LosA, a Key Glycosyltransferase Involved in the Biosynthesis of a Novel Family of Glycosylated Acyltrehalose Lipooligosaccharides from Mycobacterium marinum

Members of the genus Mycobacterium are characterized by cell envelopes rich in unusual free lipids, interacting with a covalently anchored mycolyl-arabinogalactan matrix. Previous studies have shown that Mycobacterium marinum produces large amounts of a diacylglycosylphenolphthiocerol, “phenolic” glycolipid. When cultivated on liquid Sauton medium, traces of a polar lipooligosaccharide (LOS) glycolipid antigen were also previously indicated. In this study, it was found that growth of the type strain of M. marinum on solid Sauton or Middlebrook 7H10 agar gave substantial, but different, amounts of a family of four major trehalose-based LOSs. The core pentasaccharide LOS-I was a rhamnosyl diglucosyl-acylated trehalose. The heptasaccharide, LOS-II, was derived from LOS-I by adding xylose accompanied by a novel sugar (X); repeated addition of this sugar unit X gave the octasaccharide LOS-III. LOS-IV has a decasaccharide component with two additional unusual sugar units, YZ. In a recent study (Alexander, D. C., Jones, J. R., Tan, T., Chen, J. M., and Liu, J. (2004) J. Biol. Chem. 279, 18824–18833), chromatographically similar glycolipids were assigned to the family

 Members of the genus Mycobacterium include the major human pathogens Mycobacterium tuberculosis and Mycobacterium leprae but also some less common opportunists, such as Mycobacterium malmoense, Mycobacterium marinum, Mycobacterium kansasii, Mycobacterium gastri, Mycobacterium gordonae, and Mycobacterium szulgai.

All these mycobacteria possess a cell envelope structure based on long-chain mycolic acids esterified to an arabinogalactan polysaccharide, which is attached to a peptidoglycan backbone. This mycolyl-arabinogalactan-peptidoglycan complex intercalates with an array of unusual free lipids, resulting in an effective external permeability barrier (1). This organelle also contributes to the intrinsic resistance of mycobacterial pathogens to antimicrobial drugs (2).

The free lipids of mycobacterial cell envelopes include a selection of species and subspecies-specific antigenic glycolipids, which may contribute to the external relationships of the organism (1–7). There are three major classes of these extractable glycolipids. First, the “phenolic glycolipids” (PGLs) possess similar aglycone structures, formed from polymethyl-branched fatty acids esterified to a phenolphthiocerol diol unit (7). Variations in the PGL sugar moieties allow the expression of the inherent antigenicity of these glycolipids. Second, the glycopeptidolipids found in the members of the Mycobacterium avium complex as well as in Mycobacterium smegmatis (4–6) can display subspecies specificity because of subtle variations in their oligosaccharide units. Finally, there is a broad class of trehalose-based lipids (7), ranging from apolar pen-tacyl trehaloses, through to amphiphilic tri- and diacyltrehaloses and sulfolipids to highly polar lipooligosaccharides (LOSs) (8). LOSs were found and described in M. kansasii (9–11), M. gastri (11, 12), M. szulgai (13), M. malmoense (14), M. gordonae (15), and certain representatives of M. tuberculosis (16).

In addition to the complex glycolipids outlined above, all mycobacterial species produce families of polar glycosphospholipids, the phosphatidylinositolmannosides (PIMs) (1, 4–6). Four major PIMs are usually expressed, mono- and diacylmannosides (AcPIM₁, Ac₂PIM₁, Ac₃PIM₁) and mono- and diacylmannosides (AcPIM₃ and Ac₂PIM₃) (6). In addition, a minor PIM₄ lipid has been shown to be a natural antigen for CD1d-restricted T cells (17). Furthermore, the characteristic mycobacterial lipidoglycans, lipoarabinomannan and lipomannan are both multiglycosylated versions of PIMs. We initially proposed the following biosynthetic pathway: phosphatidylinositol → PIM → lipomannan → lipoarabinomannan (18), which is now supported by biochemical and genetic evidence (19–22). PimA catalyzes the addition of Manₙ provided by GDP-mannose to the 2-position of the myo-inositol of phos-
and show that the proposed PIM5 and PIM7 (23) are in fact have reinvestigated the PIMs and other highly polar glycolipids of glycolipid isolated previously from M. marinum (24). In this study, we have reinvestigated the PIMs and other highly polar glycolipids of M. marinum and show that the proposed PIM5 and PIM7 (23) are in fact members of a novel family of LOSs. In addition, analyses based on M. marinum strains with a reported genetic disruption of Rv1500 and subsequent complementation with a plasmid expressing Rv1500, allows us to draw the conclusion that the product of this open reading frame, which we have termed LosA, is involved in the final assembly of the M. marinum LOS-IV glycolipid.

**MATERIALS AND METHODS**

**Strains and Culture Conditions**—The type strain of M. marinum 1218R (ATCC 927, MS425, GA 411) and strain MRS2521 (Rv1500::pMycMar) generated by transposon mutagenesis of 1218R (23) were grown at 30°C both on Sauton agar and Middlebrook 7H11 agar, supplemented with 10% oleic acid/albumin/dextrose/catalase (Difco). Escherichia coli DH5α was used for standard manipulations and propagation of plasmid DNA. E. coli DH5α Δpir116 was used for isolation of transposon-containing plasmid. Antibiotics were added as required: kanamycin, 50 μg/ml for E. coli and 25 μg/ml for M. marinum 1218R; hygromycin B, 150 μg/ml for E. coli and 75 μg/ml for M. marinum 1218R.

**Generation and Screening of M. marinum ΔMycMar Insertion Library**—Propagation of the ΔMycMar transposon phage and propagation of phage lysates were carried out as previously described (25). For phage infection, M. marinum 1218R cells were washed and resuspended in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM MgSO4, and 2 mM CaCl2. Phage were added at a multiplicity of infection of 10:1 and incubated at 37°C for 3 h to allow infection to occur. Bacteria were then plated on Middlebrook 7H11 agar supplemented with kanamycin and incubated at 30°C. Kanamycin-resistant (i.e., transposon-containing) M. marinum colonies were picked onto Middlebrook 7H11 agar to obtain a library of 7680 (i.e. 80 plates × 96 colonies/plate) clones. Colonies with unusual morphology were identified by visual inspection as reported previously (23). One colony, M. marinum MRS2521, was chosen for further characterization.

**Localization of M. marinum MRS2521 ΔMycMar Insertion**—M. marinum MRS2521 chromosomal DNA was isolated as described previously (23). BamHI, a restriction endonuclease, which does not cut within the MycoMar element, was used to cleave total chromosomal DNA. Such digestion generates a restriction fragment containing the kanamycin resistance cassette and R6K γ ori of the MycoMar element plus flanking chromosomal DNA. Self-ligation of this restriction fragment generates a plasmid that can replicate in E. coli strains containing the pir gene. Digested DNA was self-ligated with T4 DNA ligase and transformed into competent E. coli DH5α Δpir116 and plasmid DNA was isolated (23). Oligonucleotide primers MAR1 (5′-CCGAAAG-TGCCACCGTGA AAAGCCCG-3′) and MAR2 (5′-CGCTTCTCTGGT- GCTTTACGGATCG-3′) were used to determine the position of the transposon, which was inserted at a TA dinucleotide, 412 bp downstream, from the ATG start codon of an open reading frame previously termed pirF (23). The DNA sequence was compared with the genome sequences of M. marinum and M. tuberculosis at the Sanger Institute and analyzed with NTI Suite software (Informax) revealing that the predicted protein was homologous to the Rv1500 of M. tuberculosis H37Rv.

**Molecular Cloning**—The complementing pPMT1-Rv1500 plasmid was generated as described previously (23) by cloning DNA fragments containing the homologous Rv1500 gene from a M. tuberculosis H37Rv BAC library (obtained from S. Cole, Institut Pasteur, Paris) (26) into the E. coli-Mycobacterium shuttle vector pNBV1, which contains a hygromycin resistance cassette (27).

**Lipid Extraction and Analysis**—Polar lipids and apolar lipids were extracted from freeze-dried M. marinum cells grown either on Sauton or Middlebrook 7H11 agar according to the procedures of Dobson et al. (28) by stirring in 220 ml of methanolic saline (20 ml of 0.3% NaCl and 200 ml of CH3OH) and 220 ml of petroleum ether for 2 h. The cells were centrifuged at 3000 × g for 5 min. The resulting biphasic solution was separated and the upper layer containing apolar lipids was recovered. An additional 220 ml of petroleum ether was added, mixed, and harvested as described above. The two upper petroleum ether fractions were combined and dried under reduced pressure.

To extract polar lipids, 260 ml of chloroform, methanol, 0.3% NaCl (9:10:3, v/v/v), were added to the lower aqueous methanol layer and the solution stirred for 4 h. This mixture was filtered and the filter cake re-extracted twice with 85 ml of chloroform, methanol, 0.3% NaCl (5:10:4, v/v/v). Chloroform (145 ml) and 0.3% NaCl (145 ml) were added to the combined filtrates. This mixture was stirred for 1 h, allowed to settle, and the lower layer containing the polar lipids recovered and dried under reduced pressure. The polar lipid extract was examined by two-dimensional TLC on aluminum backed plates of Silica Gel 60 F254 (Merck 5554), using chloroform/methanol/water (60:30:6, v/v/v) in the first direction and chloroform/acetic acid/methanol/water (40:25:36, v/v/v) in the second direction (28). Glycolipids were visualized by either spraying plates with α-naphthol/sulfuric acid followed by gentle char ring of the plates, or visualized using the Dittmer and Lester reagent that is specific for phospholipids and glycoprophospholipids.

**Purification of Glycolipids**—The crude polar lipid extract (250 mg) was dissolved in chloroform/methanol (2:1, v/v) and applied to a DEAE cellulose column (2 × 15 cm) for purification. The column was eluted with chloroform/methanol (100 ml; 2:1, v/v) and increasing concentrations of ammonium acetate (5–50 mM) in chloroform/methanol (2:1, v/v). The purification process was monitored by TLC using either chloroform/methanol/water (60:30:6, v/v/v) or chloroform/acetic acid/methanol/water (40:25:36, v/v/v) (28). Glycolipids were visualized by spraying plates with α-naphthol/sulfuric acid followed by gentle char ring of the plates. Phospholipids and glycoprophospholipids were stained and visualized using the Dittmer and Lester reagent. Glycolipids were further purified into individual species by preparative TLC on 10 × 20-cm plastic backed TLC plates of Silica Gel 60 F254 (Merck 5735, Darmstadt, Germany), run in chloroform/methanol/water (60:30:6, v/v/v). The plates were then sprayed with 0.01% 1,6-diphenyl-1,3,5-hexatriene dissolved in petroleum ether/aceton (9:1, v/v) and the glycolipids visualized under UV light. Following detection the plates were re-developed in toluene to remove diphenyl-1,3,5-hexatriene and the corresponding glycolipid bands were scraped from the plates and extracted from the silica gel using chloroform/methanol (2:1, v/v).

**Sugar Compositional Analysis**—Samples were hydrolyzed in 1 M methanolic hydrogen chloride at 80°C for 16 h and the reagent was
removed under a stream of nitrogen. Hexosamines were re-N-acetylated in 500 μl of methanol/pyridine/acetic anhydride (50:1:5, v/v/v) for 15 min at room temperature, then dried under nitrogen. Trimethylsilyl derivatization was performed in 200 μl of Tri-Sil “Z” (Pierce) at room temperature for 30 min, after which the reagent was removed under nitrogen. Derivatized monosaccharides were resuspended in 1 ml of chloroform and washed several times with water before drying under a stream of nitrogen. Trimethylsilyl derivatization was performed in 200 μl of 2,6-di(-ethylated and purified on a Sep-Pak C18 cartridge as described above.

**Order of Linkages—**Samples were incubated in 100 μl of 50 mM sodium periodate in 50 mM ammonium acetate buffer, pH 4.5, for 48 h at 4 °C in the dark. The reaction was terminated by adding 4 μl of ethylene glycol and incubating for a further 60 min in the dark and the reaction mixtures were freeze-dried. Samples were reduced with 10 mg/ml sodium borohydride in 2 M aqueous ammonium hydroxide at room temperature for 2 h, then the reaction was terminated with a few drops of acetic acid. Excess borates were removed by loading directly onto Dowex columns (Merck) and samples were eluted with 2 column volumes of 5% acetic acid (~2 × 2 ml). Samples were dried and per-O-methylated using the sodium hydroxide procedure described above.

**Absolute Configuration of Sugars—**Samples were prepared as described for trimethylsilyl derivatives, except hydrolysis was performed in 1 M (S)-(+) -2-butanol-HCl prior to re-N-acetylation and trimethylsilyl derivatization. Samples were analyzed by GC-MS using temperature program D (see below).

**GC-MS Analysis—**This was carried out using a PerkinElmer Clarus 500 instrument, fitted with a RTX-5 (30 m × 0.25-mm internal diameter, Restek Corp.) for sugar analysis or a Stabilwax (30 m × 0.32 mm internal diameter, Restek Corp.) for lipid analysis. For temperature program A the oven was held at 65 °C for 1 min before being increased to 140 °C at a rate of 25 °C/min, then to 200 °C at a rate of 5 °C/min, and finally to a temperature of 300 °C at a rate of 10 °C/min. For temperature program B the oven was held at 90 °C for 1 min before being increased to 150 °C at a rate of 20 °C/min, then to 250 °C at a rate of 3 °C/min. For temperature program C the oven was held at 60 °C for 1 min before being increased to 300 °C at a rate of 8 °C/min. For temperature program D the oven was held at 65 °C for 1 min before being increased to 160 °C at a rate of 25 °C/min, then to 250 °C at a rate of 3 °C/min, finally to a temperature of 300 °C at a rate of 25 °C/min.

**Matrix-assisted Laser Desorption Ionization-Mass Spectrometry (MALDI-MS) Analysis—**MALDI-MS was performed using a PerSeptive Biosystems Voyager DE STR mass spectrometer in the reflectron mode with Delayed Extraction. Per-O-methylated samples were dissolved in methanol and 1-μl aliquots were loaded onto a metal plate with 1 μl of the matrix 2,5-dihydroxybenzoic acid. Native glycolipids were analyzed using the matrix 2-(4-hydroxypheinylazo)benzoic acid. Sequazyme peptide mass standards were used as external calibrants (Applied Biosystems).

**Electrospray Ionization-MS Analysis—**Per-O-methylated samples were dissolved in methanol and sequenced by tandem mass spectrometry (MS/MS) using a hybrid quadrupole orthogonal acceleration time of flight mass spectrometer (Micromass, UK). MS and MS/MS spectra were collected in the positive ion mode. Collision energies typically were 50–90 eV. Data were acquired and processed using Masslynx software (Micromass, UK). The instrument was pre-calibrated using a 1-pmol/μl solution of [Glu1]fibrinopeptide B in acetonitrile, 5% aqueous acetic acid (1,3, v/v).

**RESULTS**

Effect of Growth Conditions on TLC Patterns of *M. marinum* Glycolipids—Polar lipids were extracted from *M. marinum* 1218R bacilli grown on both Sauton or Middlebrook 7H11 agar and glycolipid profiles were recorded by two-dimensional TLC (Fig. 1). It is clearly evident that the growth condition affects the two-dimensional TLC pattern of polar glycolipids as shown by α-naphthol-sulfuric acid-stained TLCs. The lipid pattern when *M. marinum* was grown on Sauton agar appears to be more complex than the one obtained for *M. marinum* grown in Middlebrook 7H11. The two-dimensional TLC obtained under the latter growth conditions and stained with α-naph-
Figure 1. Two-dimensional TLC patterns of *M. marinum* polar lipids. *M. marinum* 1218R, *M. marinum* MRS2521, and *M. marinum* MRS2521 transformed with either pNBV1 or pPMT1-Rv1500 were grown on Sauton agar and Middlebrook 7H11 agar. Polar lipids were extracted as described under "Materials and Methods" and visualized on two-dimensional TLC. Solvent system for the first direction (arrow 1) was chloroform/methanol/water (60:30:6, v/v/v) and the second direction (arrow 2) was chloroform/acetic acid/methanol/water (40:25:3:6, v/v/v/v). Glycolipids were detected with α-naphthol/sulfuric acid, and phospholipids/glycolipids with Dittmer-Lester stain. AcPIM$_2$ and Ac$_2$PIM$_2$, mono- and diacyl phosphatidylinositol dimannosides; AcPIM$_6$ and Ac$_2$PIM$_6$, mono- and diacyl phosphatidylinositol hexamannosides; LOS-I–IV, lipooligosaccharides; PI, phosphatidylinositol; DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; and P, unknown phospholipids.
**M. marinum Lipooligosaccharides**

**Table One**

| Elution time | Characteristic fragment ions | Relative abundance | Equivalent chain length | Assignment |
|--------------|------------------------------|--------------------|------------------------|------------|
|              |                              | I          | II         | III        | IV         |                      |
| 15.86        | 74, 87, 256                  | 0.05      | 0.01      | 0.00       | 0.00       | 15.0                 | C15:0                 |
| 16.25        | 88, 101, 284                 | 0.04      | 0.03      | 0.04       | 0.05       | 15.1                 | C17:0 (2,4 dimethyl)   |
| 18.05        | 86, 101, 298                 | 1.00      | 1.00      | 1.00       | 1.00       | 16.1                 | C18:0 (2,4 dimethyl)   |
| 18.23        | 74, 87, 270                  | 0.10      | 0.18      | 0.20       | 0.14       | 16.0                 | C16:0                 |
| 19.45        | 74, 87, 284                  | 0.02      | 0.01      | 0.03       | 0.01       | 17.0                 | C17:0                 |
| 19.77        | 86, 101, 312                 | 0.10      | 0.12      | 0.14       | 0.12       | 17.1                 | C19:0 (2,4 dimethyl)   |
| 21.85        | 74, 87, 298                  | 0.09      | 0.05      | 0.22       | 0.10       | 18.0                 | C18:0                 |
| 25.04        | 74, 87, 326                  | 0.06      | 0.01      | 0.05       | 0.01       | 20.0                 | C20:0                 |

tol/sulfuric acid was similar to the one recorded by Alexander et al. (23) with similar growth conditions on Middlebrook 7H9 or 7H11 agar.

Analysis of M. marinum Polar Lipids by Two-dimensional TLCs Using Stain Specificity—Polar lipids were also extracted from the mutant strain M. marinum MRS2521, and the mutant strain complemented with the empty plasmid pNV1 and pPMT1-Rv1500 grown on Middlebrook 7H11. Lipid profiles were revealed by two-dimensional TLCs stained with Dittmer and Lester reagent specific for phospholipids and glycolipids and α-naphthol/sulfuric acid for glycolipids (Fig. 1). Not all the glycolipids gave a positive response with the phospho reagent (Fig. 1). For all strains, the major lipid phosphate spots corresponded to diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, mono- and diacylphosphatidylglycerol dimannosides (AcPIM2 and Ac2PIM2), and mono- and diacylhexamannosides (AcPIM6 and Ac2PIM6).

As observed by Alexander et al. (23), the M. marinum MRS2521 mutant accumulated large amounts of one lipid (labeled LOS-III) and failed to produce another (labeled LOS-IV). In contrast to the previous report, our analysis here shows that these two lipids are in fact phosphorus-free glycolipids (Fig. 1). These and the two remaining phosphorus-free glycolipids observed in all strains had the chromatographic behavior of LOSs characteristic of, for example, M. kansasii (9–11) and the Canetti variants of M. tuberculosis (24) and also to the putative PIM7 described by Alexander et al. (23), whereas LOS-III corresponds to the putative PIM of M. kansasii (9–11) and the Canetti variants of M. tuberculosis (31). The major components of these glycolipids have been labeled LOS-I–IV, in order of increasing polarity (Fig. 1). The LOS-IV corresponds to the previously isolated glycolipid antigen from M. marinum (24) and also to the putative PIM described by Alexander et al. (23), whereas LOS-III corresponds to the putative PIM observed after disruption of Rv1500 (23). Treatment of the wild type polar lipid extracts with mild base and subsequent two-dimensional TLC demonstrated that these unique glycolipids grown either in Sauton or Middlebrook agar were degraded. Hence these glycolipids were alkali-labile and phosphate-negative and similar in this respect to the LOS class of antigens from mycobacteria (8–16). Moreover, the complementation of the M. marinum MRS2521 mutant strain with plasmid pPMT1-Rv1500 confirmed that the disruption of Rv1500 was responsible for the altered glycolipid profile of MRS2521, restoring the wild type chromatographic behavior, whereas the profile of the mutant transformed with the empty vector remained the same as the mutant (Fig. 1). As a result, the protein product of Rv1500, which we have termed LosA, is a glucosyltransferase involved in the transfer of sugar residues to LOS-III to form LOS-IV.

Characterization of LOS-I–IV—The LOS-I–IV glycolipids were isolated from M. marinum bacilli grown on Sauton agar; LOS-IV was also isolated from M. marinum grown on Middlebrook 7H10 agar. Ion-exchange chromatography through a DEAE cellulose column gave total glycolipids in the chloroform/methanol eluate, which were then subjected to preparative TLC to afford LOS-I, LOS-II, LOS-III, and LOS-IV from bacilli grown on Sauton agar and LOS-IV from Middlebrook 7H10 agar.

Analysis of the released fatty acid methyl esters indicated that each LOS had a similar lipid profile (TABLE ONE), with the predominating component being a branched chain acid, tentatively identified as 2,4-dimethylhexadecanoate (C18:0; 2,4-dimethyl); this is in accordance with previous fatty acid analyses of M. marinum (29, 30). Other acids are present in minor amounts ranging from C15 through to C20, including two 2,4-dimethyl fatty acids (C17 and C19). These dimethyl-branched acids were partly identified from their distinctive fragment ions at m/z 88 and 101, indicating the presence of a 2-methyl substituent. The equivalent chain lengths of these fatty acid methyl esters (TABLE ONE) showed that the three branched acids were a homologous series.

The sugar composition of the LOSs was determined by GC-MS of trimethylsilyl derivatives. LOS-I–IV were shown to be composed predominately of the hexose (Hex) sugar d-glucose (Glc), with a small amount of the 6-deoxyhexose (dHex) sugar, L-rhamnose (Rha). In addition, the pentose sugar L-xylene (Xyl) was present in LOS-II, -III, and -IV.

Per-O-methylated LOSs were analyzed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (Fig. 2). Per-O-methylated LOS-I gave a prominent signal at m/z 1059 [M + Na]+, which is consistent with a glycan of the composition dHexHexHex. The spectrum shows product ions that are consistent with the glycan composition dHexHexHex. The derivation of the product ions is shown schematically in Fig. 2A. Further evidence for this assignment was obtained by collisionally activated decomposition (CAD) ES-MS/MS of the signal at m/z 1059 [M + Na]+ (Fig. 2B). The resulting data show a series of product ions that are consistent with the glycan composition dHexHexHex. The derivation of the product ions is shown schematically in Fig. 2B. Analysis of per-O-deuteriomethylated material revealed a signal shift to m/z 1104 (data not shown), consistent with a total of 16 hydroxyl groups, one of which is naturally O-methylated.

MALDI-MS analysis of per-O-methylated LOS-II gave a signal at m/z 1567 [M + Na]+ (Fig. 2C) consistent with the addition of a pentose residue and an unidentified sugar moiety (X) onto LOS-I glycan. CAD MS/MS of this per-O-methylated LOS-II signal at m/z 1567 gave the data shown in Fig. 2D. The spectrum shows product ions that are consistent with the assignment shown schematically in Fig. 2D. Further support for this assignment is obtained from per-O-deuteriomethylated LOS-II (data not shown), which indicated the presence of 23 functional groups and the retention of the native O-methyl group.

The analysis of per-O-methylated LOS-III by MALDI-MS (Fig. 2E) afforded the signal at m/z 1915 [M + Na]+. CAD ES-MS/MS of the corresponding doubly charged signal m/z 969 [M + 2Na]+ (Fig. 2F)
FIGURE 2. Mass spectrometric analysis of LOS-I, -II, -III, and -IV. A, MALDI-MS of per-O-methylated LOS-I, 75% aqueous acetonitrile Sep-Pak fraction; B, ES CAD MS/MS of m/z 1059; C, LOS-II, 75% aqueous acetonitrile Sep-Pak fraction; D, CAD MS/MS of m/z 1567; E, LOS-III, 100% aqueous acetonitrile Sep-Pak fraction; F, CAD MS/MS of m/z 969 [M + 2Na]2⁺; G, LOS-IV, 100% aqueous acetonitrile Sep-Pak fraction; H, CAD MS/MS of m/z 1178.5 [M + 2Na]2⁺. The observed masses are [M + Na]⁺. Per-O-methylated LOS-I-IV were selected for fragmentation. Fragmentation patterns are shown schematically. Arrows labeled # result from concomitant fragmentation across two glycosidic bonds.
resulted in product ions consistent with the assignment shown schematically in Fig. 2F. The mass increments observed suggest the likely addition of a further X sugar moiety to form of LOS-III. The assignment is further supported by CAD MS/MS data obtained from per-O-deuteriomethylated LOS-III (data not shown), which indicated the presence of 28 functional groups, where X has 5 methylatable groups.

Analysis of per-O-methylated LOS-IV by MALDI-MS (Fig. 2G) afforded the signal at $m/z$ 2334 [M + Na]$^+$, CAD ES-MS/MS of the corresponding doubly charged signal $m/z$ 1178.5 [M + 2Na]$^{2+}$ (Fig. 2H) resulted in a series of product ions that are tentatively assigned to a LOS-IV structure shown schematically in Fig. 2H. The assignment of the novel sugar residues (YZ) is based on the mass increments observed between product ions. CAD MS/MS data obtained from per-O-deuteriomethylated LOS-IV (data not shown), indicated the presence of 31 functional groups, where [YZ] have a total of 3 methylatable groups.

Per-O-methylated and per-O-deuteriomethylated LOS samples were taken through linkage analysis. The linkage data for the per-O-methylated LOS-I sample (TABLE TWO) revealed the presence of terminal Rha, terminal Glcp, 3-linked Glcp, and 4-linked Glcp. The per-O-deuteriomethylated LOS-I linkage data (data not shown) were consistent with those obtained for per-O-methylated LOS-I and additionally indicated that the terminal Rha carries the native O-acetyl group at the 3-hydroxy position. Linkage analysis of LOS-II, -III, and -IV (TABLE THREE) indicated the presence of a 4-linked Rhap and a 4-linked Xylp in addition to the 3-linked, 4-linked, and terminal Glcp residues observed previously for LOS-I. No linkage data were obtained for the putative residues X, Y, and Z.

Analysis of native LOS by MALDI-MS gave clusters of signals consistent with the presence of three acyl residues on the core oligosaccharide. For example, analysis of LOS-I gave a cluster of signals around $m/z$ 1631 separated by 14 mass units (data not shown). To establish the location of the acyl components on the carbohydrate backbone of LOS-I, the native glycolipid was subjected to the neutral alkylation conditions of the Prehm procedure (30), followed by de-O-acylation, then per-O-ethylation. Partially per-O-methylated/ethylated samples were then subjected to linkage analysis (TABLE FOUR). The sites of acylation on LOS-I could be inferred from the location of the ethyl groups on individual sugars. Ethyl groups were shown to be present at the 4- and 6-positions on the terminal Glcp residue and also at the 2-position on the 4-linked Glcp residue.

The existence of a trehalose core was established as follows. LOS-I was de-O-acylated prior to being reduced by sodium borodeuteride, and then per-O-methylated. MALDI-MS analysis of the derivatized product retained the signal at $m/z$ 1059. The absence of a reduced product is indicative of an oligosaccharide core lacking a free reducing end.

Finally, periodate oxidation of vicinal hydroxyl groups was used to ascertain the order of the linkages within the oligosaccharide. Periodate oxidation of the native glycolipid resulted in the cleavage of one C-C bond as shown by a mass shift of the molecular ion from $m/z$ 1059 to 1061 consistent with C-C bond breakage between C2 and C3 of the terminal trehalose Glc residue. In a second experiment LOS-I was de-O-acylated prior to oxidation and per-O-methylation. Analysis of the resulting sample by ES-MS revealed a series of new signals at $m/z$ 1061, 1017, 827, and 651, suggesting the core oligosaccharide had been oxidized and partially degraded. The nature of these signals was further investigated by CAD MS/MS and assignments for each are shown schematically in Fig. 3, panels A–D. The sequence data derived from CAD MS/MS of $m/z$ 1061 and 1017 was consistent with the breakage of one C-C bond and two adjacent C-C bonds on the terminal Hex residue of the glycan $d$Hex$\_n$ respectively. These data are consistent with the loss of the protective acyl groups. CAD MS/MS of $m/z$ 651 indicated that this oxidation product had the composition $d$Hex$\_m$Hex, and had not been oxidized. The data are consistent with the linkage sequence $d$Hex(1→3)Hex(1→3)$\_n$Hex, which lacks vicinal hydroxyl groups and would therefore not be expected to oxidize. CAD MS/MS of $m/z$ 783 gave sequence data that can only be attributable to the sequence $d$Hex(1→3)Hex(1→3)$\_n$Hex(1→4)$\_m$Hex, consistent with C-C bond breakage between C2 and C3 of the internal Hex residue of the trehalose core.

In summary, it is proposed that the sequence of LOS-I is 3-O-Me-$d$-Rhap(1→3)-$d$-Glcpc(1→3)-$d$-Glcpc(1→4)-$d$-Glcpc(1→4)-$d$-Glcpc. Strikingly, the oligosaccharide backbone of LOS-I–IV from *M. marinum* appears to be essentially the same as that of LOS from *M. kansasii*: (1→4)-$d$-Xylp(1→3)-3-O-Me-$a$-$d$-Rhap(1→3)-$d$-Glcpc(1→3)-$d$-Glcpc(1→4)-$\_n$-Glcpc(1→1)-$\_n$-Glcpc (9–11).

**DISCUSSION**

Many of the mycobacteria examined to date are characterized by species- or type-specific glycolipid antigens, which fall into three broad categories: the phenolic glycolipids, glycopeptidolipids, and trehalose-based lipids, extending in complexity from simple diacyltrehaloses (7) to LOS (8–16). The glycolipids (Fig. 1, LOS-I to LOS-IV) characterized from *M. marinum*, in the present study, belong to the latter class of alkaline-labile phosphorus-free glycolipids. Representatives of *M. marinum* fall into a select group of mycobacteria, which produce both LOSs and PGLs (32). In addition to *M. marinum*, this group is limited to *M. kansasii*, *M. gastri*, and the Canetti variants of *M. tuberculosis*. It is interesting that LOSs have not yet been characterized from *Mycobacterium ulcerans*, a close relative of *M. marinum* (33).

### Table Two: GC-MS analysis of partially per-O-methylated alditol acetates obtained from LOS-I

| Elution time (min) | Characteristic fragment ions | Assignment   |
|-------------------|-----------------------------|--------------|
| 16.75             | 115, 118, 131, 175          | Terminal Rha |
| 18.70             | 118, 129, 145, 162, 205     | Terminal Glcp |
| 19.94             | 118, 129, 161, 234          | 3-Linked Glcp |
| 20.08             | 113, 118, 162, 233          | 4-Linked Glcp |

### Table Three: GC-MS analysis of partially per-O-methylated alditol acetates obtained from LOS-II, -III, and -IV

| Elution time (min) | Characteristic fragment ions | Assignment   |
|-------------------|-----------------------------|--------------|
| 18.52             | 118, 143, 203, 247          | 4-Linked Rha |
| 18.55             | 118, 129, 189, 233          | 4-Linked Xylp |
| 19.05             | 118, 129, 145, 162, 205     | Terminal Glcp |
| 20.29             | 118, 129, 161, 234          | 3-Linked Glcp |
| 20.41             | 113, 118, 162, 233          | 4-Linked Glcp |

### Table Four: GC-MS analysis of partially per-O-methylated/ethylated alditol acetates obtained from LOS-I

| Elution time (min) | Characteristic fragment ions | Assignment   |
|-------------------|-----------------------------|--------------|
| 17.14             | 115, 118, 131, 175          | Terminal Rha |
| 19.93             | 118, 143, 173, 189, 233     | Terminal 4,6-Eth-Glcpc |
| 20.32             | 118, 129, 161, 234          | 3-Linked Glcp |
| 20.75             | 113, 116, 132, 233, 291     | 4-Linked 2-Eth-Glcpc |
One of the polar glycolipids (LOS-IV), identified here, corresponds to a lipid antigen described previously (24), but only found as a variable minor component in *M. marinum* grown in liquid Sauton medium. In the recent study of Alexander et al. (23), a family of glycolipids was apparent in *M. marinum*, in addition to the expected major amounts of the ubiquitous phosphatidylinositol di- and hexamannosides (PIM<sub>2</sub> and PIM<sub>6</sub>). These authors designated two of these lipids as PIM<sub>5</sub> and PIM<sub>7</sub> and proposed that a glycosyltransferase PimF was instrumental in the biosynthetic transformation of the former to the latter. No investigation of the phosphorus content (or glycosyl compositional analysis) of these glycolipids was reported. The two-dimensional TLC system used by Dobson et al. (28) to clearly distinguish PIMs from phosphorus-free LOS glycolipids; the LOSs run as a series of spots below, but parallel to, the PIMs.

The polar lipid patterns recorded here and by Alexander et al. (23) fall into this pattern, other published examples being the LOSs of *M. kansasi* (28) and *M. tuberculosis* Canetti (31).

The essential character (Fig. 4) of four novel LOSs (I–IV) from *M. marinum* has been determined, but further studies will be needed to elucidate the fine structure and stereochemistry of some of the unknown components, such as the novel sugars X, Y, and Z. However, the structures exhibit structural features in common with the previously characterized LOS antigens of *M. kansasi* (9–11), *M. gastri* (11, 12), *M. szulgai* (13), *M. malmoense* (14), and *M. gordonae* (15), and *M. tuberculosis* Canetti (16). All uniformly possess an acylated trehalose terminus, which is further glycosylated by several variable sugar residues. *M. marinum* LOS-I appears to be superficially the same as a LOS from *M. kansasi* (9–11). The principal fatty acyl substituents in both these...
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 lipids appear to be 2,4-dimethylhexadecanoate in the former case and 2,4-dimethyltetradecanoate in the latter (9–11). The absolute stereochemistry of these branched chain acids also was determined; the availability of synthetic routes to such 2,4-dimethyl branched acids (34) will facilitate this. The 2,4-dimethylhexadecanoate was found previously (35, 36) to be a characteristic biomarker fatty acid for M. marinum and this study shows the natural location of this acid, for the first time.

Although LOSs are present in most atypical mycobacterial species, the Canetti strain of M. tuberculosis appears to be the only LOS-producing M. tuberculosis strain reported to date. The Canetti variant has a smooth and moist texture (37), unlike the vast majority of field isolates of M. tuberculosis, which are of rough and dry texture. M. kansasii also exhibits analogous colony variations and it was demonstrated that all rough variants of M. kansasii are devoid of LOS (38). In this regard, it is noteworthy that the MRS2521 mutant strain of M. marinum bearing a transposon insertion in the Rv1500 gene produced an altered morphology, characterized by a wide, filmy, and translucent border around colonies on agar plates (23).

Analysis and comparison of the respective regions surrounding the Rv1500 gene in M. marinum and M. tuberculosis showed that the Rv1500 region of M. marinum was analogous to the Rv1496–Rv1505 region of the M. tuberculosis genome, and that in addition to Rv1500, genes homologous to Rv1496, Rv1497, Rv1501, Rv1502, Rv1503, Rv1504, and Rv1505 are present. However, the M. marinum region is much larger than the corresponding M. tuberculosis region, spanning at least nine additional genes. The fact that M. tuberculosis is devoid of LOS is intriguing and deserves consideration. It is therefore tempting to speculate that, the lack of LOS biosynthesis is attributable to one or several genes, which are missing in M. tuberculosis. Alternatively, a frameshift mutation may have occurred in M. tuberculosis, in a gene conserved in both species and involved early in the LOS biosynthetic pathway. This is in line with the recent demonstration that strains of M. tuberculosis deficient in the production of phenolphthiocerol derivatives (which are precursors of PGLs) are natural mutants with a frameshift mutation in pks15/1 (39). A single open reading frame for pks15/1 is found in Mycobacterium bovis BCG, M. leprae, and the Canetti strains of M. tuberculosis, which produce PGLs (36). The presence of LOS in the Canetti strain could be explained by the assumption that M. tuberculosis Canetti is either the common ancestor for the M. tuberculosis strains or it diverged before the appearance of other members of the M. tuberculosis complex (40). However, confirmation of this frameshift hypothesis awaits sequencing of the whole gene cluster in these various species.

Careful inspection of the M. tuberculosis genomic region downstream of Rv1500 revealed the presence of at least 7 genes encoding putative glycosyltransferases (Rv1514c, Rv1516c, Rv1518, Rv1520, Rv1524, and Rv1526c), as well as a possible rhamnosyltransferase WbbL2 (Rv1525). All these enzymes belong to the GT2 family in the CAZy classification, except Rv1524 and Rv1526c, which are members of the GT1 family. In addition, gmdA (Rv1511) and epdA (Rv1512) encoding a GDP-d-mannose dehydratase and a possible nucleotide-sugar epimerase, respectively, are other candidates for this LOS “biosynthetic cluster.” The inactivation of these genes and further biochemical characterization of the resulting mutants should elucidate whether this gene cluster is required for the modification of the carbohydrate moiety of M. marinum LOS-I to LOS-IV.

The M. marinum MRS2521 mutant strain of M. marinum, inactivated in Rv1500, exhibited comparable growth rates of replication in macrophages during the course of infection, compared with the wild type strain (23). However, this mutant was less efficient in entering macrophages, suggesting that inactivation of Rv1500 affected the initial step of cell entry (23). Altogether, these results suggest that failure of the mutant to produce LOS-IV alters the capacity of this strain to enter the host macrophage. Thus, the hypothesis that mycobacterial cell surface LOS may interact with a receptor(s) present at macrophage surface is attractive. Further experiments in animal models will help to define the role of LOSs in the mycobacterial invasion process. Efforts are underway to generate a strain of M. tuberculosis Canetti deficient in LOS production by disrupting Rv1500. This mutant strain, which will be of great interest to dissect the role of LOS in the pathogenicity of M. tuberculosis Canetti.

REFERENCES

1. Minnikin, D. E. (1982) in The Biology of the Mycobacteria (Ratledge, C., and Stanford, J. L., eds) pp. 95–184, Academic Press, Ltd., London
2. Glickman, M. S., and Jacobs, W. R., Jr. (2001) Cell 104, 477–485
3. Cosma, C. L., Sherman, D. R., and Ramakrishnan, L. (2003) Annu. Rev. Microbiol. 57, 641–667
4. Brennan, P. J. (1984) in Microbiology (Leive, L., and Schlesinger, D., eds) pp. 366–375, American Society for Microbiology, Washington, D. C.
5. Brennan, P. J. (1988) in Microbial Lipids (Ratledge, C., and Wilkinson, S. G., eds) Vol.
I, pp. 203–298, Academic Press, London
6. Brennan, P. J., and Nikaido, H. (1995) Annu. Rev. Biochem. 64, 29–63
7. Minnikin, D. E., Kremer, L., Dover, L. G., and Besra, G. S. (2002) Chem. Biol. 9, 545–553
8. Besra, G. S., and Brennan, P. J. (1994) in Mass Spectrometry for the Characterization of Microorganisms (Fenselau, C., ed) pp. 203–232, American Chemical Society, Washington, D.C.
9. Hunter, S. W., Murphy, R. C., Clay, K., Goren, M. B., and Brennan, P. J. (1983) J. Biol. Chem. 258, 10481–10487
10. Hunter, S. W., Jardine, I., Yanagihara, D. L., and Brennan, P. J. (1985) Biochemistry 24, 2798–2805
11. Gilleron, M., and Puzo, G. (1995) Glycoconj. J. 12, 298–308
12. Minnikin, D. E., Kremer, L., Dover, L. G., and Besra, G. S. (2002) Chem. Biol. 9, 545–553
13. Hunter, S. W., Barr, V. L., McNeil, M., Jardine, I., and Brennan, P. J. (1988) Biochemistry 27, 1549–1556
14. McNeil, M., Tsang, A. Y., McClatchy, J. K., Stewart, C., Jardine, I., and Brennan, P. J. (1987) Biochemistry 27, 1549–1556
15. Besra, G. S., McNeil, M. R., Khoo, K. H., Dell, A., Morris, H. R., and Brennan, P. J. (1993) Biochemistry 32, 12705–12714
16. Daffe, M., McNeil, M., and Brennan, P. J. (1991) Biochemistry 30, 378–388
17. Fischer, K., Scotet, E., Niemeyer, M., Koebenick, H., Zerrahn, J., Maillet, S., Hurwitz, R., Kursar, M., Bonneville, M., Kaufmann, S. H., and Schaible, U. E. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 10685–10690
18. Besra, G. S., Morehouse, C. B., Rittner, C. M., Waechter, C. J., and Brennan, P. J. (1997) J. Biol. Chem. 272, 18460–18466
19. Schaeffer, M. L., Khoo, K. H., Besra, G. S., Chatterjee, D., Brennan, P. J., Belisle, J. T., and Inamine, J. M. (1999) J. Biol. Chem. 274, 31625–31631
20. Kremer, L., Gurcha, S. S., Bifani, P., Hitchen, P. G., Baulard, A., Morris, H. R., Dell, A., Brennan, P. J., and Besra, G. S. (2002) Biochem. J. 363, 437–447
21. Kordulakova, J., Gilleron, M., Mikusova, K., Puzo, G., Brennan, P. J., Gicquel, B., and Jackson, M. (2002) J. Biol. Chem. 277, 31335–31344
22. Gurcha, S. S., Baulard, A. R., Kremer, L., Locht, C., Moody, D. B., Muhlecker, W., Costello, C. E., Crick, D. C., Brennan, P. J., and Besra, G. S. (2002) Biochem. J. 365, 441–450
23. Alexander, D. C., Jones, J. R., Tan, T., Chen, J. M., and Liu, J. (2004) J. Biol. Chem. 279, 18824–18833
24. Minnikin, D. E., Reedell, M., Wallerstrom, G., Besra, G. S., Parlett, J. H., Bolton, R. C., and Magnusson, M. (1989) Acta Leprol. 7, Suppl. 1, 51–54
25. Sassetti, C. M., Boyd, D. H., and Rubin, E. J. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 12712–12717
26. Brosch, R., Gordon, S. V., Billaud, A., Garnier, T., Eiglmeier, K., Sorrano, C., Barrell, B. G., and Cole, S. T. (1998) Infect. Immun. 66, 2221–2229
27. Howard, N. S., Gómez, J. E., Ko, C., and Bishai, W. R. (1995) Gene (Amst.) 166, 181–182
28. Dobson, G., Minnikin, D. E., Minnikin, S. M., Parlett, M., Goodfellow, M., Risell, M., and Magnusson, M. (1985) in Chemical Methods in Bacterial Systematics (Goodfellow, M., and Minnikin, D. E., eds) pp. 237–265, Academic Press, London
29. Dell, A., Reason, A. J., Khoo, K. H., Panico, M., McDowell, R. A., and Morris, H. R. (1994) Methods Enzymol. 203, 108–132
30. Prehm, P. (1980) Carbohydr. Res. 78, 373–374
31. Minnikin, D. E., Khoo, K. H., and Magnusson, M. (1990) FEBS Microbiol. Lett. 55, 55–57
32. Dobson, G., Minnikin, D. E., Besra, G. S., Mallet, A. L., and Magnusson, M. (1990) Biochim. Biophys. Acta 1042, 176–181
33. Stamm, L. M., and Brown, E. J. (2004) Microbes Infect. 6, 1418–1428
34. Wallace, P. A., and Minnikin, D. E. (1996) Chem. Phys. Lipids 82, 141–146
35. Torun, T., Welty, D. B., Jantzen, E., and Small, P. L. (1998) J. Clin. Microbiol. 36, 918–925
36. Jantzen, E., Tangen, T., and Eng, I. (1989) APMIS 97, 1037–1045
37. Papa, F., Laszlo, A., David, H. L., and Daffe, M. (1989) Acta Leprol. 7, Suppl. 1, 98–101
38. Belisle, J. T., and Brennan, P. J. (1989) J. Bacteriol. 171, 3465–3470
39. Constant, P., Perez, E., Malaga, W., Lanneelle, M. A., Saurel, O., Daffe, M., and Guilhot, C. (2002) J. Biol. Chem. 277, 38148–38158
40. Brosch, R., Gordon, S. V., Marmiesse, M., Brodin, P., Buchrieser, C., Eiglmeier, K., Garnier, T., Gutierrez, C., Hewinson, G., Kremer, K., Parsons, L. M., Pym, A. S., Samper, S., van Soolingen, D., and Cole, S. T. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 3684–3689

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