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Methylation of GPLs in *Mycobacterium smegmatis* and *Mycobacterium avium*

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Several species of mycobacteria express abundant glycopeptidolipids (GPLs) on the surfaces of their cells. The GPLs are glycolipids that contain modified sugars including acetylated 6-deoxy-talose and methylated rhamnose. Four methyltransferases have been implicated in the synthesis of the GPLs of *Mycobacterium smegmatis* and *Mycobacterium avium*. A rhamnosyl 3-*O*-methytransferase and a fatty acid methyltransferase of *M. smegmatis* have been previously characterized. In this paper, we characterize the methyltransferases that are responsible for modifying the hydroxyl groups at positions 2 and 4 of rhamnose and propose the biosynthetic sequence of GPL trimethylrhamnose formation. The analysis of *M. avium* genes through the creation of specific mutants is technically difficult; therefore, an alternative approach to determine the function of putative methyltransferases of *M. avium* was undertaken. Complementation of *M. smegmatis* methyltransferase mutants with *M. avium* genes revealed that MtfC and MtfB of the latter species have 4-*O*-methyltransferase activity and that MtfD is a 3-*O*-methyltransferase which can modify rhamnose of GPLs in *M. smegmatis*.

Mycobacteria are acid-fast bacteria that have characteristic lipid-rich cell envelopes that afford the cells protection from desiccation, chemical disinfectants, and some antibiotics. The cell envelopes of pathogenic mycobacteria are one of the major virulence factors that assist the bacteria to live within host macrophages and withstand the bactericidal defenses of such cells. The mycobacterial cell envelope has a multilaminar structure, comprising an inner layer of peptidoglycan and arabinogalactan polysaccharides and mycolic acids and an outer layer made of species-specific glycolipids and phospholipids. Among the lipids of the outer layer are the glycopeptidolipids (GPLs) that are characteristic of some nontuberculosis mycobacteria such as *Mycobacterium avium* and *Mycobacterium smegmatis*. The core structure of most GPLs comprises a long-chain fatty acid (3-hydroxy and 3-methoxy $C_{26–34}$) amidated with a tripeptide-amino alcohol (D-Phe-D-*allo*-Thr-D-Ala-L-alaninol). In most cases, the hydroxyl groups of the *allo*-Thr and/or alaninol are modified with 6-deoxytalose (dTal) or rhamnose (Rha), respectively. In *M. smegmatis*, the dTal is variably acetylated, while the Rha residue can be modified with up to three methyl groups. Heterogeneity in the methylation of both amide-linked fatty acids and the Rha residue results in the appearance of four main GPL species in *M. smegmatis*. GPL-1 and GPL-2 have 3-methoxy $C_{26–34}$ fatty acids but differ from each other in the degree of methylation of the terminal Rha, in that GPL-1 has 2,3,4-tri-*O*-Me-Rha and GPL-2 has 3,4-di-*O*-Me-Rha. GPL-1a and GPL-2a have tri- and di-Me-Rha as above; however, these GPLs have 3-hydroxy $C_{26–34}$ fatty acids. We have simplified our nomenclature of the GPLs from that used in Patterson et al. (9).

M. avium GPLs have the same lipopeptide core as *M. smegmatis* GPLs, but the alaninol is glycosylated with 3-*O*-Me-Rha or 3,4-di-*O*-Me-Rha, and the dTal can be extended with additional sugars. A locus designated *ser2* contains some of the genes encoding enzymes required for synthesis of the haptenic disaccharide of GPLs of serotype 2 *M. avium*. Eckstein et al. showed that the simpler GPLs naturally found in *M. smegmatis* could serve as intermediates in the biosynthesis of a *ser2*-like locus encoding GPL in recombinant *M. smegmatis* (3). We have identified an equivalent region in *M. smegmatis*, shown that the *mps* gene encodes a peptide synthetase that makes the GPL peptide, and assigned the roles to two of the four potential methyltransferases encoded in the GPL biosynthetic locus (1, 7, 9). Mtf1 is a rhamnosyl 3-*O*-methyltransferase, and *mtf2* encodes a fatty acid *O*-methyltransferase that modifies the hydroxyl at C_3 of the GPL fatty acid (7, 9). For the purposes of clarity, we propose that the *M. smegmatis* genes formerly designated *mtf1*, *mtf2*, *mtf3*, and *mtf4* (GenBank accession no. AY138899) be renamed *rmt3* for rhamnosyl 3-*O*-methyltransferase, *fmt* for fatty acid *O*-methyltransferase, *rmt4* for rhamnosyl 4-*O*-methyltransferase, and *rmt2* for rhamnosyl 2-*O*-methyltransferase, respectively. As we show, this nomenclature reflects the functions of the respective methyltransferases. In this paper, we define the roles of the remaining two putative methyltransferase genes, *rmt4* and *rmt2*, and identify the functional homologues from *M. avium*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and DNA manipulation. Bacterial strains and their derivatives are listed in Table 1. *Escherichia coli* was grown on Luria medium, and *M. smegmatis* and *M. avium* were grown on Middlebrook 7H9 or 7H10 medium (Difco). Antibiotics were added at the following concentrations as required: ampicillin, 100 μg/ml; kanamycin, 20 μg/ml; hygromycin, 200 μg/ml; and streptomycin, 20 μg/ml. Sucrose (10% [wt/vol]) was included in media for *sacB* counter selection. DNA manipulation was performed according to standard protocols (12). PCRs were carried out with an MJ Research Peltier Thermal

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant feature	Source
Strains		
M. smegmatis		
mc^{2}155	Parent strain used for mutagenesis	14
Myco29	*rmt3*::Tn*611*, Kan^r Str^r	9
Myco493	*fmt*::*str*	7
Myco657	Δ*rmt4*, Kan^r	This study
Myco694	Δ*rmt2*, Kan^r	This study
E. coli DH5α	Host for cloning	Stratagene
M. avium TMC 724	Source of *mtfA* to *-D*	
Plasmids		
pGEM-T Easy	Vector for efficient cloning of PCR products	Promega
pBluescript SK(+)	Cloning vector	Stratagene
pK18mobsacB	*aph sacB*	13
pK18mob	*aph*	13
pHBJ395	pBluescript SK(+) containing *aph*	This study
pHBJ428	pBluescript SK(+) containing *str* and *sacB*	This study
pHBJ430	pHBJ428, Δ*rmt4 aph*	This study
pHBJ488	pHBJ428, Δ*rmt2 aph*	This study
pVV16	Mycobacterial-*E. coli* shuttle vector, *hyg*	T. Eckstein
pHBJ415	pVV16, *rmt4*	This study
pHBJ409	pVV16, *rmt2*	This study
pHBJ475	pVV16, *mtfA* (*M. avium*)	This study
pHBJ512	pVV16, *mtfB* (*M. avium*)	This study
pHBJ505	pVV16, *mtfC* (*M. avium*)	This study
pHBJ502	pVV16, *mtfD* (*M. avium*)	This study

cycler. Plasmid DNA was introduced into bacterial cells with a Bio-Rad gene pulser. The manufacturer's recommendations were followed for preparation and transformation of *E. coli*, whereas the protocols of Jacobs et al. were used for *M. smegmatis* (6).

Preparation of mycobacterial genomic DNA. Genomic DNA was extracted from mycobacteria as follows. Mycobacterial cells were harvested from liquid cultures by centrifugation and then incubated for 1 h at 37°C in 1 ml of Tris-EDTA buffer containing 200 μg of proteinase K/ml, 10 mg of lysozyme/ml, and 100 μg of RNase A/ml. The suspensions were centrifuged, and the cell pellet was resuspended in 750 μl of 4 M guanidine thiocyanate, 25 M sodium citrate, and 0.5% Sarkosyl; 200 mg of 100-μm diameter glass beads was added to each tube. Cells were lysed with a tissue homogenizer (Biospec), and then the beads and unlysed cells were removed by centrifugation. The lysate was extracted three times with equal volumes of phenol:chloroform:isoamyl alcohol (25:24:1 [vol/vol]) and then once with chloroform:isoamyl alcohol (25:1 [vol/vol]). The genomic DNA was precipitated with sodium acetate and ethanol and then resuspended in Tris-EDTA buffer.

Southern blotting. Purified genomic DNA was digested with restriction endonucleases and resolved by electrophoresis in 1% (wt/vol) agarose in 1× TAE buffer and then transferred to nylon membranes (Hybond) for Southern blotting (12). DNA probes for hybridization were prepared by PCR amplification of the *rmt4* open reading frame (ORF) with primers 383 and 384 on an *M. smegmatis* genomic DNA template (Table 2). The PCR product was purified with a QIAquick PCR purification kit (QIAGEN) and digoxigenin labeled with a DIG DNA labeling kit (Roche). Membranes were incubated with labeled probes and processed according to the manufacturer's protocols.

Construction of *sacB-str* suicide vector. The vector for delivery of allelic exchange cassettes was based on pBluescript SK(+) (Stratagene) which is unable to replicate in mycobacteria. This suicide vector contained a streptomycin resistance marker (*str*) for positive selection of transformants in which the plasmid had integrated into the host chromosome. It also contained the levansucrase gene *sacB* so that sucrose could be used for counter selection of single-crossover (SCO) mutants (10). The vector was constructed as follows. The *sacB* gene was PCR amplified from pK18mobsacB (13) with primers 382 and 310 (Table 2), ligated into pGEM-T Easy (Promega) and then subcloned as an EcoRI fragment into pBluescript SK(+). A *str* gene was excised from pHP45 (11) with BamHI and was cloned into the BamHI site of the above plasmid, resulting in the *sacB-str* vector, pHBJ428.

Deletion of *rmt4* and *rmt2*. The *rmt4* and *rmt2* genes were separately deleted from *M. smegmatis* by homologous recombination between the chromosome and allelic exchange cassettes carried by the *sacB-str* vector pHBJ428. The cassettes comprised two 1-kb sequences corresponding to regions from the genome on either side of the target gene, and a kanamycin resistance marker (*aph*) replaced the target gene.

For deletion of *rmt4*, the two flanking sequences were amplified and spliced together by splice overlap extension (SOE) PCR with primers 299, 300, 301, and 302 (Table 2) (16). An MscI site was created at the junction of the fragments, and XbaI sites occurred at the termini. The spliced product was cloned into pGEM-T Easy (Promega). The *aph* gene was excised from pK18mob by digestion with NheI and BstBI, cloned, and ligated into the MscI site of the cassette. The cassette was excised from pGEM-T Easy by digestion with XbaI and cloned into an XbaI site on the *sacB-str* vector, resulting in the final construct, pHBJ430. For the deletion of *rmt2*, the upstream and downstream sequences were amplified

TABLE 2. Oligonucleotide primers

Primer	Target or purpose	Sequence (5′–3′)
299	SOE to delete *rmt4*	CCCTCTAGACGTTTCACACTGACCGTAC
300	SOE to delete *rmt4*	CGTGCTTGGCACGGTAATGGCCATAACGTGCGCGTGCTCG
301	SOE to delete *rmt4*	ACGAGCACGCGCACGTTATGGCCATTACCGTGCCAAGCACG
302	SOE to delete *rmT4*	AGCTCTAGACAAACTGAGCTGATCATCG
310	*sacB*	GGGGTACCGCGTCGCTTGGTCGGTCA
382	*sacB*	TTGGTACCTTCTGAGCGGACTCT
383	*rmt4*	TTTGGATCCGACTGATCGCGATAGCCGA
384	*rmt4*	CCCGGATCCATCTCGGCGATCTTCACTC
388	*rmt2*	TTTGGATCCGGGGTGGGCTCGGTCGTC
389	*rmt2*	GCTGGATCCAATATATGAGCGGGCAGACC
428	PCR to delete *rmt2*	ATCTAGAGGGTTCCGGCCTCTCA
442	PCR to delete *rmt2*	TTTCTAGAGTGGTGGGTACAACGCAACTCCTC
443	PCR to delete *rmt2*	CTACATTCGTCGACTGGCCAAACCTCGGCTGTATCACCAAG
444	PCR to delete *rmt2*	ATACAGCCGAGGTTTGGCCAGTCGACGAATGTAGCGATGAA
458	*mtfA*	TGAATCGGTGGGCATTGTCG
459	*mtfA*	GTGCGGCACGGCGAGTTTG
496	*mtfB*	GGATCCGACTGATCGTGACATGCGATC
497	*mtfB*	AAGCTTGCGCCACAGCACGCCG
498	*mtfC*	GGATCCGACCGATCACGACACGCGG
499	*mtfC*	AAGCTTGGGCTTGCGCCACAGCACAC
500	*mtfD*	GGATCCGCACGCCGATGTTATGGTTG
501	*mtfD*	AAGCTTGCGGGCCGGCGTTGATGAG

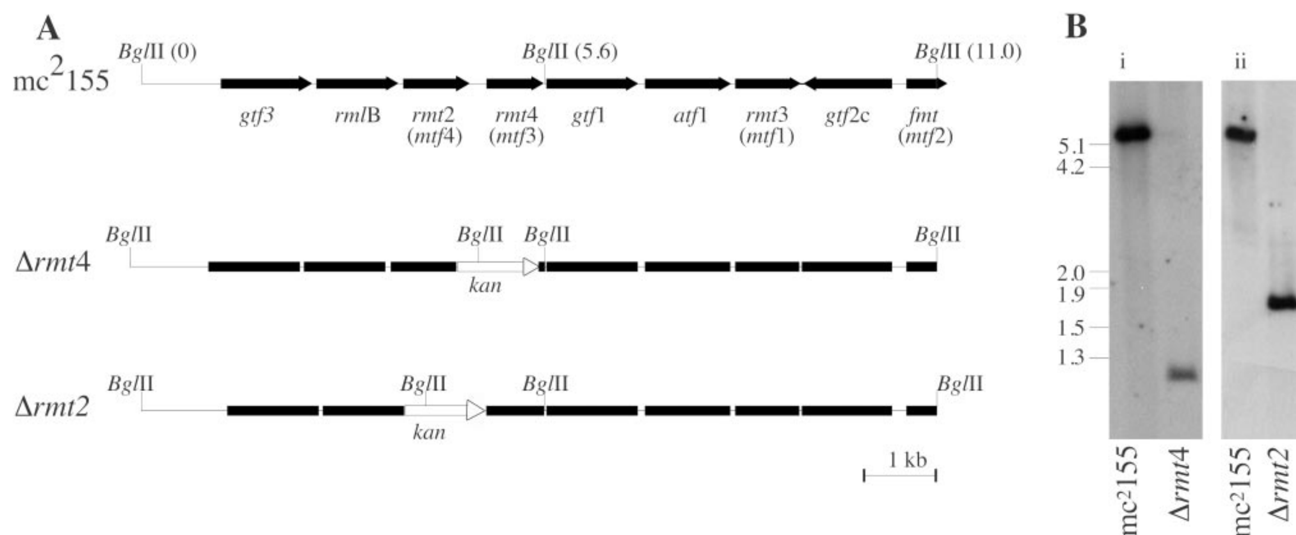

FIG. 1. Restriction maps and Southern blot of GPL locus of Myco657 (Δ*rmt4*) and Myco694 (Δ*rmt2*). (A) Genetic arrangement of part of the GPL locus containing *rmt2*, *rmt4*, *rmt3*, and *fmt*. Positions of the BglII site are shown. The gene names used previously are shown in parentheses. The position where *aph* was inserted is designated *kan*. (B) Southern blot of BglII-digested genomic DNA extracted from *M. smegmatis* mc^{2}155, Myco657 (Δ*rmt4*), and Myco694 (Δ*rmt2*). The blot was probed with digoxigenin-labeled DNA corresponding to *rmt4*. Gene replacement with *aph* introduced an additional BglII site, resulting in smaller hybridizing bands for the deletion mutants.

with primers 442 and 443 and with 444 and 428, respectively (Table 2), joined with an MscI site at the junction, and cloned into pGEM-T Easy. The *aph* gene was cloned into the MscI site, and then the cassette was excised from the pGEM-T Easy by digestion of NotI and cloned into the NotI-digested *sacB-str* vector, resulting in pHBJ488. Both pHBJ430 and pHBJ488 had the target gene replaced with *aph*, which was cloned so that the ORF was in the same direction as the original target gene had been, allowing transcription to proceed beyond the cassette to minimize the chances of polar effects on downstream genes.

The plasmids pHBJ430 and pHBJ488 were introduced into *M. smegmatis*, and then SCO mutants were isolated by selection with kanamycin and streptomycin in 7H10 agar. An SCO mutant was grown in 7H9 broth without antibiotics for 3 days and then plated on 7H10 agar containing kanamycin and sucrose. Survivors were screened for sensitivity to streptomycin, and double-crossover (DXO) mutants (kanamycin-sucrose resistant, streptomycin sensitive) were isolated. The DXO mutants were confirmed by Southern blotting as follows. BglII-digested genomic DNA from a putative DXO mutant was blotted onto a nylon membrane. A probe was prepared by PCR amplification of *rmt4* with primers 383 and 384 (Table 2) and digoxigenin labeled as described above for Southern hybridization. The resulting hybridization pattern (Fig. 1) showed that *rmt4* occurred on a 5.6-kb BglII fragment in the parent strain, mc^{2}155, whereas the band from the DXO mutant was smaller (~0.95 kb), corresponding to the size expected if *rmt4* was replaced by *aph* and a BglII site was introduced (Fig. 1A). Hybridization occurred between the DXO mutant and the *rmt4* probe because the mutant retained 75 bp of *rmt4*. The mutant was named Myco657 (Δ*rmt*4). A putative DXO *rmt2* deletion mutant was obtained and Southern blotted as described for Myco657. Replacement of the *rmt2* gene with *aph* introduced a BglII site; consequently, the hybridizing BglII band was smaller (~1.7 kb) than in the parent strain (5.6 kb). Southern blotting confirmed that *rmt2* had been deleted, and the mutant was designated Myco694 (Δ*rmt2*).

Complementation. A plasmid carrying *rmt4* was created to test complementation of Myco657. The *rmt4* ORF was PCR amplified from *M. smegmatis* genomic DNA with primers 383 and 384, which incorporated BamHI sites at the ends (Table 2). The PCR product was cloned into the BamHI site of pVV16 to produce plasmid pHBJ415. Myco657 (Δ*rmt4*) and mc^{2}155 were then transformed with pHBJ415. The vector pVV16 is a derivative of pMV261 (15) and carries a hygromycin resistance marker. It allows expression of recombinant proteins from cloned genes from the GroEL promoter as fusions with a carboxyl-terminal hexahistidine epitope tag, although the tag was not utilized in these experiments. A plasmid for complementation of Myco694 (Δ*rmt2*) was constructed as follows. The *rmt2* ORF was PCR amplified with primers 388 and 389, which incorporate terminal BamHI sites (Table 2). The PCR fragment was cloned into the BamHI site of pVV16 to create plasmid pHBJ409, which was then introduced into mc^{2}155 and Myco694 (Δ*rmt2*). Controls were transformed with pVV16. Transformants were used in cell compositional analyses.

Cloning *M. avium mtf* genes. The genes *mtfA* to *mtfD* of *M. avium* were PCR amplified from genomic DNA of *M. avium* TMC 724 with the primer pairs 458 and 459 (*mtfA*), 496 (BamHI) and 497 (HindIII) (*mtfB*), 498 (BamHI) and 499 (HindIII) (*mtfC*), and 500 (BamHI) and 501 (HindIII) (*mtfD*) (Table 2). Restriction sites incorporated into the primers are indicated above. In the cases of *mtfB*, *mtfC*, and *mtfD*, the PCR product was cloned into BamHI-HindIII-digested pVV16, whereas the *mtfA* fragment was blunt cloned into the PvuII site. Each plasmid and the vector control were transferred into mc^{2}155, and each *M. smegmatis* methyltransferase mutant, specifically Myco29 (*rmt3*::Tn*611*) (9), Myco493 (*fmt*::*str*) (7), Myco657 (Δ*rmt4*) (this study), and Myco694 (Δ*rmt2*) (this study). The GPL profile of each of the transformed strains was analyzed by high-performance thin-layer chromatography (HPTLC) to determine which of the *M. avium* methyltranfserase genes could complement the *M. smegmatis* mutations and therefore indicate which methyltransferases were functional homologues.

Sequence analysis. A neighbor-joining tree showing the relationship of authentic and putative methyltransferases was constructed using the protdist and neighbor-joining tree programs from PHYLIP (Phylogeny Inference Package) with the default parameters (J. Felsenstein, PHYLIP [Phylogeny Inference Package] version 3.5c, Department of Genetics, University of Washington, Seattle). Bootstrap values were determined using the seqboot program with1,000 bootstraps.

Extraction and compositional analysis of cell wall components. Lipids were extracted from mycobacteria by Folch partitioning as previously described (4, 9). Cell wall lipids were recovered in the chloroform phase and subjected to alkaline methanolysis to cleave ester-linked fatty acids followed by partitioning between water-saturated 1-butanol (4 volumes) and water (2 volumes). The alkali-stable GPLs were analyzed by HPTLC with aluminum-backed Silica Gel 60 HPTLC plates (Merck) and a liquid phase containing chloroform:methanol (9:1 [vol/vol]). Plates were developed with orcinol-H_2SO_4 and charring at 100°C. For monosaccharide compositional analysis, fractions containing GPLs were hydrolyzed in 2 M trifluoroacetic acid (2 h at 100°C), and the acid was removed under a stream of nitrogen. Released monosaccharides were subsequently converted to their alditol acetate derivatives by reduction in sodium borodeuteride ($NaBD_4$) and acetylation in acetic anhydride with 1-methyl-imidazole as a catalyst (5). For gas chromatography-mass spectrometry (GC-MS) analysis, conditions were as previously described (9).

RESULTS

Deletion of *rmt2* and *rmt4*. The *rmt2* and *rmt4* genes were individually deleted from *M. smegmatis*, resulting in two mu-

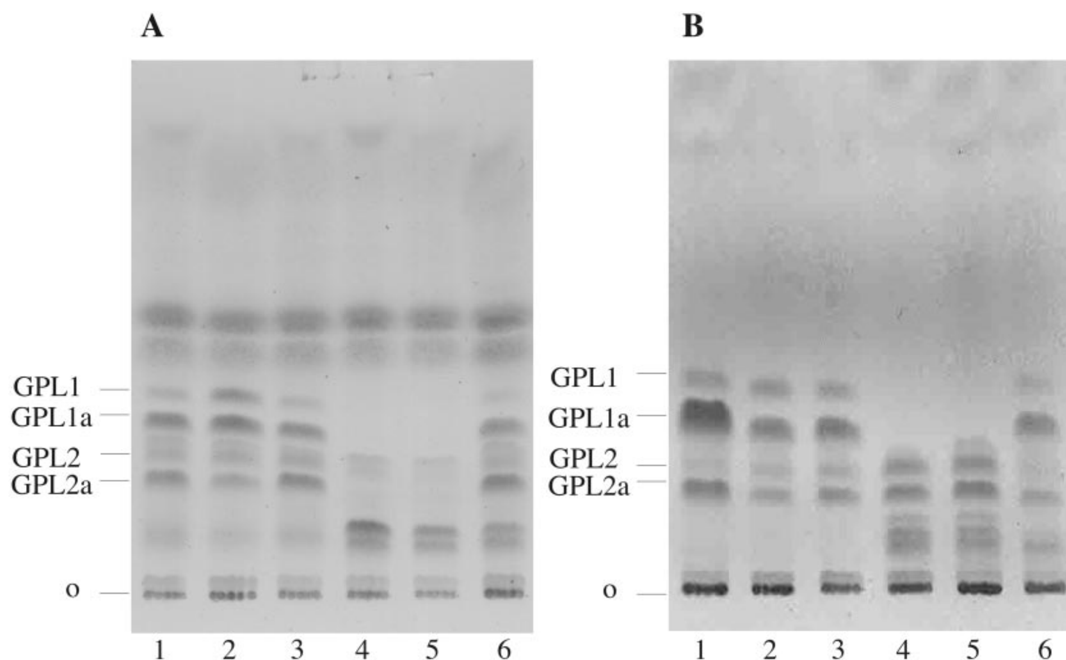

FIG. 2. HPTLC analysis of base-treated GPLs. Lipid extracts of wild-type *M. smegmatis* (lanes 1 to 3) and *rmt* mutants (lanes 4 to 6) were base treated and analyzed by HPTLC. (A) GPL extracts of parent strain *M. smegmatis* mc^{2}155 (lane 1), mc^{2}155 pVV16 vector control (lane 2), mc^{2}155 complementation plasmid bearing *rmt4*, pHBJ415 (lane 3), Myco657 (Δ*rmt4*) (lane 4), Δ*rmt4* pVV16 (lane 5), and Δ*rmt4* pHBJ415 (lane 6). (B) GPL extracts of parent strain *M. smegmatis* mc^{2}155 (lane 1), mc^{2}155 pVV16 vector control (lane 2), mc^{2}155 complementation plasmid bearing *rmt2*, pHBJ409 (lane 3), Myco694 (Δ*rmt2*) (lane 4), Δ*rmt2* pVV16 (lane 5), and Δ*rmt2* pHBJ409 (lane 6). Each of the mutants (lanes 4) has a novel GPL profile compared to the parent strain (lanes 1), and complementation restored mature GPL synthesis to the mutants (lanes 6).

tants, Myco657 (Δ*rmt4*) and Myco694 (Δ*rmt2*). Each mutant had deep rough-colony morphology compared to the parent strain, which is fairly flat and waxy when grown on Middlebrook 7H10 agar. This phenotype is typical of mutants that have defects in GPL synthesis (1). Complementation of these mutants with *rmt4* and *rmt2*, respectively, restored the wild-type colony morphology and mature GPL synthesis (see below) suggesting that the phenotype is directly related to the gene deletion rather than to any polar effects on downstream genes.

Analysis of GPL content of *M. smegmatis* Myco657 (Δ*rmt4*) and Myco694 (Δ*rmt2*). To determine whether the colony morphologies of Myco657 and Myco694 were due to changes in GPL levels or structures, the base-treated GPLs of the wild-type and mutant strains were analyzed by HPTLC (Fig. 2). As shown previously (9) wild-type *M. smegmatis* expresses two major GPL species that migrate as doublets on HPTLC, due to chain length heterogeneity in the amide-linked fatty acid (Fig. 2A, lane 1). Myco657 no longer expressed the GPL-1 doublet and contained very low levels of the GPL-2 doublet. However, a new GPL doublet with slower HPTLC mobility accumulated in this mutant (Fig. 2A, lane 4). Complementation of Myco657 with *rmt4* largely restored synthesis of GPI-1 and GPL-2 to wild-type levels, although low levels of the polar GPL were still detected in the complemented cell line (Fig. 2A, lane 6). To further characterize the nature of the GPL defect in Myco657, the base-treated GPLs were acid hydrolyzed, and the released monosaccharides were analyzed as their alditol acetates by GC-MS. As previously shown, the major GPL species of wild-type *M. smegmatis* contained dTal; 3,4-di-*O*-Me-Rha; and 2,3,4-tri-*O*-Me-Rha (Fig. 3A) (9). In contrast, the Myco657 GPLs contained dTal and 3-*O*-Me-Rha as major sugars and 2,3-di-*O*-Me-Rha as a minor sugar (Fig. 3B). No 2,3,4-tri-*O*-Me-Rha was detected in the Myco657 GPLs (Fig. 3B). Complementation of Myco657 with *rmt4* resulted in the appearance of GPLs with 3-*O*-Me-Rha, 2,3-di-*O*-Me-Rha, and 2,3,4-tri-*O*-Me-Rha (Fig. 3C). These data strongly suggest that the polar GPLs in Myco657 are modified with 3-*O*-Me-Rha and that the enzyme activity associated with Rha 4-*O*-methylation is missing in this Myco657. The presence of low levels of 2,3-di-*O*-Me-Rha in the Myco657 GPLs is consistent with the presence of low levels of GPL-1–GPL-1a in the mutant strain (Fig. 2A). The decrease in dimethylated GPLs in this mutant was consistently observed, suggesting that abrogation of Rha 4-*O*-methylation may reduce the efficiency of Rha 2-*O*-methylation.

The Myco694 (Δ*rmt2*) mutant also lacked the GPL-1 doublet but expressed GPLs that comigrated with GPL-2 and a complex of bands that had a HPTLC mobility similar to that of the polar GPLs of Myco657. GC-MS analysis confirmed that Myco694 lacked trimethylated Rha and that it also contained 3-*O*-Me-Rha. However, levels of 3-*O*-Me-Rha (relative to that of dTal) were lower than in Myco657, and a 3,4-di-*O*-Me-Rha species was observed, rather than 2,3-di-*O*-Me-Rha (Fig. 3D). Traces of GPLs with 3-*O*-Me-Rha were still apparent in the complemented strains, suggesting that partial complementation may have occurred, possibly due to low levels of expression from the complementation plasmids. Complementation restored the synthesis of mature GPLs with trimethylated Rha (Fig. 2B, lane 6, and 3E). These data suggest that Myco694 is deficient in the enzyme activity required for Rha 2-*O*-methyl-

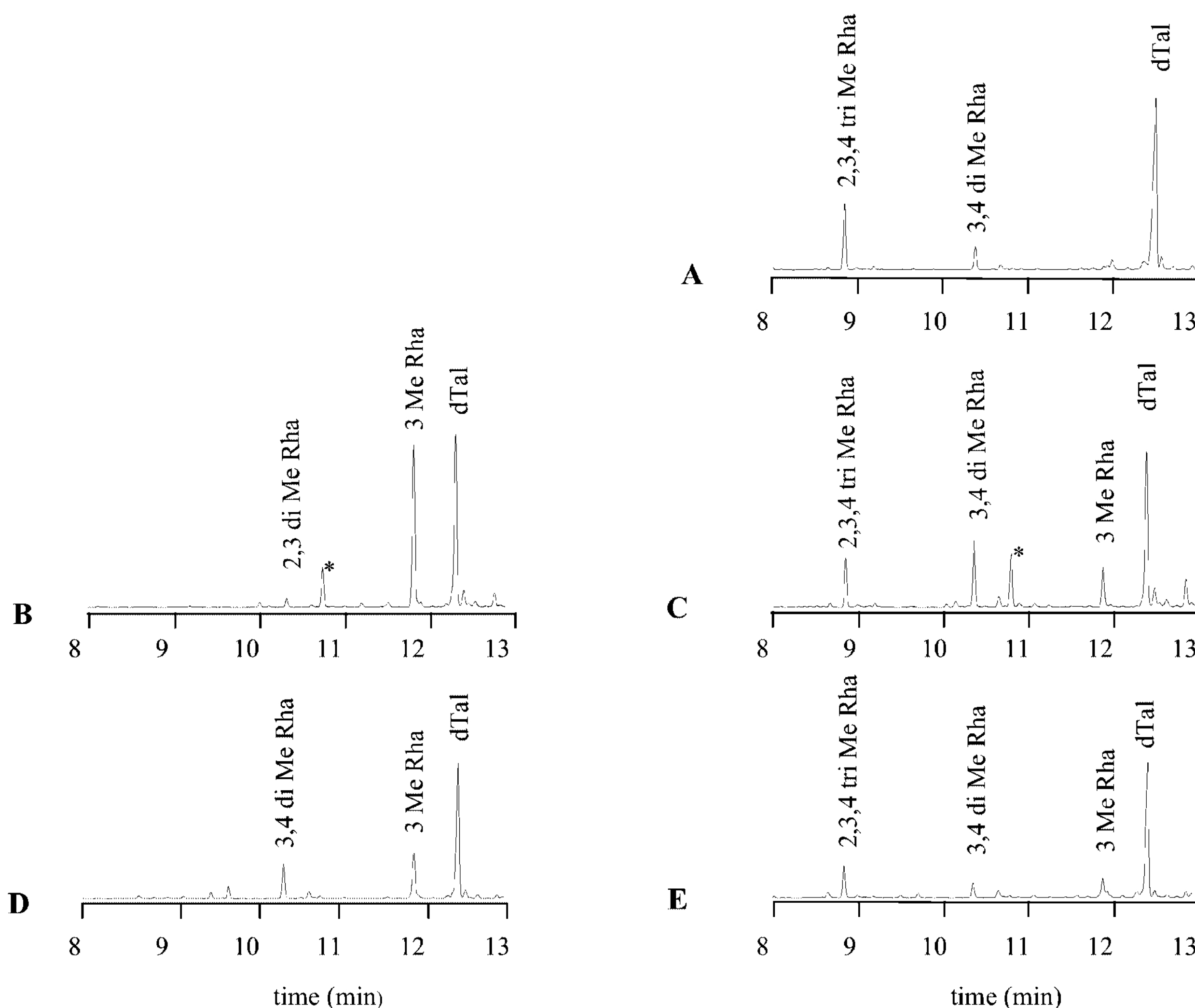

FIG. 3. Monosaccharide analysis of mutant GPLs. Based-treated GPLs from wild-type and mutant cell lines were subjected to acid hydrolysis, and released monosaccharides were analyzed as their corresponding alditol acetate derivatives by GC-MS. (A) *M. smegmatis* mc^{2}155, (B) Δ*rmt4*, (C) Δ*rmt4* pHBJ415, (D) Δ*rmt2*, and (E) Δ*rmt2* pHBJ409. dTal and methylated Rha alditol acetates were identified based on their retention time compared to authentic standards and their mass spectrum. Diagnostic ions for methylated Rha derivatives were as follows: 3-*O*-Me-Rha, *m/z* 101, 130, 143, 190, 203; 2,3-di-*O*-Me-Rha, *m/z* 102, 118, 143, 203; 3,4-di-*O*-Me-Rha, *m/z* 89, 131, 190; and 2,3,4-tri-*O*-Me-Rha, *m/z* 102, 118, 131, 162, 175. Asterisks indicate reagent peaks.

ation. Interestingly, loss of Rha 2-methylation had only a minor affect on the formation of dimethylated GPLs, suggesting that Rha 2-*O*-methylation is not critical for Rha 3- or 4-*O*-methylation.

Methyltransferase gene sequence analysis. The *ser2* locus of *M. avium* contains four ORFs predicted to encode methyltransferases that were annotated *mtfA* through *mtfD* (GenBank accession no. AF143772). Four methylation events are required to make mature *ser2*-encoded GPLs containing 3,4,di-*O*-Me-Rha and 2,3-di-*O*-Me-fucose. Methylation of Rha C_2 does not seem to occur in *M. avium*. We hypothesized that the rhamnosyl 3-*O*-methyltransferase and rhamnosyl 4-*O*-methyltransferases might be conserved between *M. avium* and *M. smegmatis*. The *S*-adenosyl methionine binding site and three other motifs associated with methyltransferases are evident in the *M. smegmatis* methyltransferases and MtfB, MtfC, and MtfD of *M. avium* (7, 9). The methyltransferases can be grouped on the basis of similarity of motifs IV, V, and VI to create a neighbor-joining tree (Fig. 4A). The polypeptides encoded by *M. avium mtfB* and *mtfC* resemble *M. smegmatis* Rmt4, whereas MtfD and Rmt3 are similar. Fmt is dissimilar to the rest of the rhamnosyl methyltransferases but is similar to two putative methyltransferases of *Mycobacterium tuberculosis* encoded by *RV2952* and *RV1523* (2). *M. avium* MtfA is dissimilar to any of the above sequences and is not included in the tree. Other authentic rhamnosyl transferases (ElmMI, ElmMII, and ElmMIII) which methylate Rha during elloramycin synthesis in *Streptomyces olivaceus* were included for comparison (8). ElmMIII, a 4-*O*-methyltransferase, clusters with Rmt4, MtfB, and MtfC. We were interested in determining if the sequence similarity between the methyltransferases correlated with similar enzymatic activities. *M. avium* is difficult to genetically modify, so rather than make *M. avium* mutants, we tested each of the *M. avium ser2*-encoded methyltransferases

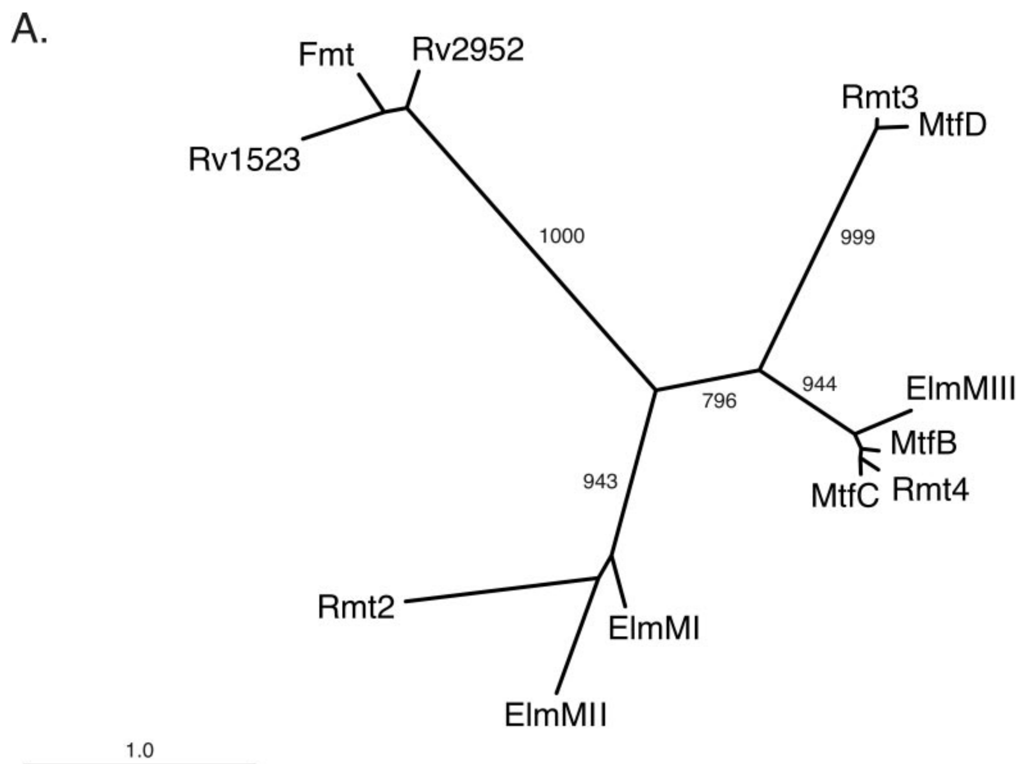

FIG. 4. Neighbor-joining tree showing the similarity between methyltransferase motifs IV and V in the *M. smegmatis* GPL rhamnosyl methyltransferases (Rmt2, Rmt3, and Rmt4), *M. avium ser2* putative methyltransferases (MtfB, -C, and -D), *S. olivaceus* elloramycin rhamnosyl methyltransferases (ElmMI, ElmMII, and ElmMIII), and an authentic fatty acid methyltransferase (Fmt) and two putative methyltransferases from *M. tuberculosis* (Rv2952 and Rv1523). Amino acid sequences used for the tree are shown below. The bootstrap values are shown on the branches.

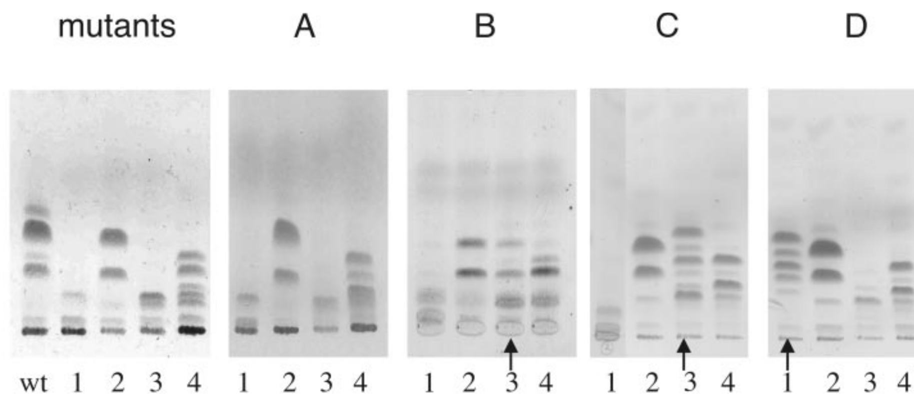

FIG. 5. HPTLC analysis of base-treated GPL extracts from *M. smegmatis* mutants transformed with *M. avium* methyltransferase genes. Lanes 1, host strain Myco29 (*rmt3*::Tn*611*); lanes 2, Myco493 *fmt*::*str*; lanes 3, Myco657 (Δ*rmt4*); and lane 4, Myco694 (Δ*rmt2*). Plasmids containing *M. avium* putative methyltransferase genes (listed in parentheses after the plasmid) were introduced into each strain. (A) Strain transformed with pHBJ475 (*mtfA*); (B) strain transformed with pHBJ512 (*mtfB*); (C) strain transformed with pHBJ505 (*mtfC*); (D) strain transformed with pHBJ502 (*mtfD*). The "mutants" panel shows extracts from untransformed mutants and the parent strain (lane wt). Lanes showing a significant difference from the untransformed control are indicated with arrows.

for their ability to functionally complement our *M. smegmatis* methyltransferase mutants, namely Myco29 (*rmt3*::Tn*611*) (9), Myco493 (*fmt*::*str*) (7), Myco657 (Δ*rmt3*), and Myco694 (Δ*rmt2*).

Cross-species complementation. The ORFs of *mtfA*, *-B*, *-C*, and *-D* were PCR amplified from *M. avium* genomic DNA, and the amplicons were cloned into pVV16. The recombinant plasmids were introduced into *M. smegmatis* mc^{2}155 and into each of the *M. smegmatis* methyltransferase mutants by transformation. The phenotypes of transformants were assessed by HPTLC analysis of base-treated GPL extracts. *M. avium mtfA* did not complement any of the *M. smegmatis* mutants (Fig. 5A), and the GPL profiles for each of the transformed mutant strains appeared the same as those for the untransformed mutant strains (Fig. 5, panel "mutants"). *M. avium mtfB* complemented *M. smegmatis* with an *rmt4* defect (Myco657) (Fig. 5B, lane 3) and restored a wild-type GPL profile. The untransformed mutant makes only GPLs with monomethylated Rha (Fig. 5, panel "mutants," lane 4) whereas the Myco657 mutant containing *mtfB* had mature GPLs. Interestingly, GPL-1a and GPL-2a were more abundant than GPL-1 and GPL-2 in the complemented strain. The result suggests that *mtfB* encodes a methyltransferase that can methylate the hydroxyl group at C_4 of Rha. The result from *mtfC* transformations gave similar results in that it complemented the rhamnosyl 4-*O*-methyltransferase mutant (Δ*rmt4*) but had no effect on the other mutants (Fig. 5C). *M. avium mtfD* complemented the *M. smegmatis rmt3*::Tn*611* mutant previously characterized (9) and was shown to be a rhamnosyl 3-*O*-methyltransferase mutant. In each case where complementation was observed, the morphology of the complemented strains returned to the smooth-colony type. These data show that *M. avium mtfB* and *mtfC* have rhamnosyl 4-*O*-methyltransferase activity and that *mtfD* has rhamnosyl 3-*O*-methyltransferase activity.

DISCUSSION

It has previously been shown that Rmt3 is a rhamnosyl 3-*O*-methyltransferase, while Fmt methylates the hydroxylated fatty acid of the GPL (7, 9). In this study, we provide genetic and biochemical evidence that the genes *rmt4* and *rmt2* encode rhamnosyl 4-*O*-methyl- and rhamnosyl 2-*O*-methyltransferases, respectively. Interestingly, deletion of *rmt2* completely inhibited 2-*O*-methylation of GPL Rha, but only had a minor affect on the formation of GPLs with either 3- and 3,4-di-*O*-methylated Rha. In contrast, deletion of *rmt4* not only inhibited Rha 4-*O*-methylation, but also appeared to prevent efficient 2-*O*-methylation of Rha. Deletion of *rmt2* and *rmt4* had no apparent affect on 3-*O*-methylation of Rha. Finally, deletion of *rmt3* results in complete inhibition of Rha 3-*O*-methylation as well as the accumulation of GPLs with unmethylated Rha (9). Collectively, these results suggest that methylation of the core GPL Rha residue is initiated by the 3-*O*-methyltransferase and that the product of this reaction is sequentially methylated by 4-*O*-methyltransferase and 2-*O*-methyltransferase. As the *rmt3* mutant contained small amounts of a novel GPL with 4-*O*-methylated Rha, it is likely that the 4-*O*-methyltransferase can initiate GPL methylation in the absence of the 3-*O*-methyltransferase, but this reaction is very inefficient (9).

The only other characterized natural product containing trimethylated Rha is elloramycin from *S. olivaceus* (8). The methyltransferases ElmMI, ElmMII, and ElmMIII are responsible for consecutive methylation of the hydroxy groups at the 2′, 3′, and 4′ positions of Rha, respectively. All three of the *S. olivaceus* enzymes have some relaxed substrate specificity in that both ElmMII and ElmMIII can act on unmethylated or partially methylated Rha (8). The *M. smegmatis* enzymes may also have relaxed substrate specificity. Previously, it was observed that GPLs of the *rmt3* mutant had unmethylated Rha and a novel 4-*O*-Me-Rha not observed in the parent strain (9). This mutant is the only mutant studied here that accumulated GPLs with unmodified Rha in detectable quantities. It appears that a 4-*O*-methyltransferase, possibly Rmt4, can use unmodified Rha as a substrate if it is available. Our data show that Rmt4 carries out methylation of the C_4 hydroxy on 3-*O*-Me-Rha and that in the absence of Rmt4, GPLs containing a small amount of novel residue 2,3-di-*O*-Me-Rha can be generated, as well as the expected 3-*O*-Me-Rha. The presence of 2,3-di-*O*-Me-Rha

suggests that a 2-*O*-methyltransferase, possibly Rmt2, can use 3-*O*-Me-Rha as a substrate, whereas Rmt2 usually acts on 3,4-di-*O*-Me-Rha.

The terminal alaninol of GPLs encoded by *ser2 M. avium* is glycosylated with 3,4-di-*O*-Me-Rha and the haptenic side chain comprised comprising 2,3-di-O-Me-fucosyl-(1→3)-rhamnosyl-(1→2)-6-deoxy talose. Four methylation events would be required for full modification of the Rha and fucose. Indeed, there are four predicted methyltransferase genes in the *ser2* locus of *M. avium*. The polypeptide sequence of *M. avium* MtfD is most similar to that of *M. smegmatis* Rmt3, whereas MtfB, MtfBC, and Rmt4 are very similar to each other. None of the *M. avium* genes are similar to *fmt* (8). We cloned each of the *M. avium* methyltransferase genes (*mtfA*, *mtfB*, *mtfC*, and *mtfD*) into a shuttle vector and introduced them into wild-type *M. smegmatis* and each of the mutants defective in GPL methylation. MtfD expression functionally complemented our *rmt3* mutant, suggesting that MtfD is a rhamnosyl 3-*O*-methyltransferase. Both MtfB and MtfC complemented the *rmt4* mutant, suggesting that they have rhamnosyl 4-*O*-methyltransferase activity. These enzymes would be sufficient to fully methylate terminal Rha on *M. avium* GPLs. MtfA did not complement any of the *M. smegmatis* mutants, raising the possibility that it is involved in methylating the terminal fucose in *M. avium* GPLs. It is possible that MtfB, MtfC, or MtfD also has fucosyl methyltransferase activity that would not be detected in our experiments. None of the *M. avium* genes complemented the *fmt* mutant, which was not unexpected because the fatty acid is not methylated in *M. avium*.

Only a few *O*-methyltransferases that modify sugars have been identified, and those are involved in macrolide synthesis (89). The *O*-methyltransferases that modify Rha during GPL synthesis in *M. avium* and *M. smegmatis* expand this group considerably. Further characterization of the activities of these enzymes may show how they could be used as tools to generate bioactive compounds.

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