Identification of a Methyltransferase from *Mycobacterium smegmatis* Involved in Glycopeptidolipid Synthesis*

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Glycopeptidolipids (GPLs) are major components of the cell walls of several species of mycobacteria. We have isolated a transposon mutant of *Mycobacterium smegmatis* that is unable to synthesize mature GPLs and that displays a rough colony morphology. The disrupted gene, *mtf1*, shares a high degree of homology with several S-adenosylmethionine-dependent methyltransferases. The enzyme encoded by *mtf1* is required for the methylation of a single rhamnose residue that forms part of the conserved GPL core structure. This conclusion is supported by the finding that (a) the mutant synthesized only GPLs with undermethylated (either mono- or nonmethylated instead of di- or trimethylated) rhamnose residues; (b) complementation of the mutant with a wild-type copy of *mtf1* restored high levels of synthesis of GPLs containing di- and trimethylated rhamnose; and (c) S-adenosylmethionine-dependent methylation of rhamnosylated GPLs could be detected in cell lysates of wild-type cells and *mtf1*-complemented mutant cells, but not in mutant cells lacking intact *mtf1*. Structural analysis of wild-type and mutant GPLs suggests that disruption of *mtf1* specifically inhibits addition of O-methyl groups to the 3 (or 2)-position of the rhamnose. In the absence of 3-O-methylation, further methylation of GPL rhamnose is apparently inhibited, and overall GPL synthesis is down-regulated by 90%.

Several species of mycobacteria cause important human diseases. For example, *Mycobacterium tuberculosis* is the causative agent of tuberculosis, the leading cause of death from a single bacterial infection, whereas species of the *Mycobacterium avium* complex cause intractable infections in immunocompromised individuals (1). Many features of mycobacteria, including their ability to proliferate within phagolysosomes of host macrophages and their general resistance to a wide range of antibiotics, have been attributed to the fact that all these organisms synthesize distinctive lipid-rich cell walls (1–3). In addition to forming a highly effective permeability barrier, specific components in this wall have been shown to contribute to pathogenesis and/or to mediate specific host-bacterial interactions (4). The mycobacterial cell wall is composed of a core peptidoglycan-arabinogalactan layer surrounded by an outer lipid bilayer. The inner leaflet of the lipid bilayer is composed of mycolic acids, whereas several distinct classes of glycolipids and phospholipids form the outer leaflet (2, 3). The outer layer glycolipids are thought to contribute to the distinct surface properties of the different mycobacterial species and are also important surface antigens. The predominant outer layer glycolipids in members of the *M. avium* complex are glycopeptidolipids (GPLs), which characteristically contain a tripeptide-alcohol core that is modified with an amide-linked fatty acid, a 6-deoxytalose, and a variably O-methylated Rha residue (Fig. 1) (5–7). GPLs having this core structure are termed non-serovar-specific GPLs (nsGPLs) and are found in most isolates of the *M. avium* complex as well as in several other mycobacterial species (5–7). Most strains of *M. avium* further modify nsGPLs by addition of haptenic oligosaccharides to produce the antigenically important serovar-specific GPLs (5–7). Although the role of mycobacterial GPLs in pathogenesis is still unclear, defects in GPL biosynthesis are commonly associated with major changes in colony morphology and virulence (8, 9).

Several genes involved in the biosynthesis of the haptenic oligosaccharides of serovar-specific GPLs of *M. avium* have been identified by Belisle et al. (10–12), but less is known about the synthesis of the GPL core structures. To identify mycobacterial genes involved in the synthesis of the GPL core, we have adopted a transposon mutagenesis approach in *Mycobacterium smegmatis*. This species synthesizes nsGPLs as major outer layer glycolipids and is considerably more amenable to both genetic and biochemical analyses than the slow growing pathogenic species. Using this approach, we have previously identified a gene that encodes a large multimodular peptide synthetase involved in the synthesis of the GPL lipopeptide core (13). In this study, we report the identification of an S-adenosylmethionine (AdoMet)-dependent methyltransferase that initiates methylation of nsGPLs and that is required for normal levels of expression of these glycolipids.

**EXPERIMENTAL PROCEDURES**

*Bacterial Strains and Plasmids—Wild-type* *M. smegmatis* mc²155 (14) and mutants derived from it were grown on LB medium. The

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF192151.

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The abbreviations used are: GPLs, glycopeptidolipids; nsGPLs, non-serovar-specific glycopeptidolipids; 6-dTal, 6-deoxytalose; Rha, rhamnose; MeRha, methylrhamnose; AdoMet, S-adenosylmethionine; Tn, transposon; PCR, polymerase chain reaction; GC-MS, gas chromatography-mass spectrometry; HPTLC, high performance thin-layer chromatography; ESI-MS, electrospray ionization-mass spectrometry; MeTase, methyltransferase.
**Mycobacterial Glycopeptidolipid Biosynthesis**

**Compositional Analyses**—Monosaccharide analyses of crude GPL extracts or purified species were performed after hydrolysis with 2 M trifluoroacetic acid (2 h, 100 °C). Released monosaccharides were reduced with NaBD₄ and acetylated with an acetic anhydride/1-methylinidazole mixture (9:1, v/v) (21). The resultant alditol acetates were separated and analyzed by gas chromatography-mass spectrometry (GC-MS) on a Hewlett-Packard HP-1 column using a Hewlett-Packard Model 6890 gas chromatograph and a Model 5973 mass detector (13). In some cases, monosaccharides were analyzed after solvolysis in 0.5 M methanolic HCl and trimethylsilyl derivatization of the corresponding methyl esters (15).

**Electrospray Ionization-Mass Spectrometry**—HPTLC-purified GPL species were analyzed with an ion-trap mass spectrometer equipped with an electrospray ionization (ESI) source. Samples were introduced into the mass spectrometer using a microflow syringe pump operating at a constant flow rate as described previously (13).

**HPTLC Analyses and Purification of GPLs**—HPTLC was performed using aluminum-backed Silica Gel 60 HPTLC sheets (Merck). For separation of GPLs, HPTLC sheets were developed in chloroform followed by chloroform/methanol (9:1, v/v) in the same dimension (solvent system A). Individual GPL species were purified using the same solvent system. Silica bands (2 mm) were scraped and extracted twice in chloroform/methanol (2:1, v/v) with sonication. Partially permethylated Rha standards and methyl-¹³C-labeled sugars were resolved in benzene, acetone, water, and 30% ammonium (50:200:3:1.5, v/v; solvent system B) (22). GPLs and unlabeled sugar standards were visualized by spraying with orcinol/H₂SO₄.³¹M-Labeled methyltransferase assay products were detected by fluorography after HPTLC sheets were sprayed with EN³HANCE (NEN Life Science Products) and exposed to Biomax MR film (Eastman Kodak Co.).

**Methyltransferase Assay**—Mid-log phase liquid cultures were harvested by centrifugation and washed twice in 50 ml HEPES/NaOH (pH 7.4) (buffer A). Washed cell pellets were resuspended in buffer A containing 15% glycerol, 0.1 mM phenylmethylsulfonyl fluoride, 2 mM leupeptin, 0.2 mM N-tosyl-l-lysine chloromethyl ketone, and 2 mM EGTA (Sigma) (buffer B) at 1 g of cells/ml. Cell suspensions were disrupted (MSE Sonoprep 150 ultrasonic disintegrator) with 10 × 30-s bursts with 15-s intervals at 4°C (23). The lysate was subsequently spun at 4000 × g to remove intact cells. Cell lysate (100 µl) was assayed in a final volume of 125 ml of buffer B containing 10 µM MgCl₂ and 1 µCi of [methyl-¹³C]AdoMet (NEN Life Science Products). The mixture was incubated at 25°C for 1 h, and the assay was stopped by addition of 2.5 ml of chloroform/methanol (2:1, v/v). Lipids were extracted for 2 h at room temperature with intermittent sonication and vortex mixing. GPLs were separated by centrifugation as described above.

**Isolation and Analysis of Polyamylmanno and Polyamylglucose**—Mycobacterial cell pellets were twice extracted with acetone (16 and 4 h at room temperature), and the poly-3-O-methylmannose and poly-6-O-methylglucose polysaccharides were extracted as described by Gray and Ballou (24).

**Preparation of Partially Methylated Rha Standards**—A series of partially methylated rhamnose standards was prepared using a procedure adapted from Doares et al. (25). Rha (22 mg) was dissolved in Me₂SO (400 µl) and 2 m potassium methylsulfinylmethanide (75 µl) under an argon atmosphere and partially methylated by addition of CH₃I (100 µl) and incubated for 10 min at room temperature. The partially methylated sugars were peracylated by addition of 1-methylinidazole (20 µl) and acetic anhydride (2 ml) for 10 min at room temperature and recovered after biphasic partitioning between dichloromethane and water. Sugars were deacetylated with 2 M trifluoroacetic acid (2 h, 100°C), dried under N₂, and resuspended in 50% methanol.

**Results**

**Analysis of Wild-type and Mutant Strain Cell Walls**—Two rough colony mutants, Myco28 and Myco29, were independently isolated and shown to have Tn insertions in the same gene (Fig. 2A). The gene mt⁷ encodes a protein that is similar to several known AdoMet-dependent methyltransferases (Fig. 2A). As one or more methyltransferases are predicted to be involved in the synthesis of Mycobacterial GPLs, the gene mutational analyses investigated whether the mutants contained altered GPL profiles. As shown in Fig. 3, Myco29 cells lacked the mature nGPLs of wild-type cells, but synthesized a number of novel, base-resistant glycolipids with slower HPTLC mobility. The same phenotype was seen in Myco28 cells (data not shown). In contrast, the cellular levels of other cell wall glyco-

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**Fig. 1. The structures of M. smegmatis GPLs.** The locations of variable substituents (boxed) that are linked to the common GPL core are shown. Additional heterogeneity due to O-acetylation of the 6- deTal residue is not shown here.
lipids (phosphatidylinositol mannosides and lipoarabinomannan), lipids (mycolic acids), and polysaccharides (arabinogalactan) were essentially the same in both wild-type and *mtf1* mutant cells.

To further characterize the nature of the defect in GPL biosynthesis in the Myco29 mutant, we undertook a detailed analysis of the mature nsGPLs of wild-type cells and putative polar GPLs of Myco29 cells. GC-MS analyses of the four major GPL species of wild-type cells, which were resolved and purified by HPTLC, showed that GPL-1 and -2 contain 6-dTal and 2,3,4-tri-O-MeRha, whereas GPL-3 and -4 contain 6-dTal and 3,4,6-di-O-MeRha (Table I). Previous studies have shown that the 6-dTal and methylated Rha residues are linked to d-allo-Thr and the l-alaninol residues in the tetrapeptide backbone, respectively (5–7). Positive ion ESI-MS revealed that each of these bands comprised a series of molecular species that differed from each by intervals of 14 atomic mass units, suggesting heterogeneity in the chain length (CH₂ units) of the fatty acids. The major [M + H]⁺ molecular ions in the upper bands (i.e. GPL-1 and -3) corresponded to GPL species with monohydroxylated fatty acids containing 31–36 carbon atoms and three to four double bonds (Table I and Fig. 4). A similar range of molecular ions was also found in the lower bands (i.e. GPL-2 and -4) of each doublet (Fig. 4). However, the
The mass difference may reflect the presence of an additional methyl group on the fatty acid hydroxyl, accounting for the faster HPTLC mobility of GPL-1, -3, and -5 relative to GPL-2, -4, and -6, respectively.

**TABLE I**

| GPL species | Monosaccharide | Major molecular species (N:Z) |
|-------------|----------------|-------------------------------|
|             | 6-dTal | Rha | 4-MeRha | 3,4-MeRha | 2,3,4-MeRha | m/z |
| GPL-1 | 1 | | 1.0 | 1185.8<sup>a</sup> | 1199.8<sup>a</sup> | 1211.7 | 1225.7<sup>a</sup> | 1277.7 | 1239.7 | 1253.7 | (31:3) | (32:3) | (33:4) | (34:4) | (34:3) | (35:4) | (36:4) |
| GPL-2 | 1 | | 1.2 | 1185.6<sup>a</sup> | 1187.6 | 1211.5 | 1235.6 | 1239.6 | 1241.6 | (31:3) | (32:1) | (33:4) | (34:3) | (35:4) | (35:3) |
| GPL-3 | 1 | | 2.3 | 1183.6 | 1185.6<sup>a</sup> | 1187.7 | 1211.6 | 1213.7 | 1239.6 | (32:2) | (32:2) | (33:4) | (34:3) | (36:4) |
| GPL-4 | 1 | | 1.8 | 1169.8 | 1171.8<sup>a</sup> | 1173.8 | 1197.8<sup>a</sup> | 1199.8 | 1225.7 | (31:4) | (31:3) | (31:2) | (33:4) | (33:3) | (35:4) |
| GPL-5 | 1 | | 0.6 | 1171.5<sup>a</sup> | 1173.7<sup>a</sup> | 1185.5 | 1199.5 | 1201.7 | 1203.5 | 1213.5 | (32:2) | (32:2) | (33:3) | (34:3) | (34:2) | (34:1) | (35:3) |
| GPL-6 | 1 | | 0.7 | 1157.8<sup>a</sup> | 1159.8<sup>a</sup> | 1181.4 | 1183.6 | 1197.7 | 1211.7 | (31:3) | (31:2) | (33:3) | (34:3) | (34:4) | (35:4) |
| GPL-7 | 1 | | 1.7 | 1129.7 | 1143.7 | 1157.6<sup>a</sup> | 1169.6 | 1183.6 | 1197.6 | 1211.7 | (28:3) | (29:3) | (31:3) | (32:4) | (33:4) | (34:4) | (34:4) |

<sup>a</sup>The major ion in each spectrum.

**FIG. 4.** Positive ion ESI-mass spectra of purified GPLs. A, GPL-3; B, GPL-4; C, GPL-5; D, GPL-6; E, GPL-7.

The major ions in the spectra of the lower bands were 14 atomic mass units lower than those found in the upper bands (Table I). This mass difference may reflect the presence or absence of an O-Me substituent on the fatty acid hydroxyl group, which would also account for the different HPTLC mobilities of the upper and lower bands in each doublet. Heterogeneity in fatty acid O-methylation has previously been reported in *M. smegmatis* GPLs (7). These data suggest that the four major GPL species in wild-type cells contain a heterogeneous long chain fatty acid and only differ from each other in having either two (GPL-3 and -4) or three (GPL-1 and -2) O-Me groups on the Rha residue and either an unsubstituted (GPL-2 and -4) or three (GPL-1 and -2) O-Me groups on the fatty acid ap-
dependent methyltransferases (Fig. 2B). The proven methyltransferases with primary sequence similarity to MeTase1 are 3-O-methyltransferases that modify sugars on the polyketides avermectin, mycinamicin, and tylosin (26–31). Four putative methyltransferases from the *M. avium* ser2 cluster were also similar to MeTase1; in particular, there was 84.2% amino acid sequence identity between the *M. smegmatis* mtf1 and *M. avium* mtfD gene products (Fig. 2B).

The similarity between sequences that aligned with MeTase1 was restricted to several motifs that are characteristic of AdoMet-dependent methyltransferases (32). We have numbered the motifs using the nomenclature of Posfai et al. (32). The motifs whose primary sequences were most strongly conserved in MeTase1 are shown in Fig. 2B. Motif I forms part of the secondary structure that binds the methionine moiety of AdoMet and has a VI/E/D/UXXXGX consensus sequence in DNA methyltransferases (32). It is interesting to note that the group of 3-O-methyltransferases that aligned with MeTase1 all had unusual substitutions of the central Gly residue in Motif I.

Two open reading frames encoding a putative glycosyltransferase (gtf) and a second putative methyltransferase (mtf2) were found by sequencing downstream of mtf1 (Fig. 2A). The amino acid sequence of MeTase2 aligned more closely with a different subset of AdoMet-dependent methyltransferases than those in the MeTase1 alignment. The predicted amino acid sequence identity between MeTase1 and MeTase2 is low (28%), whereas MeTase2 was up to 59% identical to putative methyltransferases from *M. tuberculosis* (Rv1523 and Rv2952). Among the proven methyltransferases similar to MeTase2 are AveD, MitM, and RapM, which *O*-methylate the macroyclic rings of polyketides produced by *Streptomyces* species (30–32).

**Complementation of Myco29 Restores Synthesis of Methylated GPLs**—To investigate whether expression of the wild-type *mtf1* gene in Myco29 could restore synthesis of mature GPLs, a complementation plasmid was constructed. The wild-type *mtf1* open reading frame plus 418 base pairs of upstream sequence were PCR-cloned into a shuttle vector (pEP3) to create the plasmid pEP3-*mtf1* (Fig. 2A). Although transcription of the *mtf1* gene may be controlled by the native promotor, we cannot eliminate the possibility that vector-encoded promoters may control transcription of *mtf1*. GPLs were extracted from untransformed Myco29, Myco29 transformed with pEP3 alone, and Myco29 transformed with pEP3-*mtf1*, and the methylation pattern of GPL-associated Rha was determined by GC-MS analyses. Transformation of Myco29 with pEP3-*mtf1*, but not with the vector alone, restored synthesis of di- and tri-O-methylated GPLs (Fig. 5, D–F). Although the methylated sugar profile was essentially the same as that of wild-type GPLs, the total levels of GPL biosynthesis were restored only to ~65% of wild-type levels. Incomplete restoration of GPL levels may account for the observation that *mtf1*-complemented strains retained the rough colony morphology (data not shown). Interestingly, the GC-MS analyses revealed the presence of a low level of monomethylated GPLs in both untransformed and transformed wild-type strains, indicating the presence of additional GPL species (possibly precursors) that were not detected by HPTLC. In the wild-type cells, the monomethylated rhamnose was exclusively 3-O-MeRha, whereas the complemented strain contained both 3-O-MeRha and 4-O-MeRha (Fig. 5, A–C and D). In contrast, the only monomethylated sugar in Myco29 or Myco29-pEP3 was 4-O-MeRha (Fig. 5, A–C and D), consistent with the analyses of purified GPL-5 and -6 from these mutants. These data show that complementation of Myco29 with *mtf1* effectively restores the normal GPL profile, although not to wild-type cellular levels. Moreover, they provide strong evidence that MeTase1 catalyzes addition of the first methyl residue to the 3-OH of Rha in the GPL core. In the presence of this defect, a low level of Rha methylation at the 4-OH position was maintained, suggesting the presence of a second distinct methyltransferase activity in *M. smegmatis*.

**Myco29 Is Unable to Methylate GPLs in Vitro**—To confirm that MeTase1 has a direct role in GPL biosynthesis, we devel-
opposed an in vitro assay for GPL-specific methyltransferase activity. Wild-type M. smegmatis mc\(^{155}\), Myco29, and transformed bacterial strains were lysed by sonication, and a fraction containing both cytosolic and membrane/cell wall fractions was used as a source of enzyme activity and endogenous GPL acceptors. [methyl-\(^3\)H]AdoMet was added to this fraction, and the labeled GPLs were recovered by organic solvent extraction and base treatment. Incubation of mc\(^{155}\) lysates with [methyl-\(^3\)H]AdoMet resulted in the incorporation of \(^3\)H]methyl groups into a number of endogenous GPLs that comigrated with wild-type GPLs containing di- and tri-\(^O\)-MeRha and tri-\(^O\)-MeRha on HPTLC (Fig. 6, lane 1). These putative GPL species were purified by HPTLC (Fig. 7A, lanes 2–4) and subjected to strong trifluoroacetic acid hydrolysis to release the acid-released glycans of the labeled species. As expected, the acid-released glycans of the putative GPL-1/2 doublet, GPL-3/4 doublet, and GPL-5 comigrated with tri-\(^O\)-MeRha, di-\(^O\)-MeRha, and mono-\(^O\)-MeRha, respectively (Fig. 7B, lanes 2–4). These data suggest that all the methyltransferases are active in these in vitro assays and provide further evidence that the 3-\(^O\)-Me group is the first methyl group to be transferred to the Rha residue. When a similar cellular fraction was obtained from Myco29 and incubated with \(^3\)H]AdoMet, none of these bands were labeled (Fig. 6, lane 2). However, incorporation of \(^3\)H]methyl groups into GPL-1–4 was restored in lysates from Myco29 complemented with pEP3-mtf1 (Fig. 6, lane 4). Transformation of mc\(^{155}\) with pEP3-mtf1 did not affect normal incorporation of \(^3\)H]AdoMet radioactivity into GPLs (Fig. 6, lane 3). Collectively, these data suggest that the protein encoded by mtf1 is directly involved in transferring a 3-\(^O\)-Me group to the GPL rhamnose. Moreover, inhibition of this step appears to inhibit addition of other methyl groups to the 2- and 4-positions of the Rha residue, both in vivo and in vitro.

Disruption of mtf1 Does Not Affect the Methylation of Intracellular Polysaccharides—M. smegmatis synthesizes two other classes of \(^O\)-methylated polysaccharides, the poly-3-\(^O\)-methylmannose and poly-6-\(^O\)-methylglucose polysaccharides, which are thought to be involved in regulating lipid metabolism (33). To investigate the possibility that MeTase1 may also be involved in the methylation of these polysaccharides, they were isolated from wild-type and Myco29 cells and subjected to GC-MS compositional analysis. These analyses showed that both the cellular levels and methylation pattern of the methylated polysaccharides were identical in both the wild-type and Myco29 mutant cells (data not shown). These data suggest that MeTase1 is specifically involved in GPL biosynthesis and is not required for methylation of other M. smegmatis glycoconjugates.

DISCUSSION

In this study, we provide evidence that the M. smegmatis mtf1 gene encodes a 3-\(^O\)-methyltransferase that initiates the \(^O\)-methylation of the terminal Rha residue in nsGPLs. This conclusion is supported by (a) comparison of the predicted mtf1 amino acid sequence with other methyltransferases, (b) the presence of undermethylated GPLs in the Myco29 mutant, (c) the lack of in vitro AdoMet-dependent \(^O\)-methyltransferase activity in cell lysates from the mutant, and (d) the restoration of in vivo and in vitro methyltransferase activities following complementation of the Myco29 mutant with the wild-type mtf1 gene. Significantly, disruption of mtf1 was associated with a massive down-regulation of GPL biosynthesis, whereas the levels of synthesis of other cell wall glycolipids and intracellular polymethylated polysaccharides were unaffected. Thus, as shown previously (13), the down-regulation of GPL biosynthesis does not appear to be offset by the compensatory changes in the synthesis of other cell wall glycoconjugates.

The mtf1 gene product was initially identified as an AdoMet-dependent methyltransferase based on comparison of the predicted amino acid sequence with other methyltransferases. These include TylF, MycF, and AveBVII, which have been shown to be 3-\(^O\)-methyltransferases that modify sugars attached to macrolide antibiotics. Several putative methyltransferases from M. avium also showed similarity to MeTase1. In particular, the M. avium mtfD product was almost identical to MeTase1, suggesting that mtf1 and mtfD are homologs and that...
the two methyltransferases may catalyze similar reactions.

Complementation of the M. smegmatis Myco29 mutant with an intact mtf1 gene restored the ability to synthesize mature GPLs containing fully methylated Rha, thereby confirming that the Tn disruption of mtf1 had been responsible for the defect in GPL biosynthesis. Disruption of the mtf1 gene generates a rough colony morphology phenotype, which is indicative of a major perturbation in cell wall structure and/or surface properties (6, 10, 13). This phenotype may be the result of the 10-fold down-regulation of the GPL biosynthesis that is associated with mtf1 disruption, rather than the subtle changes associated with exposure of less hydrophobic glycopeptide head groups on the cell wall. We note that complementation of Myco29 with mtf1 failed to restore smooth colony phenotype despite the fact that the GPL profile of the complemented strain was essentially the same as that of the wild-type cells. However, further analysis showed that GPL levels in this strain were ~65% of those in wild-type cells. If the rough phenotype is related to GPL levels, these results suggest that a threshold level of GPL biosynthesis (above 65% of wild-type levels) is required for smooth colony morphology.

The down-regulation of GPL biosynthesis in this mutant was surprising given that MeTase1 is likely to act near the end of the GPL biosynthetic pathway. For example, mtf1 disruption does not prevent formation of the glycopeptide core, addition of Rha and 6-dTal residues, or modification of 6-dTal with O-acetyl groups (data not shown). These data raise the intriguing possibility that precursors in this pathway undergo substrate channeling and that a block in one of the terminal steps can reduce the flux of GPLs into the cell wall matrix. This finding is of particular interest, as it indicates that inhibitors of this enzyme may have potential as therapeutic agents against members of the M. avium complex.

Our data suggest that M. smegmatis MeTase1 is likely to be the first methyltransferase to act on GPL precursors and is specifically involved in adding a methyl group to the 3-position of the Rha residue. This is based on the finding that all GPL species in wild-type M. smegmatis contain a 3-O-MeRha that may be further, but variably, modified with methyl groups at the 2- and 4-positions. The absence of GPLs with monomethylated 2- or 4-O-MeRha in wild-type cells is consistent with the hypothesis that the 3-O-methyl group is added first. In contrast, none of the GPLs receive the 3-O-Me group in the Myco29 mutant, whereas addition of the other methyl groups is either greatly reduced (in the case of the 4-O-methyl group) or completely inhibited (in the case of the 2-O-methyl group). Further evidence indicating that mtf1 encodes a 3-O-methyltransferase was provided by the in vitro assay. Interestingly, we have found that the MeTase1 activity is largely associated with the membrane fraction, rather than the cell wall or cytosolic fractions (data not shown). As MeTase1 lacks an obvious transmembrane domain, it may be targeted to the appropriate membrane by binding to its substrate or alternatively to other proteins associated with this pathway.

A distinct methyltransferase, possibly encoded by mtf2, must be responsible for addition of the second methyl group to the 4-position, as this activity was still detected in the Myco29 mutant. Although a third methyltransferase may be required for addition of a methyl group to the 2-position, we cannot discount the possibility that MeTase1 may also catalyze this step. Alternatively, mtf2 may be required for methylation of the intracellular poly-3-O-methylmannose and poly-6-methylglucose polysaccharides or for the methylation of the GPL amide-linked fatty acid, as none of these methylation steps were affected in the Myco29 mutant.

In summary, we have identified a 3-O-methyltransferase gene that plays a crucial role in the biosynthesis of GPLs in M. smegmatis and have established an in vitro assay for this enzyme. These results have provided new insights into the regulation of the biosynthesis of GPL core structure and suggest that terminal steps in this pathway may be targets for inhibitors of GPL biosynthesis.

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