The Role of MmpL8 in Sulfatide Biogenesis and Virulence of Mycobacterium tuberculosis*

Received for publication, January 12, 2004, and in revised form, March 3, 2004
Published, JBC Papers in Press, March 4, 2004, DOI 10.1074/jbc.M400324200

Pilar Domenech‡, Michael B. Reed‡, Cynthia S. Dowd‡, Claudia Manca‡, Gilla Kaplan‡, and Clifton E. Barry III‡‡

From the §Tuberculosis Research Section, NIAID, National Institutes of Health, Rockville, Maryland 20852 and the §Public Health Research Institute, Newark, New Jersey 07103

To study the role of MmpL8-mediated lipid transport in sulfatide biogenesis, we insertionally inactivated the mmpL8 gene in Mycobacterium tuberculosis. Characterization of this strain showed that the synthesis of mature sulfolipid SL-1 was interrupted and that a more polar sulfated molecule, termed SL-N, accumulated within the cell. Purification of SL-N and structural analysis identified this molecule as a family of 2,3-diacyl-alpha, alpha'-d-trehalose-2'-sulfates. This structure suggests that transport and biogenesis of SL-1 are coupled and that the final step in sulfatide biosynthesis may be the extracellular esterification of two trehalose 6-positions with hydroxyphthioceraric acids. To assess the effect of the loss of this anionic surface lipid on virulence, we infected mice via aerosol with the MmpL8 mutant and found that, although initial replication rates and containment levels were identical, compared with the wild type, a significant attenuation of the MmpL8 mutant strain in time-to-death was observed. Early in infection, differential expression of cytokines and cytokine receptors revealed that the mutant strain less efficiently suppresses key indicators of a Th1-type immune response, suggesting an immunomodulatory role for sulfatides in the pathogenesis of tuberculosis.

Despite more than 100 years of research, tuberculosis continues to be a serious global health problem, and the bacterial factors that facilitate the intracellular survival and pathogenesis of this disease remain largely unknown. The unique mycobacterial envelope, rich in diverse biologically active lipids, not only provides a physical barrier from environmental factors and host damage but also presents multiple lipid species that can contribute directly to the pathology of mycobacterial disease (1–3).

The genome of Mycobacterium tuberculosis (MTb) contains 12 genes that encode RND (resistance, nodulation, and cell division) proteins designated MmpL (Mycobacterial membrane protein Large) (4). These proteins are characterized by the presence of 12 transmembrane domains and two extracytoplasmic loops and have been reported in the genomes of organisms from all major kingdoms of life. In Gram-negative bacteria, these proteins facilitate the transport of a large variety of drugs, heavy metals, aliphatic and aromatic solvents, bile salts, fatty acids, detergents, and dyes (5). In Gram-positive bacteria, an ActII-ORF3 mutant (a member of the same family of proteins) in Streptomyces coelicolor has been shown to be impaired for gamma-actinorhodin production (6). In this case, both synthesis and transport of this complex polyketide were affected. The co-localization of some of the mmpL genes with genes involved in polyketide biosynthesis (pks genes) and genes involved in lipid metabolism (papA and fadD) suggests a similar role of these proteins in complex lipid transport in MTb (7, 8). Indeed, the MmpL7 protein has been shown to be involved in transport of phthiocerol dimycolates (PDIM) (9, 10).

One of the mmpL genes, mmpL8, is positioned 8 kbp downstream from the pks2 gene. Pks2 is involved in the synthesis of heptamethyl- and octamethyl-branched fatty acids (known as phthioceranic acids) present in the major sulfolipid of M. tuberculosis, SL-1 (11). Structural analysis of SL-1 was performed by Goren and co-workers (12–14), who identified it as 2-palmitoyl(stearoyl)-3-phthioceranol, 6,6′-bis(hydroxyphthioceroneryl) trehalose 2'-sulfate. The low abundance of this molecule in cultured MTb, its unique presence in the pathogenic human tubercle bacillus, and numerous experimental studies over the past 40 years strongly suggest a role for SL-1 in virulence. Some studies have reported a significant correlation between virulence of different strains of M. tuberculosis in guinea pigs and the amount of SL-1 produced by these strains cultured in vitro (15, 16). Administration of M. tuberculosis sulfatides to cultured macrophages prevents phagosome-lysosome fusion (14), although this effect has been questioned since many anionic lipids could interact similarly with cationic sites on lysosomal hydrolases with resultant immobilization and/or inactivation of the enzymes (15). A role for SL-1 in blocking human macrophage and neutrophil activation by modulation of superoxide release and secretion of IL-1beta and TNF-alpha has also been observed (17–21). In addition, in vivo and in vitro studies have shown that SL-1 and cord factor may synergize in terms of mouse toxicity and attack on mitochondrial structure and function (22). In contrast to these studies, two different groups (23, 24) have recently reported that pks2 disruption and SL-1 deficiency do not significantly affect the replication, persistence, or pathogenicity of M. tuberculosis in mice, guinea pigs, or cultured macrophages.

In this study, we confirm and extend the recent observation by Converse et al. (23) that the synthesis of SL-1 is interrupted...
in an MTb mmpL8 mutant. This mutant accumulates a more polar molecule, termed SL-N, which is a likely precursor of SL-1. As a consequence, the cell surface charge appears to have been significantly altered. Purification and extensive analytical characterization of SL-N lead us to propose that, in contrast with the structure suggested by Converse et al. (23), this molecule is a family of 2,3-diacyl-α,α′-trehalose-2′-sulfates. Finally, we show that although loss of the MmpL8 protein does not affect in vivo replication rate or bacterial numbers during chronic infection, it does attenuate virulence of MTb in murine survival studies.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Cultures, and Plasmids**—The ElectroMAX DH5α E. coli strain (Invitrogen) used for cloning was grown in Luria-Bertani medium with hygromycin (200 μg/ml) (Invitrogen) or gentamicin (Invitrogen) (5 μg/ml) when indicated. MTb strains were grown in Middlebrook 7H9 broth (Difco) supplemented with ADC (NaCl, 8.1 g/liter; bovine albumin fraction V (Calbiochem), 50 g/liter; glucose, 20 g/liter; 0.02% glycerol, and 0.05% Tween 80 (Sigma) or on Middlebrook 7H11 agar (Difco) supplemented with OADC enrichment (as ADC but including also 0.6 ml/liter oleic acid (ICN Biochemicals) and 3.6 mM sodium hydroxide). Where indicated hygromycin (50 μg/ml) or gentamicin (100 μg/ml) was added (25). H37Rv (Pasteur) was used as the parental strain of the H37Rv mmpL8:hyg mutant and H37Rv (ATCC, Manassas, VA) for the generation of the pks2:hyg.

**Nucleic Acid Techniques**—E. coli transformations, cloning, and PCR were based on standard conditions (26). Southern blotting and hybridization procedures were performed as described previously (27). Mycobacterial DNA was isolated using the protocol of Pelicic et al. (25). Transformation of MTb was carried out as described previously (28).

**Construction of the mmpL8 and pks2 Disrupted Mutants**—Generation of the MTb disrupted mutants (mmpL8::hyg and pks2::hyg) was accomplished by homologous recombination using the system developed by Pelicic et al. (25). A 2.1-kbp fragment containing the mmpL8 gene (nucleotides 538–2388) was generated by PCR and cloned into the SpeI site of the vector pCDNA1.1 (Invitrogen). A 1.6-kbp fragment carrying the hyg gene was cloned into the mmpL8 gene at the HpaI site (3052–3302) (MS/MS), (nucleotide positions 538–2388). This fragment was cloned into the vector pCRBlunt (Invitrogen), and a 1.6-kbp fragment carrying the hyg gene was substituted for a 1-kbp internal Nhel (position 4457)-BglII (position 4775) segment. The 2.6-kbp fragment containing the disrupted pks2 sequence and hygromycin resistance determinant was excised and cloned into the mycobacterial shuttle vector pR27 (25). The pks2 mutant was produced by PCR amplification of a 2-kbp fragment containing the pks2 gene (positions 3302–5310). This fragment was cloned into the vector pCRBlunt (Invitrogen), and a 1.6-kbp fragment carrying the hyg gene was substituted for a 1-kbp internal Nhel (position 4457)-BglII (position 4775) segment. The 2.6-kbp fragment containing the disrupted pks2 sequence and hygromycin resistance determinant was excised and cloned into the mycobacterial shuttle vector pR27 (25). The pks2 mutant was produced by PCR amplification of a 2-kbp fragment containing the pks2 gene (positions 3302–5310). This fragment was cloned into the vector pCRBlunt (Invitrogen), and a 1.6-kbp fragment carrying the hyg gene was substituted for a 1-kbp internal Nhel (position 4457)-BglII (position 4775) segment. Transformation of MTb was carried out as described previously (28).

**Lipid Analysis and Sulfolipid Purification**—100-ml cultures of the different MTb strains (wild type, mmpL8::hyg, and pks2::hyg mutants) were grown to an OD600 of 0.3. Metabolic labeling of the methyl-branched fatty acids was achieved by incubating these cultures in the presence of 1 μCi/ml sodium [1-14C]propionate (American Radiolabeled Chemicals, Inc.) at 37 °C for 24 h. Bacterial numbers for isolation of the sulfolipids were determined after 72 h. Bacterial-associated lipids were extracted by two different methods. Bacteria-associated total lipids were extracted using the Folch method (29), which includes two extractions with chloroform: methanol: 2:1 and three washes with chloroform, methanol, and 0.58% NaCl in water at ratios 3:4:8:7. Alternatively, separation of bacteria-associated apolar and polar lipids was accomplished by two petroleum ether extractions (apolar) prior to chloroform:methanol extractions (polar) according to the protocol described by Slyaden and Barry (30). Apolar lipids in the presence of a supernatant were extracted as described elsewhere (31). Thin layer chromatography (TLC) was performed using 250-μm silica gel 60 plates (EM Science, USA) with chloroform:methanol:water (85:25:4) as the developing solvent. TLC plates were visualized using a Storm 860 PhosphorImager (Amersham Biosciences).

For purification of SL-1 and SL-N, 4-liter cultures of H37Rv and mmpL8::hyg were labeled with 1 μCi of [35S]Na2SO4 (Amersham Biosciences, 100 mCi/mmol) as is indicated above. Bacteria-associated lipids (apolar for SL-1, polar for SL-N, prepared as above) were washed over a silica gel 60 column and eluted with 9:1 chloroform:methanol (3 volumes), 5:1 chloroform:methanol (3 volumes), and 1:1 chloroform: methanol (6 volumes). Fractions containing sulfolipids were identified by TLC and autoradiography and were then pooled and dried. Next, the pooled fractions were applied to an anion exchange column (PS-DVB DEAE, 10 mm × 100 mm, 5-μm beads, Vydac) on a Waters 2690 HPLC. The column was activated prior to injection of the sample by equilibrating with chloroform:methanol:acetic acid (800:200:0.6) at a flow rate of 2 ml/min. Elution of the lipids was done using a 0–35% gradient over 40 min with chloroform:methanol:triethylamine (800:200:1.39) as the eluting solvent. 2-ml fractions were collected and analyzed by liquid scintillation counting. A 1% sample of each fraction was spotted on GEArray membranes according to the protocol described by Soto et al. (32).

**GC-MS of Fatty Acid Methyl Esters**—Fatty acid methyl esters from purified SL-1 and SL-N were prepared following the protocol described previously (30). GC-MS was carried out on a Hewlett Packard 5890 instrument operated in splitless mode using an HP-5MS column (30 m × 0.25 mm × 0.25 μm). The injection port temperature was 310 °C, and the column temperature was ramped from 180 to 310 °C at 10 °C/min followed by an additional 15 min at 310 °C with 8 p.s.i. helium. MS and GC-MS analysis were performed on the Varian GC-MS model 3800 with the automatic liquid solvent transfer option. MS was carried out using the nanopray technique for both positive and negative modes. For the MS/MS spectra, the collision cell was pressurized with argon, and the collision energy was adjusted to give the optimal spectrum, which was −4 electron volts for positive ion scans and −70 electron volts for negative ion scans. Identifications were based on retention times and accurate mass data calibrated using sodium iodide in either the positive or negative ion mode.

**Proton and COSY NMR Spectra**—The 1H (one-dimensional) and COSY spectra of [35S]labeled SL-N were obtained on a Varian VXR-500S NMR spectrometer at 30 °C in CDCl3:CD3OD (2:1). Mouse Experiments—Prior to infection, well dispersed liquid cultures were adjusted to an OD600 of 0.5 and stored at −70 °C as 20% glycerol stocks. Inocula were prepared by diluting these stocks to 4 × 108 colony-forming units (CFU/ml) in PBS/Tween 80 (0.05%). Eight-week-old C57Bl/6 and B6D2/F1 mice (Taconic) were infected using a BioAerosol nebulizing generator (CH Technologies Inc., Westwood, NJ) with 2.5 × 106 CFU. Mice were euthanized by overdose of ether following the Kaplan-Meier method (33), and the log-rank test was used to determine statistical significance for 24 h post-infection survival studies (GraphPad Prism version 3.0; GraphPad Software, San Diego, CA).

**Cytokine Expression Studies**—At 14 days post-infection, lungs of euthanized mice (4 mice/group) were removed and immediately snap-frozen on dry ice/ethanol. Tissues were homogenized in 5 ml of RNAzol B (Cinna/BiotecX, Houston, TX) using a tissue Polytron homogenizer. Total RNA was extracted according to the manufacturer’s protocol and the concentration and purity were determined by measuring 1 μg of RNA from each sample was reverse transcribed into cDNA with Moloney murine leukemia virus reverse transcriptase (Ambion). Biotinylated cDNA probes were hybridized to microarray-containing cDNA spots spotted on GEArray membranes according to the manufacturer's direc-
Disruption of the MTb mmpL8 Gene Interrupts SL-1 Biosynthesis—Disruption of the mmpL8 gene in MTb H37Rv was accomplished by insertion of a hygromycin resistance cassette within the coding sequence of a cloned copy of this gene followed by homologous recombination of the inactivated allele onto the chromosome of MTb using a plasmid based on the pPR27 vector (temperature-sensitive mycobacterial origin of replication and sacB negative selection marker) (25). Fig. 1A shows the results of Southern blot analysis of chromosomal DNA from six clones that were HygR, SucR, and TR (lanes 2–7) and from MTb H37Rv (lanes 1 and 8) were digested with XhoI and hybridized with a 2-kbp fragment of the mmpL8 gene of MTb H37Rv. The expected sizes were 2.1 kbp for the wild-type fragment and 3.7 kbp for legitimate double recombinants. B, genetic organization of the mmpL8 locus in H37Rv and the mmpL8:hyg mutant.

RESULTS

Disruption of the MTb mmpL8 Gene Intermits SL-1 Biosynthesis—To analyze the structure of SL-N, we extracted and purified this metabolite from the culture supernatant (Fig. 2A, lanes 9–12). In the wild-type strain the majority of the [35S]Na2SO4 was incorporated into SL-1 (Fig. 2A, lanes 9 and 11), confirming that this was the most abundant sulfolipid produced in MTb. In contrast, the mmpL8 mutant incorporated [35S]Na2SO4 into a molecule that co-migrated by TLC with the propionate-labeled metabolite (Rf 0.5) (Fig. 2A, lane 10). These results suggest that inactivation of the mmpL8 gene of MTb interrupted the normal biosynthesis of SL-1 and led to the accumulation of a more polar molecule, designated SL-N, that contained both sulfate and methyl-branched fatty acids.

SL-N Is Localized inside the Cell Envelope—We compared lipids associated with bacterial cells (Fig. 2B, lanes 1–4) with those found in the culture supernatant (Fig. 2B, lanes 5–8) for wild-type and mmpL8:hyg mutant strains. SL-1 was present in both the apolar and polar lipid extracts of wild-type culture supernatant (Fig. 2B, lanes 5 and 7), whereas SL-N was not found in the supernatant from the mmpL8 mutant (lanes 6 and 8). While some SL-1 was also found in association with the bacterial cells, SL-N was found exclusively in association with the bacterial cells. This result suggests that SL-1 normally occupies a peripheral location within the mycobacterial envelope and can be shed into the culture supernatant, whereas SL-N apparently occupies a more integral location and may be exclusively retained within the cytosol.

An attempt to address whether SL-N was peripherally associated with the cell membrane but was not efficiently shed into the medium, we examined the neutral red binding ability of the mmpL8:hyg mutant. The neutral red assay has been used extensively to distinguish between avirulent and virulent strains of MTb because of its specificity for labeling cells producing SL-1 (15, 32, 34, 35). The capacity to bind this molecule has been interpreted as indicating a surface-accessible location of the sulfatides, whose strongly acidic sulfate interacts ionically with the cationic dye (36). We performed the neutral red assay following the method described by Soto et al. (32). As shown in Fig. 2C, the wild-type strain gave the expected red coloration, while the mmpL8:hyg mutant showed only a dim yellow color, supporting the conclusion that SL-N is not localized at the cell surface. Together, these experiments suggest that SL-N is not secreted to the mycobacterial cell surface and that the absence of this molecule from the cell surface results in a significant alteration in overall charge at the region of the cell envelope most likely to interact directly with the host during infection.

Purification and Acyl Group Composition of SL-N—To analyze the structure of SL-N, we extracted and purified this molecule and SL-1 from 4 liters of [35S]propionate-labeled MTb H37Rv cells and 0.11 mg of SL-N of MTb mmpL8:hyg mutant cells.

Initially, we attempted to define the acyl group composition of these two molecules by preparation of methyl esters of fatty acids resulting from saponification of SL-1 and SL-N. Analysis of the resulting fatty acid methyl esters by GC-MS showed chromatograms for SL-1 and SL-N that were remarkably sim-
ilar (Fig. 3). Both chromatograms showed major peaks with retention times of 6.14, 7.09, 8.17, 13.71, 15.24, 16.83, 18.80, 21.78, 26.00, and 32.12 min. The molecular ions of the first three peaks were $m/z$ 270, 284, and 298, respectively, corresponding to C16 (palmitate), C17 (14-methylhexadecanoic acid), and C18 (stearate) fatty acids. The remaining peaks all contained characteristic fragment ions of $\alpha$-methyl-branched fatty acids at $m/z$ 88 and 101. The presence of these peaks in both spectra confirmed that SL-N, like SL-1, contained $\alpha$-methyl-branched fatty acids consistent with the observed labeling by $[1^{-14}C]$propionate. The molecular ions found in the peaks eluting between 13 and 22 min were at $m/z$ 438, 480, 522, 564, and 606, respectively (data not shown). These molecular ions are identical to those described by Goren and co-workers (13) in the original description of the phthioceranyl acyl substituents of SL-1 corresponding to the series of total carbon length C28, C31, C34, C37, and C40 (palmitate extended by 4–8 propionates). The broad peak at 26 min suggested that there could be more than one molecule eluting at this retention time. The major high molecular weight ion observed for this peak (383 $m/z$) corre-
sponded to the methyl-branched portion of a C_{40} hydroxyphthioceranic ester (M-239), as was described previously (13). Thus this fragment was likely composed of eight propionic acid units. An associated peak at m/z 411 (M-240 + 29) was also observed, supporting the structure of this fatty acid. The molecular ion, however, observed in the spectrum (m/z 649) did not correspond to C_{40} hydroxyphthioceranic ester but to C_{43} phthioceranic ester (a palmitic moiety condensed with nine propionic acids). In fact, this molecular ion was most visible at the later eluting portion of the peak, not at the apex. Therefore, we conclude that this peak corresponded to a mixture of the C_{40} hydroxyphthioceranic methyl ester and the C_{43} phthioceranic methyl ester. The peak at 32.12 min showed a mass at m/z 425 (M-239), identical to that observed for the C_{43} hydroxyphthioceranic methyl ester, as well as an associated peak at m/z 453 (nine propionic acid moieties plus a hydroxymethylene unit) (13). No molecular ion was detected in this peak.

The presence of the same species of fatty acids in both SL-1 and SL-N was surprising given the differences in polarity of these molecules in TLC. In addition, the identification of non-hydroxylated phthioceranic acid as one of the fatty acids esterified to the non-sulfated glucose ring of trehalose and conflicts with the structure assigned previously by Converse et al. (23) of SL1278, the molecule accumulated within their mmpL8 mutant.

**Acylation of SL-N Is at C-2 and C-3 Positions of the Trehalose Ring**—The acylated positions of the trehalose were confirmed through COSY NMR analysis of purified SL-N (Fig. 5A). The anomic protons appear at δ = 6.06 and 5.25 ppm. The doublet at δ = 6.06 ppm (J = 3.8 Hz) formed a cross-peak with an upfield doublet of doublets (δ = 4.88 ppm, J = 9.8, 3.7 Hz), which corresponds to the proton adjacent to the 2'-sulfate, and was, therefore, assigned as the H-1' anomic proton, consistent with previous descriptions (38). The H-2' signal was observed to be coupled to the doublet at δ = 7.74 ppm (J = 9.3 Hz), which was therefore assigned as the H-3' proton. The cross-peaks from the H-3' signal revealed coupling to a multiplet at δ = 3.36–3.40 ppm (H-4'), the multiplet at δ = 3.51–3.55 ppm (H-5'), the multiplet at δ = 3.63–3.70 ppm (H-6'a), and the doublet of doublets at δ = 3.80 ppm (H-6'b, J = 12.1, 2.5 Hz). The multiplet at δ = 3.63–3.70 ppm and the signals between δ = 3.47 and 3.55 ppm integrated for three protons each, consistent with these assignments. The doublet at δ = 3.25 ppm (J = 3.5 Hz) is the H-1 anomic proton and showed a cross-peak with the doublet of doublets at δ = 4.83 ppm (H-2, J = 10.3, 3.6 Hz), which in turn formed a cross-peak with the doublet of doublets at δ = 3.39 ppm (H-3, J = 10.2, 9.3 Hz). From H-3, cross-peaks were found to the multiplet at δ = 3.50–3.51 ppm (H-4), the multiplet at δ = 3.89–3.93 ppm (H-5), and two protons within the multiplet at δ = 3.63–3.70 ppm (H-6a.
and H-6b). Supporting this assignment, Baer (39) reported the separation of the H-6’ a and H-6’ b protons in an unsubstituted ring, while the chemical shifts of these protons in an acylated ring overlapped (39). Integration of the H-1, H-1’, H-2, and H-3 protons was equivalent, indicating that each represented a single proton. The downfield chemical shifts of the H-2 and H-3 protons, relative to all other non-anomeric protons, indicated that these sites are acylated. These results demonstrate that, as suggested by the observation of only unsubstituted sulfoglucose in the MS/MS spectra, acylation of SL-N is exclusively at positions 2 and 3 of the non-sulfated glucose of the trehalose ring system (Fig. 5).

Lack of MmpL8 Alters the Virulence Properties of MTb—The role of MmpL8 in the virulence of MTb was studied using a low dose aerogenic murine model of infection. Two different mouse strains (C57Bl/6 and B6D2/F1) were infected with ~100 CFU of either the mmpL8::hyg mutant or the parental H37Rv strain. The average initial number of bacteria implanted in the first experiment using C57Bl/6 mice was 62 CFU/lung for H37Rv and 78 CFU/lung for the mmpL8::hyg mutant. In the second experiment with B6D2/F1 mice, the initial CFU were found to be 169 and 160, respectively. In both cases the growth kinetics of the two strains were identical throughout the infection, indicating that loss of MmpL8 did not affect initial bacterial replication or containment of this replication in the lungs and spleens of these animals (Fig. 6, A, B, and D). The 12 mice remaining in each group were observed until they died in survival experiments that extended over a period of 370 and 220 days, respectively. The first experiment demonstrated that although the mmpL8 mutant was not impaired for growth in C57Bl/6 mice, the mice infected with this strain survived longer (mean = 328 days) than those infected with the parental H37Rv strain (mean = 265 days, p value = 0.0006, Fig. 6C). This result was confirmed in the second experiment where wild-type infected mice showed a mean survival time of 154 days. Among mice infected with the mmpL8::hyg mutant, only three mice had died 220 days post-infection when the experiment was terminated (Fig. 6E). These results indicate that although MmpL8 is not required for in vivo growth of MTb, the lack of this protein significantly alters the final outcome of the infection.

Long term survival in such experiments has been correlated previously with granuloma structure, which may be influenced by the initial cytokine response (2, 3). We therefore performed a preliminary evaluation of the level of expression of indicator cytokine genes in mice early in infection. RNA was extracted from lungs of mice infected for 14 days, and cDNA was hybridized to cytokine-chemokine-specific GEArray membranes. Fig. 6F shows the ratio of genes that are overexpressed in mice infected with the parental strain at a level greater than 5 × the level observed in those infected with the mmpL8 mutant. The cytokines whose expression was most highly affected included...
IL-10 and IL-13, Th2 lymphocyte-derived cytokines that have potent anti-inflammatory properties.

**DISCUSSION**

In this study we show that the synthesis of the major sulfatide of *M. tuberculosis*, SL-1, is interrupted in an *mmpL8* mutant strain indicating that this membrane protein is involved in the transport of this molecule or its precursor. This strain accumulates a sulfolipid of lower molecular weight and higher polarity, SL-N, indicating that transport and biosynthesis of SL-1 are tightly coupled. Together with the inability of the *mmpL8::hyg* mutant to fix the cationic dye neutral red, the absence of this molecule in the culture supernatant and in the apolar extractable lipids of the mycobacterial envelope suggests that it accumulates within the cell envelope.

We have purified SL-N and established its structure in comparison with SL-1 using GC-MS, EI-MS, and COSY NMR. The results of these studies establish that SL-N is a 2,3-diacyl-α,α′-d-trehalose-2′-sulfate containing both short-chain acyl components and multimethyl-branched long-chain derivatives of either phthioceranic or hydroxyphthioceranic acid. While this study was in progress, others authors (23) also reported the accumulation of a novel sulfolipid in an independently generated mutant in the *mmpL8* gene in *Mtb*. These authors par-
tially purified this novel species by preparative TLC from cultures labeled with stable isotopes of sulfur and used Fourier transform ion cyclotron resonance mass spectrometry for characterization. They observed a series of isofoms ranging in size from m/z 1270 to 1450 and further characterized one of these occurring at m/z 1278 (designated SL1278) by exact mass measurements as a sulfated diacyl trehalose. By precedent with the established structure of SL-1, they assumed that the short-chain acyl group was located at the 2-position of the non-sulfated glucose ring of the trehalose. They stipulated that they could not assign the position of the predicted hydroxyphthioceranate residue they had proposed but suggested that it was esterified to the 6'-position of the sulfated trehalose. They therefore concluded that this molecule was likely to be 2,6'-diacyl-α,α'-O-trehalose-2'-sulfate with a palmitate group in the 2-position and a C42-hydroxyphthioceranic acid at position 6'. No experimental evidence has been shown so far confirming the presence of this C42-hydroxyphthioceranic acid in SL-1. In contrast our more complete analysis of the structures of this family of molecules suggests that the sulfated diacyl trehalose species observed at 1278 is in fact 2-palmitoyl-3-(C42)-phthioceranyl-α,α'-O-trehalose-2'-sulfate.

Perhaps more importantly from our data the major species observed are of slightly lower molecular weight and contain both phthioceranate and hydroxyphthioceranate moieties. Further, the COSY NMR demonstrates convincingly that the substitution pattern of the two acyl groups is 2,3 rather than 2,6'. However, like Converse et al. (23), we cannot conclusively state which acyl group occupies each position and again assign the shorter chain component to the 2-position based upon precedent (40). Although in SL-1 the major substituents reported at the 3-position are phthioceranic acids with hydroxyphthioceranic acids occurring at the two 6-positions, in some sulfatides these positions were interchanged (40) indicating that the acyltransferase that catalyzes the reaction at the 3-position can utilize either phthioceranic acids or hydroxyphthioceranic acids. The presence of hydroxyphthioceranic acids at the 3-position of SL-N supports this idea. It might also be reasonably anticipated that in the mmpL8::hyg mutant, unused hydroxyphthioceranic acids would accumulate and as a consequence appear overrepresented in SL-N as we observe.

Our proposed SL-N structure suggests that acylation at the 2- and 3-position of the trehalose-2'-sulfate occurs in the cytoplasm generating a 2,3-diacyl sulfo trehalose, SL-N, that is then transported through the mycobacterial membrane by the MmpL8 protein. The final addition of the two remaining hydroxyphthioceranic acids at positions 6 and 6' would then be an extracytoplasmic process. The acyltransferase involved in this step has not been identified yet, although a candidate could be the secreted protein Ag85C, which has been shown to be involved in the transfer of fatty acids to the 6- and 6'-positions of trehalose (41).

Besides the essential role of MmpL8 in synthesis of SL-1, we have also shown that mature sulfatides may play an important but subtle role in determining the outcome of infection with MTb. Although the mmpL8::hyg mutant was not impaired for the initial growth and containment phases of infection in the mouse model, highly significant differences in mouse survival times were observed in experiments performed in two different mice strains. These results suggest a qualitative difference in the nature of the host response that ultimately determines the fate of the infected animal. Similar differences have been observed with recent clinical isolates such as CDC1551 and HN878 (2, 3). The higher levels of cytokines and cytokine receptors expressed in mice infected with the wild-type strain compared with mice infected with the mmpL8::hyg mutant are consistent with a more pronounced suppression of a Th1-type immune response by the wild-type strain than by the mmpL8::hyg mutant. The simplest interpretation of this phenotype would be a direct effect of SL-1 on the murine immune system.

For more than 40 years, multiple studies have associated the level of SL-1 produced by specific strains with alterations in virulence in humans and in animal models (15–18, 20, 22, 36). These observational studies of isolates that were far from iso genetic in contrast to two recent reports that SL-1 mutants in pks2 were not growth-impaired in either mice or Guinea pigs (23, 24). In one of the mouse studies growth of the isolates was only analyzed for 42 days, while in a second mouse study and in Guinea pigs growth was examined for 100 days. In neither study was survival at the end of infection (typically almost a year later) measured. Our results are entirely consistent with this data and support that the production of SL-1 does not affect the ability of strains to proliferate during the initial replication period, nor does it affect the ability of the host to mount an acquired immune response sufficient to restrict bacterial growth. We have also examined a mutant in pks2 in such assays. Unfortunately, as we have discovered occurs with many mutants in both pks and unrelated loci, this mutant was found to have lost the ability to produce PDIM. Loss of PDIM synthesis appears to be a spontaneous process that occurs in a fraction of mutants generated either by electroporation or by phage-mediated gene replacement strategies. PDIM is an important component of the cell wall structure whose loss has been reported previously to attenuate MTb (10, 42). Thus far the results of long term survival experiments in mice have not been obtained with a PDIM-containing pks2 mutant, so an exact comparison cannot be made. The possibility also exists that accumulation of SL-N results in an uncharacterized effect on the mmpL8::hyg mutant that compromises long term bacterial survival or that MmpL8 transports another unidentified lipid that contributes to virulence. Indeed, Converse et al. (23) describe a slight attenuation of their mmpL8 mutant (but not the corresponding pks2 mutant) during the bacterial replication and containment phases following high dose intravenous mouse infection but did not assess the relevance of these effects to final survival times of the animals. In contrast to these authors, using the low dose aerosol model we do not see any attenuation of the mmpL8::hyg mutant prior to death of the animal. We have also examined as fully as possible the secreted lipid repertoire of the mmpL8::hyg mutant and the parental strain without observing any significant differences other than the unique production of SL-N. In any case, the function of SL-1 in human tuberculosis still remains unknown, and more experiments need to be done to clarify the contribution of this lipid to disease outcome in humans.

In summary, we have shown that inactivation of the mmpL8 gene of MTb interrupts SL-1 synthesis, resulting in accumulation of a precursor to this molecule, SL-N, which we have identified as a family of 2,3-diacyl-α,α'-O-trehalose-2'-sulfates. In the mouse model this alteration results in an apparently normal infection but shows a dramatic effect on ultimate outcome of disease. Since the unique multimethyl-branched fatty acid components of sulfatides (the phthioceranates and hydroxyphthioceranates) are not utilized in any other known molecules, are produced at too low a level to perform a structural function in the cell envelope, and require a significant biosynthetic investment by the cell, it stands to reason that their production likely plays an important role in some aspect of the disease. Studies to explore the importance of the level of sulfatide production with reference to disease outcome in human tuberculosis patients may ultimately lead to stratifying...
REFERENCES

1. Daffe, M., and Draper, P. (1998) Adv. Microb. Physiol. 39, 131–203
2. Manca, C., Tsenova, L., Barry, C. E., III, Bergtold, A., Freeman, S., Haslett, P. A., Musser, J. M., Freedman, V. H., and Kaplan, G. (1999) J. Immunol. 162, 6740–6746
3. Manca, C., Tsenova, L., Bergtold, A., Freeman, S., Tovey, M., Musser, J. M., Barry, C. E., III, Freedman, V. H., and Kaplan, G. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 7572–7577
4. Cole, S. T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S. V., Egdameh, K., Gas, S., Barry, C. E., III, Tekaia, F., Badcock, K., Basham, D., Brown, D., Chillingworth, T., Connor, R., Davies, R., Devlin, K., Feltwell, T., Gentle, S., Hamlin, N., Holroyd, S., Hornby, T., Jagels, K., Krogh, A., McLean, J., Moore, S., Murphy, L., Oliver, K., Osborne, J., Quail, M. A., Rajandream, M. A., Rogers, J., Rutter, S., Seeger, K., Skelton, J., Squares, R., Squares, S., Sulson, J. E., Taylor, K., Whithead, S., and Barrell, B. G. (1998) Nature 393, 537–544
5. Tseng, T. T., Gratwick, R. S., Collman, J., Park, D., Nies, D. H., Goffeau, A., and Saier, M. H., Jr. (1999) J. Mol. Microbiol. Biotechnol. 1, 107–125
6. Bystrykh, L. V., Fernandez-Moreno, M. A., Herrema, J. K., Malpartida, F., Hogwood, D. A., and Dijkhuizen, L. (1996) J. Bacteriol. 178, 2238–2244
7. Tekaia, F., Gordon, S. V., Garnier, T., Brosch, R., Barrell, B. G., and Cole, S. T. (1999) Tuberc. Lung Dis. 79, 329–342
8. Minskina, D. E., Kremer, L., Dover, L. G., and Besra, G. S. (2002) Chem. Biol. 9, 545–551
9. Camacho, L. R., Constant, P., Raynaud, C., Laneelle, M. A., Tricas, J. A., Gicquel, B., Daffe, M., and Guilhot, C. (2001) J. Biol. Chem. 276, 19845–19854
10. Cox, J. S., Chen, B., McNeil, M., and Jacobs, W. R., Jr. (1999) Nature 402, 79–83
11. Sirakova, T. D., Thirumala, A. K., Dubey, V. S., Sprecher, H., and Kolat-Tekade, P. E. (2001) J. Biol. Chem. 276, 16833–16839
12. Goren, M. B. (1970) Biochim. Biophys. Acta 210, 116–126
13. Goren, M. B., Brokl, O., Das, B. C., and Lederer, E. (1971) Biochemistry 10, 72–81
14. Goren, M. B., Darvay Hart, P., Young, M. R., and Armstrong, A. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 2510–2514
15. Goren, M. B., Brokl, O., and Schaefer, W. B. (1974) Infect. Immun. 9, 142–149
16. Gangadaram, P. R., Cohn, M. L., and Middlebrook, G. (1963) Tubercle 44, 452–455
17. Zhang, L., English, D., and Andersen, B. R. (1991) J. Immunol. 146, 2730–2736
18. Zhang, L., Gay, J. C., English, D., and Andersen, B. R. (1994) J. Biomed. Sci. 1, 253–262
19. Zhang, L., Goren, M. B., Holzer, T. J., and Andersen, B. R. (1988) Infect. Immun. 56, 2678–2683
20. Pabst, M. J., Gross, J. M., Bronza, J. P., and Goren, M. B. (1988) J. Immunol. 140, 634–640
21. Bronza, J. P., Horan, M., Rademacher, J. M., Pabst, K. M., and Pabst, M. J. (1991) Infect. Immun. 59, 2542–2548
22. Kate, M., and Goren, M. B. (1974) Infect. Immun. 10, 733–741
23. Converse, S. E., Mougas, J. D., Leavell, M. D., Leary, J. A., Bertozzi, C. R., and Cox, J. S. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 6121–6126
24. Rousseau, C., Turner, O. C., Rush, E., Bordat, Y., Sirakova, T. D., Kolat-Tekade, P. E., Ritter, S., Orme, I. M., Gicquel, B., and Jackson, M. (2003) Infect. Immun. 71, 4684–4690
25. Pelicic, V., Jackson, M., Reynat, J. M., Jacobs, W. R., Jr., Gicquel, B., and Guilhot, C. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 10955–10960
26. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
27. Demenech, P., Menendez, M. C., and Garcia, M. J. (1994) FEMS Microbiol. Lett. 116, 19–24
28. Snapper, S. B., Melton, R. E., Mustafa, S., Kieser, T., and Jacobs, W. R., Jr. (1990) Mol. Microbiol. 4, 1911–1919
29. Polach, J., Lees, M., and Stoane Stanley, G. H. (1957) J. Biol. Chem. 226, 457–469
30. Slayden, R. A., and Barry, C. E., III (2001) in Mycobacterium tuberculosis Protocols (Parish, T., and Stoker, N. G., eds) Vol. 54, pp. 229–245, Humane Press Inc., Totowa, NJ
31. Constant, P., Perez, E., Malaga, W., Laneelle, M. A., Saurel, O., Daffe, M., and Guilhot, C. (2002) J. Biol. Chem. 277, 38148–38158
32. Soto, C. Y., Andreu, N., Gibert, I., and Luquin, M. (2002) J. Clin. Microbiol. 40, 3021–3024
33. Kaplan, L. E., and Meier, P. (1958) J. Am. Stat. Assoc. 53, 457–481
34. Morse, W. C., Dail, M. C., and Olitzky, I. (1953) Am. J. Public Health 43, 36–39
35. Hughes, D. E., Moss, E. S., Hool, M., and Henson, M. (1954) Am. J. Clin. Pathol. 24, 621–625
36. Middlebrook, G., Coleman, C. M., and Schafer, W. B. (1959) Proc. Natl. Acad. Sci. U. S. A. 45, 1801–1804
37. Mougas, J. D., Leavell, M. D., Senaratne, R. H., Leigh, C. D., Williams, S. J., Riley, L. W., Leary, J. A., and Bertozzi, C. R. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 17037–17042
38. Aliquaghi, S., Laneelle, M. A., Larsson, L., and Daffe, M. (1995) J. Bacteriol. 177, 4566–4570
39. Baer, H. H. (1993) Carbohydr. Res. 240, 1–22
40. Goren, M. B., Brokl, O., and Das, B. C. (1976) Biochemistry 15, 2728–2735
41. Belisle, J. T., Vissa, V. D., Sievert, T., Takayama, K., Brennan, P. J., and Besra, G. S. (1997) Science 276, 1420–1422
42. Camacho, L. R., Ensergueix, D., Perez, E., Gicquel, B., and Guilhot, C. (1999) Mol. Microbiol. 34, 257–267

Sulfolipid Transport in M. tuberculosis 21265
The Role of MmpL8 in Sulfatide Biogenesis and Virulence of Mycobacterium tuberculosis
Pilar Domenech, Michael B. Reed, Cynthia S. Dowd, Claudia Manca, Gilla Kaplan and Clifton E. Barry III

J. Biol. Chem. 2004, 279:21257-21265.
doi: 10.1074/jbc.M400324200 originally published online March 4, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M400324200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 40 references, 22 of which can be accessed free at http://www.jbc.org/content/279/20/21257.full.html#ref-list-1