been fully elucidated. We report the characterization of Mycobacterium smegmatis MmpL11. We showed previously that M. smegmatis lacking MmpL11 has reduced membrane permeability that results in resistance to host antimicrobial peptides. We report herein the further characterization of the M. smegmatis mmpL11 mutant and identification of the MmpL11 substrates. We found that biofilm formation by the M. smegmatis mmpL11 mutant was distinct from that by wild-type M. smegmatis. Analysis of cell wall lipids revealed that the mmpL11 mutant failed to export the mycolic acid-containing lipids monomeromycolyl diacylglycerol and mycolate ester wax to the bacterial surface. In addition, analysis of total lipids indicated that the mycolic acid-containing precursor molecule mycolyl phospholipid accumulated in the mmpL11 mutant compared with wild-type mycobacteria. MmpL11 is encoded at a chromosomal locus that is conserved across pathogenic and nonpathogenic mycobacteria. Phenotypes of the M. smegmatis mmpL11 mutant are complemented by the expression of M. smegmatis or M. tuberculosis MmpL11, suggesting that MmpL11 plays a conserved role in mycobacterial cell wall biogenesis.

Tuberculosis infections are one of the leading causes of death due to infectious disease. The World Health Organization estimated that the mycobacterial cell wall plays a crucial role in mycobacterial intrinsic resistance to external stresses and antibiotics. Cell wall lipids contribute to mycobacterial biofilm formation and have immunomodulatory properties that are essential to the infectious strategy of pathogenic mycobacteria. The composition and architecture of the mycobacterial cell wall are unique. The outer membrane contains an inner leaflet of very long chain mycolic acids, covalently bound to the arabinogalactan-peptidoglycan layer, and an outer leaflet composed of noncovalently associated lipids such as trehalose 6,6'-dimycolate (TDM), glycopeptidolipid (GPL), phthiocerol dimycocerosate (PDIM), and sulfolipids. Mycolic acids are β-hydroxyl fatty acids with an α-alkyl side chain and require the fatty acid synthase systems FAS-I and FAS-II and the polyketide synthase Pks13 for synthesis. The resulting mycolic acid is transferred as trehalose monomycolate (TMM) to the outer leaflet of the bacterium, where it is a precursor for mycolyl arabinogalactan and TDM. Although mycolic acid side chains differ in length and oxygenation between mycobacterial species, heterologous expression of core enzymes demonstrates that their biosynthesis is largely conserved between M. tuberculosis and the fast-growing nonpathogenic species M. smegmatis.

Microbial biofilms are defined as communities of microorganisms that range from surface-attached colonies to well-developed pellicles formed at the air-liquid interface. Bacteria within biofilm communities are typically associated with a complex architecture of extracellular material that contains secreted molecules such as polysaccharides, lipids, proteins, and DNA. This matrix provides a physical barrier to environmental stresses and allows for the emergence of a drug-tolerant phenotype.
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phenotype. As such, the ability to form biofilms is associated with virulence in a number of bacterial pathogens. Although the presence and role of biofilms during M. tuberculosis infections remain unclear, biofilm formation by M. smegmatis and other environmental species has been established. The extracellular matrix associated with mycobacterial biofilms is notably rich in lipids. Free mycolic acids are associated with the formation of biofilms by M. smegmatis and M. tuberculosis (10, 11). In M. smegmatis, free mycolic acids are liberated from TDM by the hydrolase MSMEG_1529 and are linked to drug resistance in vitro (10, 12). Mutants lacking MSMEG_1529 or that are defective in mycolic acid biosynthesis have impaired biofilm formation. In M. smegmatis and Mycobacterium avium, GPLs also contribute to biofilm formation (13, 14). Reduced biofilm formation has been demonstrated in M. smegmatis lsr2 mutants, and this phenotype was attributed to a lack of mycolyl diacylglycerol (15).

The mycobacterial cell wall is a rich area for research, and biochemical and genetic approaches are being employed to further elucidate the mechanism of cell wall biosynthesis. In mycobacteria, MmpL (mycobacterial membrane protein large) proteins appear to be dedicated to the export of cell wall lipid constituents. MmpL3 was recently demonstrated to be the transporter responsible for delivery of TMM to the mycobacterial surface (16). MmpL3 is predicted to be essential in M. tuberculosis, and its essentiality was demonstrated in M. smegmatis. This is perhaps unsurprising because TDM biosynthesis and incorporation into the mycobacterial cell wall are essential for mycobacterial viability (17, 18). M. smegmatis mutants lacking the MmpL4a and MmpL4b (originally named TmpkB and TmpkC) transporters do not have GPLs on their surface. These mutants, along with mutants lacking the accessory protein MmpS4, have altered colony morphology and reduced sliding motility and biofilm formation (13, 19). In M. tuberculosis, MmpL7 and MmpL8 play respective roles in PDIM and sulfolipid export to the outer leaflet of the cell wall and are required for full virulence in a mouse model (20–24). Although substrates for M. tuberculosis MmpL4, MmpL5, and MmpL11 have not yet been described, data suggest that they also contribute to M. tuberculosis virulence (22, 25). Two independent studies found that an M. tuberculosis mmpL11 mutant is attenuated in a mouse model of infection; however, neither group determined the substrate of MmpL11 or defined the mechanism by which their mutant was attenuated (22, 25).

Our efforts have focused on characterization of the MmpL11 transporter. We showed that the loss of MmpL11 reduces the membrane permeability of M. smegmatis (26). In this work, we demonstrate that the M. smegmatis mmpL11 mutants had differences in cell wall lipid composition compared with wild-type mycobacteria. Specifically, mutant bacteria were unable to transport monomerozymacyl diacylglycerol (MMDAG) and mycolate ester wax to the bacterial surface. In addition, the mycolic acid precursor molecule mycolyl phospholipid (MycPL) accumulated in the absence of MmpL11. MmpL11 is encoded at the MmpL3/MmpL11 chromosomal locus that is conserved across mycobacteria, including Mycobacterium leprae. Expression of the M. tuberculosis MmpL11 protein complemented the M. smegmatis mmpL11 mutant phenotypes. Our data are consistent with a model in which MmpL11 plays a conserved role in mycobacterial cell wall biology and can function across species.

EXPERIMENTAL PROCEDURES

Maintenance of Bacterial Cultures and Cells—M. smegmatis mc2155 was obtained from American Type Culture Collection. Mycobacterial strains were maintained in Middlebrook 7H9 liquid medium (Difco) or on Middlebrook 7H11 agar plates (Difco) supplemented with 10% albumin/dextrose/saline. When required, cultures were incubated with the antibiotics kanamycin (25 μg/ml) and hygromycin (75 μg/ml). Planktonic M. smegmatis strains were grown in 7H9 liquid medium containing oleic acid/albumin/dextrose/catalase supplements and the detergent Tween 80 with shaking.

For biofilm growth, M. smegmatis was grown in polystyrene Petri dishes at 30 °C in modified Sauton’s medium without Tween 80. Sauton’s medium contained 0.5 g/liter K2HPO4, 0.5 g/liter MgSO4, 4.0 g/liter 1- asparagine, 0.05 g/liter ferric ammonium citrate, 4.76% glycerol, and 1.0 mg/liter ZnSO4 at a final pH of 7.0. Pellicle formation in borosilicate glass tubes was observed when M. smegmatis was grown in 7H9 medium without shaking at 37 °C for several days. To enumerate bacteria grown under biofilm-inducing conditions, Tween 80 was added to a final concentration of 0.5% to promote dissociation of biofilm-associated bacteria. The pellicle was mechanically disrupted, and the bacteria were completely dispersed by syringing through a tuberculin syringe. Serial dilutions were performed in PBS containing 0.1% Tween 80 and plated in Middlebrook 7H10 medium.

Mycobacterial Strains and Construction of Complementation Plasmids—The isolation of the M. smegmatis mmpL11 mutant GP02 was described previously (26). For complementation, the M. smegmatis mmpL11 gene was amplified using primers MSMMpl11NdeI (CATATGATGGCTTGAAGCAGC) and MSMMpl11H3 (AAGCTTGGCTTCTCACAGTTG) and cloned into pVV16 (27) in frame with a C-terminal His6 affinity tag. For heterologous complementation with M. tuberculosis mmpL11, the gene was amplified from M. tuberculosis CDC1551 genomic DNA using primers RvMpl11H1 (ATCATGATGGCCTTGAGCCGAACCTG) and RvMpl11H3 (ATACAAGCTTCCGCTGCTCAAAATCGCC) and cloned into pVV16 in frame with a C-terminal His6 affinity tag. All plasmid constructs were sequence-verified.

Scanning Electron Microscopy—M. smegmatis biofilms were cultured in a 24-well plate as described above. At the time of harvest, the medium below the pellicle was removed with a tuberculin syringe and replaced with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.4 (Electron Microscopy Sciences). A 5-mm polystyrene coverslip was used to capture a portion of the pellicle that had been in contact with the fixative. Samples were dried and subsequently gold-sputtered (PELCO 91000 sputter coater) and imaged using a Sirion XL30 scanning electron microscope (FEI, Portland, OR) at the Portland State University Center for Electron Microscopy and Nanofabrication.

Lipid Isolation and Analysis—To harvest total lipids, cultures were harvested by centrifugation and resuspended in chloro-
form/methanol (2:1, v/v). Following extraction, total lipids were dried under N₂ gas. Apolar lipids were harvested as described (11). Briefly, total lipids were resuspended in 5 ml of methanol and 0.3% NaCl (10:1, v/v) and 2.5 ml of petroleum ether. Samples were rocked for 30 min at room temperature; after centrifugation, the upper layer containing the apolar lipids was retained and dried under N₂ gas.

Surface lipids were extracted from \textit{M. smegmatis} biofilms as described previously (20). Briefly, biofilms grown in polystyrene dishes were harvested, resuspended in 5 ml of hexane, and then sonicated for 5 min. After centrifugation at 3000 rpm for 5 min, the hexane-extracted lipids were dried under N₂ gas.

The above total, apolar, and surface lipids were resuspended in chloroform/methanol (2:1, v/v), and equivalent amounts were spotted onto TLC plates. TDM, MycPL, and TMM were resolved by TLC in a chloroform/methanol/ammonium hydroxide (80:20:2, v/v/v) solvent system (28). Free mycolates were resolved by TLC in a chloroform/methanol/distilled H₂O (90:10:1, v/v/v) solvent system. Meromycolyl diacylglycerol and triacylglycerol were resolved by TLC in a toluene/acetone (99:1, v/v) solvent system. The TLC plates were visualized by spraying with 5% molybdophosphoric acid in ethanol and charred. Lipids were also purified from silica plates (Analtech) using standard preparative TLC procedures.

Radiolabeled lipids and mycolic arabinogalactan were isolated as described (29). Briefly, \textit{M. smegmatis} strains were grown to mid-log phase and then radiolabeled with 1 μCi/ml [¹⁴C]sodium acetate ([1,2-¹⁴C]acetic acid sodium salt, specific activity of 100–120 mCi/μmol, American Radiolabeled Chemicals). At each time point indicated, the cells were pelleted, resuspended in chloroform/methanol (2:1, v/v), and sonicated. TDM, MycPL, and TMM were resolved by TLC using a chloroform/methanol/ammonium hydroxide (80:20:2) solvent system. Mycolic arabinogalactan was isolated from the delipidated cells and analyzed as mycolic acid methyl esters (MAMEs) as described previously (22). The MAMEs were separated by TLC by running the plate five times using a petroleum ether/diethyl ether (9:1, v/v) solvent system. To detect radiolabeled lipids, TLC plates were exposed to a phosphor screen (GE Healthcare) and visualized using a STORM imager (GE Healthcare). Densitometry on the lipid profiles was performed using NIH Image software. The mean intensities of TDM and mycolyl arabinogalactan were normalized to the total signal from each sample, and the means ± S.D. from three independent experiments were graphed. This normalization ensured that each sample was controlled internally rather than in comparison with other samples and compensated for the reduced radiolabel incorporation that we routinely observed for the \textit{mmpL11} mutant.

[¹⁴C]Acetate Uptake Assay—\textit{M. smegmatis} strains were grown to mid-log phase and then radiolabeled with 1 μCi/ml [¹⁴C]sodium acetate. These conditions were chosen to mimic the conditions used for lipid incorporation. At the indicated time points, the cells were pelleted, washed twice with PBS, and counted in a liquid scintillation counter.

Mass Spectrometry—Both electrospray ionization (ESI) high-resolution (\( R = 100,000 \) at \( m/z \) 400) and low-energy collisionally activated dissociation tandem mass spectra were acquired with a Thermo Scientific LTQ Orbitrap Velos mass spectrometer with an Xcalibur operating system. Total lipid extracts were fractionated using a CHROMABOND Sep-Pak amino column (Merck) (Macherey-Nagel, Duren, Germany) as described previously (15, 16). Fractions were loop-injected onto the ESI source, where the skimmer was set at ground potential, the electrospray needle was set at 4.5 kV, and the temperature of the heated capillary was 275 °C. The automatic gain control of the ion trap was set to 5 × 10⁶, with a maximum injection time of 400 ms. Helium was used as the buffer with collision gas at a pressure of 1 × 10⁻³ millibars (0.75 millitorrs). The MS² experiments were carried out with a relative collision energy ranging from 30 to 40% and with an activation \( q \) value at 0.25. The activation time was set at 10 ms. Mass spectra were accumulated in the profile mode. The MALDI-TOF spectra of the total lipid and fractionated lipid extracts were acquired in the reflector mode using a Voyager-DE STR mass spectrometer (Applied Biosystems) equipped with a 337-nm nitrogen laser and delayed extraction. The final mass spectra were from an average of 5–10 spectra, in which each spectrum was a collection from 200 laser shots. 2,5-Dihydroxybenzoic acid was used as the matrix.

RESULTS

\textit{M. smegmatis} Mutant \textit{mmpL11} Forms Altered Biofilms—We demonstrated previously that the \textit{M. smegmatis} \textit{mmpL11} transposon mutant GP02 is hyper-resistant to host antimicrobial peptides and has decreased membrane permeability relative to wild-type \textit{M. smegmatis} (26). Our further characterization of the mutant indicated that there was no difference in the growth rate of wild-type \textit{M. smegmatis} and GP02 grown planktonically in 7H9 medium containing the detergent Tween 80 to prevent clumping (Fig. 1A). However, we noted differences when the two strains were cultivated in standing culture in medium lacking Tween 80. Surfactants and detergents are known inhibitors of biofilm formation (30, 31). Wild-type \textit{M. smegmatis} grown under biofilm-inducing conditions formed a pellicle or film at the air-liquid interface 3 days post-inoculation that developed further into a reticulated biofilm (Fig. 1B). The \textit{mmpL11} mutant GP02 had delayed pellicle formation relative to wild-type \textit{M. smegmatis} upon standing culture in borosilicate glass tubes. At later time points, the \textit{mmpL11} mutant defect in biofilm formation was seen as a reduced ability of the pellicle to attach and extend upwards along the glass tube (Fig. 1B, middle and lower).

The defect in biofilm formation is better appreciated when cultured under biofilm-inducing conditions in polystyrene plates. The \textit{mmpL11} mutant GP02 formed a less textured, or reticulated, biofilm compared with wild-type and complemented strains (Fig. 1D). We also noted that the pellicle formed by the \textit{mmpL11} mutant was easily disturbed upon handling either the standing cultures in glass tubes or polystyrene plates. In contrast, the pellicle formed by wild-type or complemented strains remained intact.

To ensure that reduced biofilm formation did not result from reduced bacterial numbers in the mutant biofilm cultures, biofilms were mechanically disrupted, and the number of bacteria were determined by plating serial dilutions. This enumeration, which included planktonic bacteria at the bottom of the culture
tube and biofilm-associated bacteria, indicated that there was no significant difference in the number of bacteria present in each culture (Fig. 1C). Therefore, replication of the mmpL11 mutant is not impaired in 7H9 medium lacking Tween 80.

The GP02/pVV16Sm complemented mutant formed a robust biofilm that initiated sooner than compared with the wild type (Fig. 1B, upper). We believe that this phenotype likely resulted from our complementation strategy, in which mmpL11 is expressed constitutively from the hsp60 promoter of the pVV16 plasmid. Quantification of mmpL11 expression by quantitative RT-PCR indicated that there was 8-fold more mmpL11 transcript in the GP02/pVV16Sm4 complemented strain relative to wild-type M. smegmatis (mc²155/pVV16) (data not shown).

Scanning electron microscopy analysis of M. smegmatis biofilms revealed reduced extracellular material surrounding the mmpL11 mutant bacteria compared with the wild-type and complemented strains (Fig. 1E). Consistent with the inability of GP02 to form a textured biofilm, the mmpL11 mutant samples appeared flatter and less structured than wild-type biofilms. At the higher magnification, distinct mmpL11 mutant bacteria were discernible, whereas the wild-type and complemented bacteria were enveloped in extracellular material and closely associated with one another.
Development of mycobacterial biofilms is promoted in the presence of iron and copper (31, 32). At least 1 µM ferrous sulfate is required for *M. smegmatis* biofilm formation, and our standard biofilm culture medium contains 178 µM iron. Addition of 100 µM copper sulfate to the biofilm culture medium did not restore wild-type biofilm formation of the *mmpL11* mutant (data not shown). These results indicate that the inability of the *mmpL11* mutant to form biofilms is not a result of metal ion deprivation.

*MmpL11 Transporter Function Is Likely Conserved across Nonpathogenic and Pathogenic Mycobacteria*—*MmpL11* is present in a conserved chromosomal locus in nonpathogenic and pathogenic mycobacteria, and the *M. smegmatis* protein MmpL11 shares 69% identity with the *M. tuberculosis* protein MmpL11. To determine whether MmpL11 function is conserved among mycobacteria, we assessed whether the *M. tuberculosis* *mmpL11* gene could complement the *M. smegmatis* *mmpL11* mutant. *M. tuberculosis* MmpL11 restored both texted biofilm formation (Fig. 1D) and levels of extracellular matrix material to those of the wild type (Fig. 1E), consistent with conserved MmpL11 function.

**The Cell Wall Lipid Composition Is Altered in the *M. smegmatis mmpL11* Mutant**—The reduced cell wall permeability and impaired biofilm formation suggested that the *mmpL11* mutant had altered cell wall lipid composition compared with wild-type *M. smegmatis*. GPLs and free mycolic acids are required for *M. smegmatis* biofilm formation (10, 11, 13, 33). Impaired biofilm formation by the *mmpL11* mutant GP02 suggested that these surface-associated lipids might be absent. Therefore, lipids from biofilm-grown cultures of *M. smegmatis* mc^{2}^{155} and the *mmpL11* mutant GP02 were examined by TLC. GPLs were present in total lipid samples extracted from the wild type and the *mmpL11* mutant (Fig. 2A). To examine free mycolates, apolar lipids from biofilm cultures were examined as described previously (11). No significant differences were observed in the presence of free mycolates between the wild-type, *mmpL11* mutant, and complemented strains (Fig. 2B). MS analysis also confirmed that there were no differences in the composition of GPLs or free mycolates (supplemental Figs. S1 and S2). To identify other potential differences in lipid composition, total and surface-exposed lipids were extracted and analyzed by TLC using a range of solvent polarities. We noted the absence of apolar lipids A–C in the hexane-extracted lipids of the *mmpL11* mutant compared with wild-type *M. smegmatis* and the complemented strain using the toluene/acetone (99:1, v/v) solvent system (Fig. 2C).

Following preparative TLC purification of these lipids, structural analysis with high-resolution multiple-stage MS identified lipids B and C as mycolate ester wax and MMDAG, respectively (Fig. 3 and supplemental Figs. S3 and S4) (34). However, we were unable to obtain structural information on the apolar lipid that ran at the solvent front (lipid A) using ESI/MS, MALDI-TOF-MS, or GC/MS. The data presented in Fig. 2C support a model in which MmpL11 transports MMDAG and mycolate ester wax. In the absence of MmpL11, MMDAG and mycolate ester wax were synthesized but were not transported to the surface, where they would be readily extracted by hexane treatment. Instead, these lipids were retained in the bacterium and were present only in the total lipid extract.

In addition, closer examination of the TLC analysis depicted in Fig. 2 (A and B) revealed a relative accumulation of MycPL in the *mmpL11* mutant compared with the wild-type and complemented strains. Besra *et al.* (28) identified MycPL as a mycolic acid-containing precursor molecule in *M. smegmatis* and demonstrated that the mycolic acid from MycPL incorporates into TMM and TDM using *in vitro* assays with purified cell extracts. The identity of MycPL was confirmed by MS analysis following preparative TLC purification. On the basis of our data, we suggest a working model in which MmpL11 is the dedicated transporter for MMDAG and mycolate ester wax. Furthermore, the accumulation of MycPL when MmpL11 is absent suggests that...
this mycolic acid-containing precursor molecule is also a biosynthetic intermediate for MMDAG and mycolate ester wax.

We also compared the lipid profiles of wild-type and mmpL11 mutant strains grown planktonically. When mycolic acid-containing species were examined, we observed a relative accumulation of MycPL in the mutant compared with the wild type (Fig. 4A). We quantified the relative levels of TMM, MycPL, and TDM at four time points of bacterial growth. The relative levels of MycPL were consistently higher in mid-logarithmic and late-logarithmic cultures of the mmpL11 mutant relative to the wild type (supplemental Fig. S5). In contrast, we did not observe significant levels of MMDAG or mycolate ester wax in lipids extracted from planktonic bacteria (Fig. 4B). These results are consistent with the biosynthesis and/or accumulation of these lipid classes in late stationary phase or dormancy-like conditions (35, 36).

Results from MS analysis of Sep-Pak column-fractionated lipids from wild-type mc²155/pVv16, GP02/pVv16, and GP02/pVv16mmpL11 using MALDI-TOF-MS or ESI/MS analysis indicated that TDM, TMM, GPLs, phosphatidylinositol mannosides, cardiolipin, phospholipids, and MycPL were present in the mmpL11 mutant at levels comparable with those in wild-type bacteria. MS analysis also indicated that there were no significant changes in the lipid profiles and compositions between the mmpL11 mutant and wild type (supplemental Figs. S1 and S2).

The Absence of MmpL11 Does Not Impact TMM Transport—MmpL11 and MmpL3 appear to be closely related phylogeneti-
Our data suggest that TMM transport, measured as biosynthesis of the cell wall components TDM and mycolyl arabinogalactan, is not greatly impacted by the absence of MmpL11. These results are consistent with the primary function of MmpL3 as the TMM transporter (16) and that of MmpL11 as the MMDAG and mycolate ester wax transporter.

In the course of our radiolabel experiments, we routinely observed reduced incorporation of radiolabel by the mmpL11 mutant GP02. This is consistent with our previous observation obtained using an ethidium bromide uptake assay that the reduced membrane permeability of the mmpL11 mutant is reduced compared with that of the wild type (26). We examined the uptake kinetics of [14C]acetate by intact cells and found that the rate of acetate uptake by the mmpL11 mutant was reduced by 25% relative to the wild type (Fig. 5C). These data provide further evidence that the presence of MmpL11 impacts the cell wall permeability of M. smegmatis.

**MmpL11 Transports Mycolic Acid-containing Lipids**

In this work, we have further characterized the role of MmpL11 in M. smegmatis and demonstrated that it contributes to the biogenesis of the mycobacterial cell wall and biofilm formation. In addition, heterologous complementation of the M. smegmatis mmpL11 mutant with the M. tuberculosis mmpL11 gene suggests that the function of MmpL11 transporters in cell wall biosynthesis is conserved between non-pathogenic and pathogenic mycobacteria. On the basis of the observation that the M. smegmatis mmpL11 mutant had altered cell wall permeability and biofilm formation, we hypothesized that there were differences in cell wall lipid composition. Indeed, when cell wall lipids were examined by TLC, we observed that the M. smegmatis mmpL11 mutant accumulated MycPL and lacked MMDAG and mycolate ester wax in the surface lipid profiles, indicating that the reduced biofilm formation is likely attributable to the absence of one or both of these lipid families on the cell wall surface. Biofilm formation in mycobacteria is multifactorial, and the roles of GPLs and free mycolates in biofilm formation by M. smegmatis have been demonstrated (10, 11, 13, 33). Although the role of extracellular lipids in mycobacterial biofilm formation has been established, a recent genetic screen demonstrated that M. tuberculosis biofilm formation also requires a number of additional factors that are not limited to cell wall lipid biosynthesis or transport (37). It is unknown whether homologs of these proteins also play a role in M. smegmatis biofilm formation.

Our finding that the reduced biofilm formation of the M. smegmatis mmpL11 mutant is associated with the absence of mycolate ester wax and MMDAG is consistent with an earlier report that the absence of apolar lipids in an M. smegmatis lsr2 mutant reduces biofilm formation (15). However, our thorough structural analysis of the apolar lipid, lipid species B (Fig. 2C), using high-resolution ESI with multiple-stage MS on the similar ions previously identified as mycolyl diacylglycerol confirmed that the lipid actually belongs to the mycolate ester wax family and consists of a pentatriacontanol (35:3) linked to the mycolic acid via an ester bond to form a pentatriacontatrienyl mycolate ester (Fig. 3). Interestingly, the high-resolution ESI mass spectrum profile of this new mycolate ester wax (Fig.

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**DISCUSSION**

In this work, we have further characterized the role of MmpL11 in M. smegmatis and demonstrated that it contributes to the biogenesis of the mycobacterial cell wall and biofilm formation. In addition, heterologous complementation of the M. smegmatis mmpL11 mutant with the M. tuberculosis mmpL11 gene suggests that the function of MmpL11 transporters in cell wall biosynthesis is conserved between non-pathogenic and pathogenic mycobacteria. On the basis of the observation that the M. smegmatis mmpL11 mutant had altered cell wall permeability and biofilm formation, we hypothesized that there were differences in cell wall lipid composition. Indeed, when cell wall lipids were examined by TLC, we observed that the M. smegmatis mmpL11 mutant accumulated MycPL and lacked MMDAG and mycolate ester wax in the surface lipid profiles, indicating that the reduced biofilm formation is likely attributable to the absence of one or both of these lipid families on the cell wall surface. Biofilm formation in mycobacteria is multifactorial, and the roles of GPLs and free mycolates in biofilm formation by M. smegmatis have been demonstrated (10, 11, 13, 33). Although the role of extracellular lipids in mycobacterial biofilm formation has been established, a recent genetic screen demonstrated that M. tuberculosis biofilm formation also requires a number of additional factors that are not limited to cell wall lipid biosynthesis or transport (37). It is unknown whether homologs of these proteins also play a role in M. smegmatis biofilm formation.

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3) is nearly identical to that of the mycolic acid (supplemental Figs. S1 and S2, panel f) isolated from M. smegmatis mc²155, consistent with the notion that the apolar lipids represent mycolate esters. The presence of mycolic acid substituents in the apolar lipids (spots 1 and 2 in Fig. 4 of Chen et al. (15)) was confirmed using alkaline deacylation of the purified lipids, followed by methylation and TLC analysis of fatty acid methyl ester products. The presence of mycolic acid substituents is therefore in agreement with the identification of mycolate ester wax in our analysis.

Understanding the molecular mechanisms underlying the biogenesis of the mycobacterial cell wall not only elucidates the basic biology of pathogenic mycobacteria but also identifies potential targets for antimicrobials. Studies on mycolic acids have been particularly insightful, as these lipids are essential to mycobacterial viability and contribute to M. tuberculosis pathogenicity. In addition, mycolic acid biosynthesis is a target of important anti-tuberculosis drugs. The small molecule inhibitors AU1235, BM212, and SQ109, which target MmpL3 function, were identified recently as potential novel therapeutics for mycobacteria (16, 38, 39). MmpL3 is essential, and block of the TMM transporter MmpL3 results in reduced synthesis of TDM and mycolyl arabinogalactan (16). MmpL3 plays a crucial role in mycobacterial cell wall biosynthesis by transporting TMM to the outer membrane, and our data strongly support a role for MmpL11 in cell wall biosynthesis, specifically in the transport of the mycolic acid-containing glycoconjugate MMDAG and an ester wax mycolate. That we did not see differences in the rate of TDM or mycolyl arabinogalactan biosynthesis indicates that the MMDAG and mycolate ester wax are not intermediates in the biosynthesis of either of these lipids. However, at this time, we cannot rule out that MMDAG and mycolate ester wax are intermediates in another synthesis pathway. Our model for the roles of MmpL3 and MmpL11 is depicted in Fig. 6. MmpL3 and MmpL11 are closely related phylogenetically and share 25% protein identity in M. tuberculosis. Therefore, it is compelling that both proteins appear to transport lipids with mycolic acid functional groups.

We also noted the absence of an apolar lipid (lipid A) in both the surface and total lipids extracted from the mmpL11 mutant GP02 (Fig. 2). The identity of this lipid is also of interest to us. Experiments to define the structure of this lipid and to delineate its biosynthetic pathway are in progress.

A recent report attributed heme transport to MmpL3 and MmpL11, and the genetic locus that encodes MmpL3 and MmpL11 also encodes a protein with heme-binding activity.
acts with the polyketide synthase PpsE, which is involved in PDIM biosynthesis (21, 24, 43). If this model holds true for MmpL11, we predict that MmpL11 interacts with the enzymes responsible for catalyzing the formation of MMDAG and mycolate ester wax from MycPL and other substrates. Our current efforts are focused on identifying these biosynthetic enzymes and testing the coupled biosynthesis/transport model.

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MmpL11 Transports Mycolic Acid-containing Lipids

FIGURE 6. Model depicting MmpL3 and MmpL11 function in mycobacterial cell wall biosynthesis. Data from our group and others support a model in which MmpL3 and MmpL11 are conserved mycobacterial cell wall lipid transporters. Mycolic acid biosynthesis yields the intermediate MycPL. The mycolic acid group of MycPL incorporates into TMM, which is transported by MmpL3, and into MMDAG and mycolate ester wax (WE), which are transported by MmpL11. The enzymes responsible for the biosynthesis of these lipid species from MycPL remain unknown.

Although transport of heme or heme complexes may be one function of these transporters, our data and those of others suggest that MmpL3 and MmpL11 are lipid exporters with a primary function in cell wall biosynthesis. Reduced uptake of heme or heme complexes by the mmpL11 mutant is consistent with the changes in cell wall physiology that we described here and in previous work. The mmpL11 mutant possesses reduced membrane permeability compared with the wild type using an ethidium bromide uptake assay (26). Our present study indicates reduced uptake and incorporation of radiolabeled acetate by the mmpL11 mutant compared with wild-type bacteria (Fig. 5C). Reduced membrane permeability must be taken into consideration because this phenotype likely impacts the overall ability of small molecules including nutrients to access the bacterial cytoplasm. Similarly, M. smegmatis mspA mutants that lack the major porin protein had reduced membrane permeability as measured by uptake of ethidium bromide, radiolabeled glucose, and antibiotics. Strains lacking MspA alone or in combination with other porin proteins grow slower in both rich and minimal media (41, 42).

Further investigation of MmpL transporter function will expand our understanding of how this class of proteins contributes to mycobacterial cell wall biosynthesis. It is proposed that MmpL proteins function in complex with biosynthetic enzymes to promote coordinated synthesis and transport of lipid substrates. For example, M. tuberculosis mmpL7 mutants accumulate PDIM intracellularly, and MmpL7 directly inter-
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