Genetic Basis for the Biosynthesis of Methylglucose Lipopolysaccharides in Mycobacterium tuberculosis

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Mycobacteria produce two unusual polymethylated polysaccharides, the 6-O-methylglucosyl-containing lipopolysaccharides (MGLP) and the 3-O-methylmannosyl polysaccharides, which have been shown to regulate fatty acid biosynthesis in vitro. A cluster of genes dedicated to the synthesis of MGLP was identified in Mycobacterium tuberculosis and Mycobacterium smegmatis. Overexpression of the putative glycosyltransferase gene Rs3032 in M. smegmatis greatly stimulated MGLP production, whereas the targeted disruption of Rs3032 in M. tuberculosis and that of the putative methyltransferase gene MSMEG2349 in M. smegmatis resulted in a dramatic reduction in the amounts of MGLP synthesized and in the accumulation of precursors of these molecules. Disruption of Rs3032 also led to a significant decrease in the glycogen content of the tubercle bacillus, indicating that the product of this gene is likely to be involved in the elongation of more than one α-(1→4)-glucan in this bacterium. Results thus suggest that Rs3032 encodes the α-(1→4)-glucosyltransferase responsible for the elongation of MGLP, whereas MSMEG2349 encodes the O-methyltransferase required for the 6-O-methylation of these compounds.

Mycobacteria produce two unusual polymethylated polysaccharides (PMPS), the 3-O-methylmannosyl polysaccharides (MMP) (1–2) and the 6-O-methylglucosyl-containing lipopolysaccharides (MGLP) (3, 4). Both polysaccharides localize to the cytoplasm, where they have been postulated to regulate fatty acid synthesis by FAS-I as a consequence of their ability to form stable 1:1 complexes with long-chain fatty acids and acyl-coenzyme A derivatives (5–9) (for a review, see Ref. 10). PMPs were also proposed to protect fatty acid products from degradation and to serve as general lipid carriers facilitating the synthesis of the very large and insoluble mycolic acid esters while at the same time increasing the tolerance of mycobacteria to high cytoplasmic concentrations of long-chain acyl-CoA derivatives (10–12).

PMPs were first isolated from Mycobacterium phlei, Mycobacterium smegmatis, and Mycobacterium tuberculosis in the 1960s (2–4), and much of the information we have about the structure, biosynthesis, and biological activities of these molecules comes from this early work. Others then revised the structure of MGLP and extended the analysis of these molecules to other mycobacterial species (13–15). The structures of MGLP and MMP are shown in Fig. 1. MMP have been found in multiple nonpathogenic fast growing species of mycobacteria (16) and in Streptomyces griseus (17), whereas MGLP have been isolated from several Nocardia species as well as M. phlei, M. smegmatis, Mycobacterium bovis BCG, M. tuberculosis, M. leprae, and M. xenopi (3–4, 13–15, 18, 19).

Ballou and co-workers (2, 16, 20, 21) isolated precursors of MMP and characterized an α-(1→4)-mannosyltransferase and a 3-O-methyltransferase from cell-free extracts of M. smegmatis. These studies led to a biosynthetic model in which MMP is elongated by a linear alternating process of mannosylation followed by O-methylation, in which GDP-Man serves as the sugar donor for the mannosyltransferase and 5-adenosylmethionine serves as the source of methyl groups. Termination of the elongation reaction occurs when the length of the chain is sufficient to confer on the polysaccharide fatty acid-binding properties (11–13 3-O-methylmannoses). At this stage, the chain is terminated with an unmethylated mannose because the acyl-CoA-bound oligosaccharides are no longer available as acceptors for the 3-O-methyltransferase.

Knowledge of the initiation, elongation, and termination reactions involved in the biosynthesis of MGLP is more limited. A membrane-associated acyltransferase activity responsible for the transfer of acetyl, propionyl, isobutyryl, octanoyl, and succinyl groups from their respective acyl-CoA derivatives onto...
mycobacteria, the biosynthesis of PMPS had not been reinvestigated in the postgenomic era.

**EXPERIMENTAL PROCEDURES**

Construction of the M. tuberculosis and M. smegmatis Glucosyltransferase and Methyltransferase Mutants—The ts-sacB method (26) was used to achieve allelic replacement at the Rv3032 locus of M. tuberculosis (ATCC number 25618) and at the MSEG2349 locus of M. smegmatis mc²155. The M. tuberculosis Rv3032 gene and flanking regions was PCR-amplified from M. tuberculosis H37Rv genomic DNA using primers Rv3032.1 (5′-gggctgcagatgcggccggcgtggcgc-3′)/Rv3032.2 (5′-tgagaagctctccctccctgg-3′), and a disrupted allele, Rv3032::kan, was obtained by inserting the kanamycin resistance cassette from pUC4K (Amersham Biosciences) into the Smal restriction site of Rv3032. Rv3032::kan was then cloned into the Ntl-cut and blunt-ended pPR27-xyE (26) to obtain pPR27Rv3032KX, the construct used for allelic replacement in M. tuberculosis. The M. smegmatis MSEG2349 gene and flanking regions was PCR-amplified using the primers Ms3030f (5′-cgcttcgacaaatcacc-3′) and Ms3030r (5′-ctgctacatggagacgcctcgg-3′). A disrupted copy of MSEG2349 was obtained by substituting 152 bp of the coding region of this gene bracketed between two Sall sites by the Kan cassette from pUC4K. MSEG2349::kan was then cloned into the XbaI-cut pJQ200-xyE, yielding pJQMSMEG2349KX.

Overexpression of Rv3032 in M. smegmatis and Complementation Studies—The entire coding sequence of Rv3032 was PCR-amplified from M. tuberculosis H37Rv genomic DNA using the primers Rv3032.3 (5′-gggctgcagatgcggccggcgtggcgtg-3′) and Rv3032.4 (5′-ggagaagctctccctccctgggctctcc-3′) and cloned into the Ndel and HindIII restriction sites of the expression vector pVV16 (27), yielding pVV/Rv3032. The production of recombinant Rv3032 protein in M. smegmatis and M. tuberculosis was analyzed by immunoblotting with the monoclonal Penta-His antibody from Qiagen as described previously (27).

Whole Cell Radiolabeling Experiments—Radiolabeling of whole M. smegmatis and M. tuberculosis cells with [1,2-¹⁴C]acetate (0.5 μCi ml⁻¹; specific activity, 113 Ci mol⁻¹), MP Biomedicals Inc.) was performed at 37 °C in 7H9-ADC-Tween 80 broth for 14 h with shaking. Radiolabeling with [methyl-¹⁴C]-methionine (0.5 μCi ml⁻¹; specific activity, 68 Ci

**FIGURE 1. Structures of the mycobacterial PMPS.** A, the MMP from M. smegmatis is shown. They are composed of 10–13 (n = 10–13) α-(1→4)-linked 3-O-methyl-D-mannoses terminated at the nonreducing end by a single α-(1→4)-linked unmethylated D-mannose and at the reducing end by an α-methyl a-glucosyl side chain. MMP occur in the cells as a mixture of at least four isomers due to differences in size and degree of O-methylation. B, MGLP from M. bovis BCG. MGLP are composed of 10 α-(1→4)-linked 6-O-methylglucosyl residues with a nonreducing end made of the tetrasaccharide 3-O-methyl-D-Glc-p-(α-(1→4)-D-Glc)p-α(1→). The tetrasaccharide 3-O-methyl-D-Glc-p-(α-(1→4)-D-Glc)p-(α-(1→6)-D-Glc)p-α(1→) linked to the position 2 of α-glyceric acid constitutes the reducing end of the molecule. Position 3 of the second and fourth α-D-Glc residues are substituted by single β-D-Glc residues. The nonreducing end of the polymer is acylated by a combination of acetate, propionate, and isobutyrate (26). Since these early studies, and despite the important roles PMPS had not been reinvestigated in the postgenomic era.

purified MGLP and partially acetylated α-(1→4)-linked D-gluco-oligosaccharides has been described (22). A soluble protein fraction from M. phlei capable of catalyzing the transfer of methyl groups from S-adenosylmethionine to positions 6 and 3 of MGLP and partially acetylated α-(1→4)-D-gluco-oligosaccharides has also been reported (23, 24). Since the position of the O-methylation on the oligosaccharide acceptor was dependent on its degree of acetylation, it was suggested that acylation and O-methylation occurred together during the biosynthesis of the lipopolysaccharide, the former process exerting a control on the latter. The characterisation of weakly acidic and partially O-methylated methylglucosyl-containing polysaccharide precursors from M. smegmatis led Kamisango et al. (25) to propose a model for the biosynthesis of MGLP in which the elongation of the chain proceeds stepwise, from the reducing end toward the nonreducing end, through a sequential glucosylation-methylation reaction. Although at least one α-(1→4)-glucosyltransferase is expected to be required for the elongation of the glucan backbone of MGLP, no such enzymatic activity had been reported.

Since these early studies, and despite the important roles PMPS might play in the regulation of fatty acid metabolism in
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mol⁻¹, MP Biomedicals Inc.) was performed at 37 °C in Sauton medium for 48 h (M. tuberculosis) or 24 h (M. smegmatis).

Preparation, Purification, and Analysis of MGLP—for the preparation of PMPs from cold or radiolabeled cultures, M. smegmatis and M. tuberculosis were grown in Sauton’s medium as surface pellicles. Total lipids and PMPs were extracted with chloroform/methanol (1:2) followed by two other extractions with chloroform/methanol (2:1). The pooled and dried organic extracts were then partitioned between chloroform and water (1:1), and the MGLP and MMP contained in the aqueous phase were further purified by reverse phase chromatography on Sep-Pak® Plus tC18 columns (Waters). Fractions were eluted with increasing concentrations of methanol in water, and the MMP and MGLP content of each fraction was determined by TLC and gas chromatography analysis of alditol acetates derived from these fractions (28). TLC analyses were performed on aluminum-backed silica gel 60-precoated plates F254 (Merck) using chloroform/methanol/water (56:38:10) as the eluent.

PMPs were recovered in the fractions eluted with 40 and 70% methanol in water in which they were estimated to be 75–90% pure (supplemental Fig. 1).Dry MGLP were decylated in 1 M NaOH at 37 °C for 3 h, yielding MGP. The mixture was neutralized with 0.2 M HCl and desalted using reverse phase SepPak® Plus tC18 (Waters) chromatography as described above.

Matrix-assisted Laser Desorption Ionization Time-of-flight (MALDI-TOF) Mass Spectrometry (MS) analysis of purified MGLP, MGP, and MMP samples was performed on a 4700 Proteomics Analyzer MALDI TOF/TOF system (Applied Biosystems, Framingham, MA) equipped with a pulsed Nd:YAG laser emitting at 355 nm and operating at 200 Hz. Typically, 0.3 μl of the sample in water was mixed with 0.3 μl of matrix solution (2-(4-hydroxyphenylazo)benzoic acid at a concentration of ~10 mg ml⁻¹) in ethanol/water (1:1) directly onto the target plate. All analyses were analyzed in reflector mode using both positive and negative ion detection. Final spectra were obtained by accumulating 2,500 subspectra generated by 250 laser pulses on 10 distinct areas of the dried droplet and calibrated using the instrument’s external calibration procedure.

Analysis of Glycogen and Glucan—for the analysis of glycogen and α-d-glucan, M. tuberculosis strains were grown in Sauton’s medium as surface pellicles. High molecular weight carbohydrates and proteins were precipitated from 10-fold concentrated culture filtrates with six volumes of cold ethanol overnight at 4 °C, as described (29). Precipitates were recovered by centrifugation at 14,000 × g for 1 h, dissolved in distilled water, dialyzed for 1 day against water to eliminate traces of salts and glycerol, lyophilized, and weighted. The extracellular α-d-glucan content was determined by gas chromatography of sugar derivatives resulting from the acid hydrolysis of the lyophilized macromolecules. Further purification of α-d-glucan was carried out by anion-exchange chromatography on a DEAE-trisacryl gel, and the neutral fractions were rechromatographed on a Bio-Gel P-60 column, as previously described (30).

For the purification of glycogen, heat-killed cells were weighted, suspended in water, and centrifuged twice at 1,100 × g for 15 min to remove loosely attached compounds, among them the extracellular α-d-glucan. Washed cells were broken in a French press at 140 bars to liberate the intracellular glycogen. The material was centrifuged at 1,100 × g for 30 min to eliminate nonbroken cells, and the supernatant was recentrifuged at 27,000 × g for 15 min to remove cell walls. Glycogen was extracted as described (31) with only minor modifications.

RESULTS

Identification of a Cluster of Genes Potentially Involved in the Synthesis of MGLP—Glucosyltransferase(s) likely to be involved in the elongation of MGLP were searched within the CAZy classification of glycosyltransferases (GTs) (Carbohydrate-Active enZymes; available on the World Wide Web). The cytosolic localization of MGLP suggested that the glucosyltransferase(s) probably used ADP-β-Glc or UDP-β-Glc as β-Glc donor. The fact that these lipopolysaccharides essentially consist of α-(1→4)-linked β-Glc residues further suggested that the glucosyltransferase(s) used a retaining type of catalytic mechanism for sugar transfer, leading to a glycosidic bond in α-configuration. The CAZy GT-4 family is the largest family of NDP-sugar-dependent retaining GTs. Enzymes from this family carry a conserved motif (D/E)XXXD proposed to be involved in the binding of the sugar-nucleotide donor. M. tuberculosis H37Rv contains seven representatives of this family, among which three (PimA, PimB, and MshA) have been functionally characterized in mycobacteria or related species and shown to participate in the biosynthesis of phosphatidylinositol mannosides, glycosylated diacylglycerols, and mycolith (27, 32–34). Among the four remaining GTs, Rv1212c and Rv3032 showed the greatest level of sequence similarity with the glycogen synthase from E. coli (GlgA) (46.7 and 46% similarity at the amino acid level, respectively). Rv1212c is orthologous to the Corynebacterium glutamicum glycogen synthase gene, glgA, with which it shares 71.8% similarity (60.3% identity) at the amino acid level (35). In contrast, no functional information was available for Rv3032. Interestingly, this gene displayed a limited distribution within prokaryotes, having orthologs only in mycobacterial spp. (M. tuberculosis H37Rv and CDC1551, M. bovis, M. leprae, M. smegmatis, Mycobacterium avium, M. avium subsp. paratuberculosis, Mycobacterium ulcerans, M. flave-scens) and Nocardia farcinica. A closer examination of the genomic region encompassing Rv3032 in M. tuberculosis H37Rv revealed that, adjacent or in close vicinity to this putative glucosyltransferase gene, lay a putative acetyltransferase gene (Rv3034c), two putative S-adenosyl-methionine-dependent-methyltransferase genes (Rv3030 and Rv3037c), and a putative α-amylase/glucoside hydrolyase/GH-57 family branching enzyme gene (Rv3031) (Fig. 2). Given the likely involvement of some if not all of these genes in MGLP biosynthesis, efforts were focused on this cluster of genes and, more particularly, on Rv3032 and Rv3030.

Construction of M. smegmatis and M. tuberculosis Glucosyltransferase and Methyltransferase Knock-out Mutants and Phenotypic Analyses—The putative glucosyltransferase gene, Rv3032, and methyltransferase gene, MSMEG2349 (orthologous to Rv3030 of M. tuberculosis), were disrupted by homologous recombination in M. tuberculosis H37Rv and M. smegmatis, respectively, using the ts-sacB method (26). Allelic replacements at the Rv3032 and MSMEG2349 loci were con-
firmed by Southern hybridization (data not shown). Interestingly, the growth of H37Rv/H9004Rv3032 was slightly slower than that of wild-type H37Rv in 7H9 broth at 37 °C and completely abolished at a higher temperature (39 °C) (supplemental Fig. 2). Likewise, the mc2/H9004MSMEG2349 mutant did not grow at high temperature (42 °C) in Sauton’s medium, although its growth was comparable with that of mc2155 at 30 and 37 °C in this medium (data not shown). An analysis of the MGLP content of the wild-type and mutant strains upon metabolic labeling with [methyl-14C]L-methionine revealed that the amount of radioactivity incorporated into the MGLP of the H37Rv/H9004Rv3032 and mc2/H9004MSMEG2349 mutants was only 17 and 38%, respectively, of that incorporated by their wild-type parent (Figs. 3A and 4A). Comparison of the yields of MGLP from the different strains also supported these conclusions in that, depending on the growth phase, the amounts of lipopolysaccharides recovered from H37Rv/H9004Rv3032 were 20–60% less than that recovered from wild-type M. tuberculosis H37Rv. Recovery from mc2/MSMEG2349 was significantly less than that from wild-type M. smegmatis. However, quantification was not possible due to the important amounts of MMP co-eluting with MGLP in this Mycobacterium species (see supplemental Fig. 3A and 4A).

FIGURE 3. MGLP composition of the wild-type and Rv3032 mutant strains of M. tuberculosis H37Rv. A, TLC analysis of the MGLP from wild-type H37Rv (1), H37RvΔRv3032 (2), and H37RvΔRv3032/pVVRv3032 (3). B, partial high mass range (m/z 2750–3700) MALDI mass spectra in the negative ion detection mode of the purified MGP (deacylated MGLP) from the M. tuberculosis wild-type, mutant, and complemented mutant strains. All annotated peaks correspond to [M – H] pseudomolecular ions with the exception of those labeled with one or two asterisks attributed to [M – 2H + Na] and [M – 3H + 2Na] ions, respectively.

FIGURE 4. MGLP composition of the wild-type and MSMEG2349 mutant strains of M. smegmatis. A, TLC analysis of the MGLP from mc2155 (1) and mc2ΔMSMEG2349 (2). B, partial high mass range (m/z 2750–3700) MALDI mass spectra in the negative ion detection mode of the purified MGP (deacylated MGLP) from the M. smegmatis wild-type and mutant strains. All annotated peaks correspond to [M – H] pseudomolecular ions with the exception of those labeled with an asterisk attributed to [M – 2H + Na] ions.
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It is noteworthy that MMP could not be detected in the extracts or in the purified PMPS fractions of *M. tuberculosis*, neither by MALDI-MS nor by TLC (supplemental Fig. 1). Consistent with this observation, gas chromatography analyses of the alditol acetates derived from purified PMPS revealed the presence of 3-O-methylmannose in the O-methylated polysaccharide fractions from *M. smegmatis* but not in those from *M. tuberculosis* (strains H37Rv and Mt103) (data not shown).

Complementation of H37RvΔRv3032 with a wild-type copy of Rv3032 carried by the multicopy plasmid pVVRv3032 restored the production of MGLP in the mutant, thereby confirming the involvement of Rv3032 in MGLP synthesis (Fig. 3A). Attempts to complement the mc2ΔMSMEG2349 mutant with Rv3030 from *M. tuberculosis* carried by the multicopy expression plasmid pVV16 were not successful due to the apparent toxic effect of overexpressing this gene in expression plasmid pVV16.

The fact that significant differences in the oligosaccharide backbones of MGP19,11 and MGP20,12 (MGP containing 19 and 20 glucose residues) were not successful due to the apparent toxic effect of overexpressing this gene in expression plasmid pVV16 was not sufficient to conclude that the synthesis of the capsular glucan was unaffected in the mutant. This suggests that the complex MGLP pattern described above resulted from variable combinations of acylation of mainly two oligosaccharide backbones, MGP_{19,12} and MGP_{20,12} and, to a much lesser extent, MGP_{20,11} and MGP_{20,13}.

As shown in Figs. 3B and 4B, the disruptions of Rv3032 and MSMEG2349 had significant impacts on the MGLP patterns of *M. tuberculosis* and *M. smegmatis*. H37RvΔRv3032 accumulated polysaccharidic compounds with lower masses, consisting essentially of 17 Glc residues (MGP_{17,9}, MGP_{17,10}, MGP_{17,11}), with lesser quantities of MGP_{16,10}, MGP_{18,10}, MGP_{18,11}, MGP_{18,12}, and MGP_{19,12}. More glycosylated forms of MGLP could not be detected in this strain. Complementation of the mutant partially restored MGP synthesis, resulting in a clear shift of its MGP profile toward the mature forms of these polysaccharides (MGP_{19,12} and MGP_{20,12}) (Fig. 3B). MS analysis of the MGP from the MSMEG2349 mutant also revealed a shift in the types of MGP produced from the mature to the less glycosylated forms of these molecules, with a clear accumulation of MGP_{16,11} and MGP_{16,12} ([m/z 2851 and m/z 2865, respectively) (Fig. 4B).

In conclusion, the analyses performed on the two mutant strains indicate that Rv3032 in *M. tuberculosis* and MSMEG2349 in *M. smegmatis* participate in the biosynthesis of MGLP, although compensatory glucosyltransferase and O-methyltransferase activities exist in these species, allowing the knock-out mutants to produce residual quantities of glycosylated PMPS.

**Disruption of Rv3032 Also Affects the Glycogen Content of M. tuberculosis**—The likely involvement of Rv3032 in the formation of α-(1→4)-glucosidic bonds prompted us to further analyze the glycogen (36) and capsular α-D-glucan (29) content of H37RvΔRv3032. Analyses performed on two independent cultures of *M. tuberculosis* wild-type and Rv3032 mutant strains revealed an important decrease (44.5 ± 9.0%) in the glycogen content of the mutant relative to the wild-type strain. This tendency was partially reversed upon complementation of the mutant with pVVRv3032 (the glycogen content of the complemented mutant was 74 ± 0.6% of that of the wild-type strain).

To analyze the impact of the inactivation of Rv3032 on the production of glucan, which represents the major extracellular polysaccharide of *M. tuberculosis* (29), the ratios of Glc to the monosaccharide constituents of the other extracellular polysaccharides (i.e. arabinomannan and mannan) (29) (Glc versus Ara + Man + Glc) were determined in the culture filtrates of three or four independent batches of the wild-type and mutant strains. Very similar ratios were obtained (0.56 ± 0.07 and 0.58 ± 0.09, respectively) for the different batches and strains, indicating that the synthesis of the capsular glucan was unaffected in the mutant. This suggests that Rv3032 is not involved in the synthesis of the capsular polysaccharide or that residual α-(1→4)-glucosyltransferase activity (or activities) in the mutant cells is preferentially utilized to synthesize glucan. The glycolgen and glucan produced by the mutant were otherwise structurally identical to those of *M. tuberculosis* H37Rv as determined by 1H NMR analyses (data not shown).

Altogether, our results implicated Rv3032 in the synthesis of two different α-(1→4)-linked glucans in *M. tuberculosis*: MGLP and glycogen.

**Effect of Overexpressing Rv3032 in M. smegmatis**—To further study the role of Rv3032 in MGLP synthesis, we analyzed the
Rv3032 is the main mc2155/pVVRv3032. M. smegmatis Rv3032 catalyzes presented elsewhere, indicated that a purified recombinant form of assumption, preliminary cell-free assays, which will be pre-
supported this, suggesting that Rv3032 is the main the elongation of the lipopolysaccharides. Supporting this
tumylatory effect the overexpression of Rv3032 had on the
production of mature MGLP in M. smegmatis strongly suggest
that Rv3032 is the main α-(1→4)-glucosyltransferase committed in the elongation of those lipopolysaccharides. Likewise, the similar phenotype that arose from the genetic disruption of MSMEG2349 in M. smegmatis suggests that this gene encodes the main O-methyltransferase of the polysaccharide backbone of MGLP. Important information derived from the analysis of the M. smegmatis mutant is that a defect in O-methylation abolishes MGLP synthesis. Thus, despite both the α-(1→4)-glucosyltransferase and the 6-O-methyltransferase being active on unmethylated α-(1→4)-α-gluco-oligosaccharides in vitro (23–24), the elongation of MGLP in whole bacterial cells appears to proceed with glucosylation and O-methylation occurring hand in hand. This observation is consistent with the biosynthetic model proposed by Kamisango et al. (25) based on the structural anal-
ysis of MGLP precursors.

The residual quantities and types of MGLP found in H37RvΔRv3032 and mc2ΔMSMEG2349 imply that compensatory glucosyltransferase and O-methyltransferase activities exist allowing M. tuberculosis and M. smegmatis to synthesize basal amounts of wild-type MGLP. According to our bioinformatics analyses, an obvious α-(1→4)-glucosyltransferase gene candidate in M. tuberculosis H37Rv is Rv1212c, the ortholog of the glycogen synthase gene from Corynebacterium glutamicum (35). Work is in progress in our laboratories to study the involvement of this gene in glycogen and MGLP synthesis and to generate a double Rv3032/Rv1212c knock-out mutant of M. tuberculosis.

DISCUSSION

The availability of a growing number of mycobacterial genome sequences and the rapidly growing knowledge of the processes underlying sugar transfer in these bacteria (37) have opened the way to the elucidation of the biosynthetic pathways of complex glycoconjugates in M. tuberculosis. This work was undertaken with the aims of investigating the biosynthetic pathway of MGLP. A cluster of genes relatively conserved within the Mycobacterium genus was identified, among which the glucosyltransferase gene Rv3032 and the ortholog of the O-methyltransferase gene Rv3030 in M. smegmatis were studied. Also present within the gene cluster is Rv3031, annotated as a putative α-amylase in the genome of M. tuberculosis H37Rv, Rv3034c, annotated as a putative acetyltransferase, and Rv3037c, encoding a putative S-adenosylmethionine-dependent methyltransferase of unknown function. Sequence similarities between Rv3031 and a GH-57 family branching enzyme from Thermococcus kodakaraensis (38) suggest that this enzyme is involved in generating the α(1→6) glycosidic bond linking the first and second d-Glc residues at the reducing end of the molecule.

In M. tuberculosis, Rv3032 participates in the production of gly-
cogen and MGLP. It also stimu-
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An intriguing preliminary observation that arose from our study is the fact that, contrary to M. smegmatis, M. tuberculosis (strains H37Rv and Mt103) does not produce detectable amounts of MMP. Although more isolates will have to be tested before a definitive conclusion can be drawn, these results and the fact that MMP have so far only been reported in fast growing nonpathogenic species of mycobacteria (16) raise the interesting possibility that mannosylated PMPS may not be produced by slow growing mycobacterial species.

Due to the existence of compensatory enzymatic activities and to the dual involvement of Rv3032 in MGLP and glycogen synthesis, it is at present not possible to draw any conclusions as to the potential essentiality and physiological role(s) of MGLP in mycobacteria. Answers to these questions will require the construction of an M. tuberculosis mutant totally deficient in their synthesis, a goal that is likely to be achievable through the individual or combined inactivation of the branching enzyme, O-methyltransferase and acetyltransferase genes, Rv3031, Rv3030, Rv3037c, and Rv3034c.

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