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Polymethylated Polysaccharides from *Mycobacterium* Species Revisited*[S]

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Mary Jackson and Patrick J. Brennan[1]

From the Department of Microbiology, Immunology, and Pathology, Colorado State University, Fort Collins, Colorado 80523-1682

Mycobacteria produce two sets of unusual polymethylated polysaccharides, the 3-*O*-methylmannose polysaccharides and the 6-*O*-methylglucose lipopolysaccharides. Both polysaccharides localize to the cytoplasm, where they have been postulated to regulate fatty acid metabolism due to their ability to form stable 1:1 complexes with fatty acyl chains. Physiological evidence for this assumption is lacking, however. Recent advances in our knowledge of the processes underlying sugar transfer in mycobacteria, together with the availability of genome sequences and tools for the genetic manipulation of these microorganisms, have opened the way to the elucidation of the biosynthetic pathways and biological functions of these unique carbohydrates.

Mycobacterium spp. are a source of unique carbohydrates (1) whose structural oddities have stimulated the interest of many scientists both from a fundamental perspective and as a source of potential antigens and targets for new vaccines, diagnostics, and drugs for tuberculosis. Despite this, relatively little is known about the physiological functions and regulation of most of these compounds, and only in recent years have the genetics of their biosynthesis started to be explored.

One such family of unusual carbohydrates unique to the order Actinomycetales is the polymethylated polysaccharides (PMPS).[2] PMPS are cytoplasmic (lipo)polysaccharides of intermediate size composed of 10–20 sugar units, many of which are partially *O*-methylated, thus conferring on the molecules a slight hydrophobicity. Mycobacteria produce two such classes. One class is known as the 3-*O*-methylmannose polysaccharides (MMPs) and the second as the 6-*O*-methylglucose lipopolysaccharides (MGLPs) (Fig. 1). A remarkable property associated with MGLPs and MMPs is their ability to form stable 1:1 complexes with long-chain fatty acids and acyl-coenzyme A derivatives *in vitro*, resulting in the suggestion that they may be important regulators of lipid metabolism in mycobacteria.

PMPS were first isolated from *Mycobacterium phlei*, *Mycobacterium smegmatis*, and *Mycobacterium tuberculosis* in Dr. Clinton Ballou's laboratory (2–4), and much of our present information on these molecules comes from the early work of this group. Indeed, from the mid-1960s until the end of the 1980s, Ballou and associates spent more than 20 years characterizing the structures, exploring the biosynthetic pathways, and, in parallel with Konrad Bloch's laboratory, studying the biological activities of these unusual polysaccharides. Even though others later revised the structure of MGLPs and extended the analysis of these molecules to other mycobacterial species (5–7), since the end of the 1980s, no further work had been undertaken on the biogenesis of PMPS, and physiological evidence for a role of these molecules in the regulation of fatty acid metabolism is still lacking. The availability of a growing number of mycobacterial genome sequences combined with efficient tools for the genetic manipulation of these bacteria has prompted new research on the biosynthesis, genetics, and functions of PMPS.

Structure and Distribution of PMPS

The structures of MGLPs and MMPs are shown in Fig. 1*A*. The MMPs from *M. smegmatis* have been characterized in detail (8). They are composed of 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 aglycon. MMPs occur in the cells as a mixture of at least four isomers owing to differences in size and degree of *O*-methylation. The MGLPs from *Mycobacterium bovis* BCG 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*)$_3$-α-(1→. The tetrasaccharide →4)-(α-(1→4)-D-Glc*p*)$_3$-α-(1→6)-D-Glc*p*-α-(1→ linked to position 2 of D-glyceric acid constitutes the reducing end of the molecule (7). Position 3 of the second and that of fourth α-D-Glc*p* residues (closest to the reducing end) are substituted by single α-D-Glc*p* residues. The nonreducing end of the polymer is acylated by a combination of acetate, propionate, and isobutyrate, whereas octanoate esterifies position 1 of glyceric acid, and zero to three succinate groups esterify the glucosyl residues of the reducing end. MGLPs occur as a mixture of four main components that differ in their content of esterified succinate.

MGLPs have been isolated from several *Nocardia* species as well as *M. phlei*, *M. smegmatis*, *M. bovis* BCG, *M. tuberculosis*, *Mycobacterium leprae*, and *Mycobacterium xenopi* (2, 3, 5–7, 9–11). MMPs have been found in different nonpathogenic fast-growing species of mycobacteria (12), and closely related acetylated forms of these compounds have also been reported in *Streptomyces griseus* (13). There has been, however, no report of the production of these polysaccharides in slow-growing mycobacteria, and our inability to detect any forms of MMPs in two different strains of *M. tuberculosis* using different analyti-

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[1] To whom correspondence should be addressed. E-mail: Patrick.Brennan@colostate.edu.

[2] The abbreviations used are: PMPS, polymethylated polysaccharides; MMPs, 3-*O*-methylmannose polysaccharides; MGLPs, 6-*O*-methylglucose lipopolysaccharides; BCG, bacille Calmette-Guérin; SAM, *S*-adenosylmethionine; MGP, methylglucose polysaccharide; GTs, glycosyltransferases; FAS-I, fatty acid synthase I.

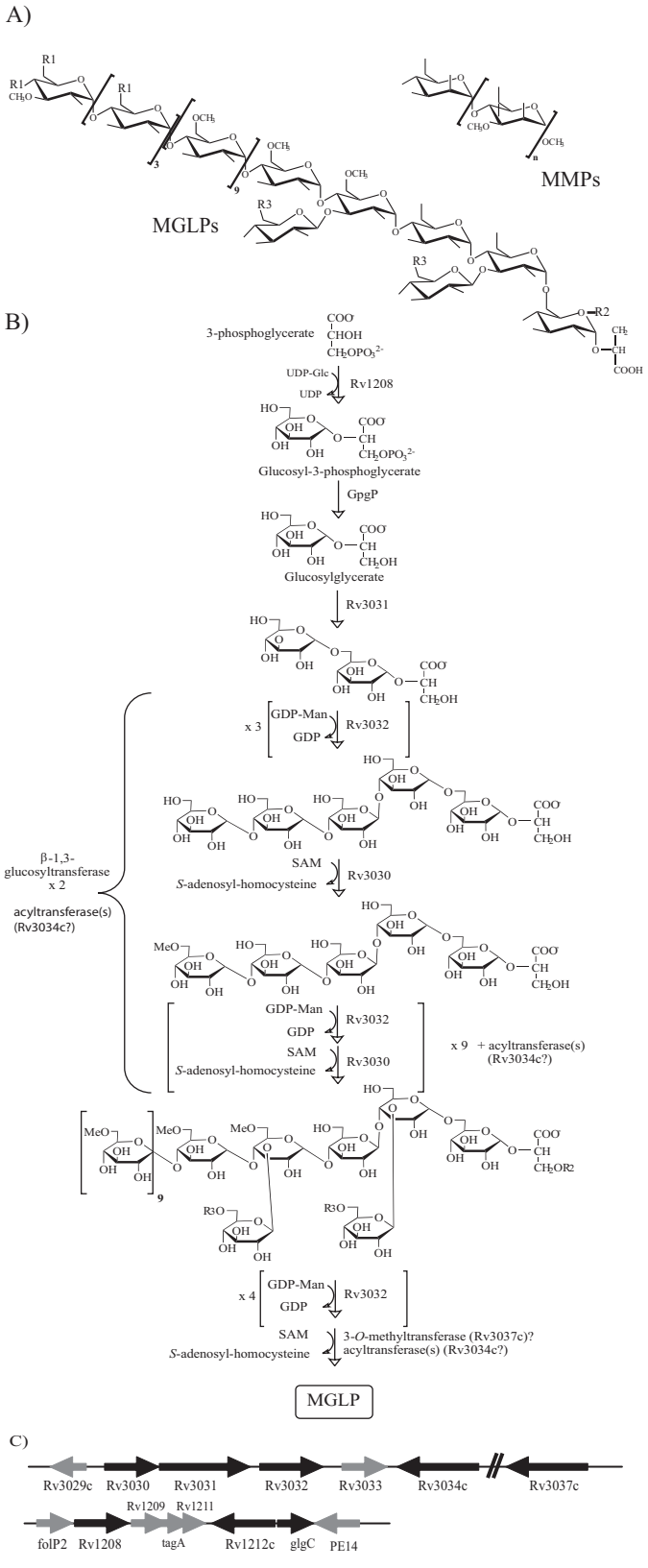

FIGURE 1. **Structure and genetics of MGLPs and MMPs.** *A*, structures of the MGLPs from *M. bovis* BCG and MMPs from *M. smegmatis* ($n = 10–13$). *R1*, *R2*, and *R3* are acyl groups: *R1*, acetate, propionate, or isobutyrate; *R2*, octanoate; and *R3*, succinate. *B*, proposed biosynthetic model of MGLPs (20) with the names of the genes thought to be involved in the different steps of their elongation and modifications now indicated. Acylation and methylation are thought to occur concurrently; the precise stage at which the two β-(1→3)-linked Glc residues are attached is not known, but the definition of early MGLP precursors (20) suggests that they are added early during the elongation process. GpgP is an unknown phosphatase. *C*, organization and proposed functions of the two gene clusters involved in the biosynthesis of MGLPs in *M. tuberculosis* H37Rv (see text for details). The genes likely to be involved in MGLP synthesis are in *black*; genes of unknown function or involved in other pathways are in *gray*. In *M. tuberculosis* H37Rv, the start codon of *Rv3031* overlaps the stop codon of *Rv3030*, and *Rv3031* and *Rv3032* are separated by an intergenic region of 32 bp. Nothing is known of the genetics of MMP synthesis.

cal techniques (14) raises the possibility that they may be restricted only to the fast-growing mycobacterial species.

Biosynthesis and Genetics of PMPS

Biosynthetic Models—Current knowledge of the biosynthesis of MMPs is limited to the early work carried out in Ballou's laboratory in the 1970s–1980s. The isolation of precursors of MMPs and the characterization of an α-(1→4)-mannosyltransferase and 3-*O*-methyltransferase from cell-free extracts of *M. smegmatis* (8, 12, 15, 16) led to the proposal of a biosynthetic model in which MMPs are elongated by a linear alternating process of mannosylation followed by methylation (supplemental Material 1). GDP-Man serves as the sugar donor for the mannosyltransferase, and *S*-adenosylmethionine (SAM) is the source of methyl groups for the methyltransferase. Termination of the elongation reaction occurs when the chain reaches a length compatible with fatty acid-binding properties (11–13 3-*O*-methylmannoses). At this stage, the chain ends with an unmethylated mannose because the K_m of the 3-*O*-methyltransferase for the polysaccharide-fatty acid complex becomes much higher than that of the α-(1→4)-mannosyltransferase. None of the genes involved in the biosynthesis of MMPs have yet been identified.

The model that emerged from Ballou's early work on the biogenesis of MGLPs resembles that proposed for MMPs. Using similar biochemical approaches for both sets of polysaccharides, Ballou and co-workers (17, 18) first reported the existence of a soluble protein fraction from *M. phlei* capable of transferring methyl groups from SAM to positions 6 and 3 of some glucosyl units in MGLPs and partially acetylated α-(1→4)-D-gluco-oligosaccharides. Because the position of methylation on the oligosaccharide acceptor was dependent on its degree of acetylation, it was suggested that acylation and methylation occurred together, the former process exerting a control on the latter. A membrane-associated acyltransferase activity catalyzing the transfer of acetyl, propionyl, isobutyryl, octanoyl, and succinyl groups from their respective acyl-CoA derivatives onto purified MGLPs and partially acetylated α-(1→4)-linked D-gluco-oligosaccharides was later reported (19). Finally, the characterization of weakly acidic and partially methylated methylglucose polysaccharide (MGP) precursors from *M. smegmatis* allowed Kamisango *et al.* (20) to propose a model for the biosynthesis of MGLPs in which the elongation of the chain proceeds stepwise, from the reducing toward the nonreducing end, through a sequential glucosylation-methylation reaction (Fig. 1*B*). Nothing is known of the termination reaction, and until our recent work on mycobacterial glycosyltransferases (GTs) (1, 14), none of the glucosyltransferase activities involved in the elongation of the glucan backbone of these lipopolysaccharides had been characterized.

Genetics of MGLP Synthesis—We recently reported the existence of two clusters of genes dedicated to the biosynthesis of MGLPs in the genomes of mycobacteria (Fig. 1*C* and supplemental Material 2) and the partial functional characterization of three of these genes: *Rv3032*, *Rv1208*, and the *M. smegmatis* ortholog of *Rv3030*, *MSMEG2349* (14).[3] Rv3032 carries the conserved motif (D/E)X_7E characteristic of the CAZy GT-4 family of NDP-sugar-dependent retaining GTs (afmb.cnrs-mrs.fr/CAZY/). The disruption of *Rv3032* by homologous recombination in *M. tuberculosis* H37Rv resulted in a dramatic decrease in all forms of MGLPs, whereas the overexpression of this gene in *M. smegmatis* mc^2155 stimulated the production of mature forms of MGLPs. Our results also indicated that Rv3032 participates in the biosynthesis of glycogen (14) and that partially purified recombinant Rv3032 has the ability to elongate short-chain α-(1→4)-D-gluco-oligosaccharides and glycogen *in vitro* using UDP-D-Glc as the glucosyl donor.[4] Altogether, results suggest that Rv3032 is the main α-(1→4)-glucosyltransferase responsible for the elongation of MGLPs, even though this enzyme also participates in the elongation of other α-(1→4)-glucans. Adjacent or in close vicinity to this glucosyltransferase gene lies a putative acetyltransferase gene (*Rv3034c*), two putative SAM-dependent methyltransferase genes (*Rv3030* and *Rv3037c*), and a putative α-amylase/glucoside hydrolase/GH-57 family branching enzyme gene (*Rv3031*) (Fig. 1*C*). The disruption of the ortholog of *Rv3030* in *M. smegmatis* resulted in a dramatic reduction in the amounts of MGLPs synthesized and in the accumulation of precursors of these molecules, suggesting that *Rv3030* encodes the main *O*-methyltransferase of the pathway, *i.e.* the one responsible for the 6-*O*-methylation of the lipopolysaccharides (Fig. 1*B*) (14). Important information derived from the analysis of this *M. smegmatis* mutant is that a defect in *O*-methylation abolishes MGLP synthesis. Thus, despite both Rv3032 and the 6-*O*-methyltransferase being active on unmethylated α-(1→4)-D-gluco-oligosaccharides *in vitro* (17, 18),[4] the elongation of MGLPs in whole bacterial cells seems to proceed with glucosylation and *O*-methylation occurring hand in hand. This observation is consistent with the biosynthetic model proposed by Kamisango *et al.* (20) based on the structural analysis of MGLP precursors (Fig. 1*B*). Whether *Rv3037c* encodes the putative SAM-dependent 3-*O*-methyltransferase responsible for the 3-*O*-methylation of the glucosyl units of the reducing end of MGLPs (Fig. 1*A*) remains to be determined. Likewise, the sequence similarities between the product of *Rv3031* and the GH-57 family branching enzyme from *Thermococcus kodakaraensis* (21) suggest that it is responsible for generating the α-(1→6)-glycosidic bond linking the first and second D-Glc*p* residues at the reducing end of the molecule in a mechanism similar to the one involved in the branching of glycogen.

As the disruption of *Rv3032* in *M. tuberculosis* and that of the ortholog of *Rv3030* in *M. smegmatis* did not totally abolish MGLP production, we had to assume on the existence of compensatory glucosyltransferase and methyltransferase activities in these species. A search for the enzyme compensating for Rv3032 in *M. tuberculosis* H37Rv led us to identify Rv1212c as a possible α-(1→4)-glucosyltransferase candidate; *Rv1212c* is the ortholog of the glycogen synthase gene from *Corynebacterium glutamicum* (22). Disruption of *Rv1212c* in *M. tuberculosis* H37Rv resulted in a significant reduction in the amount of capsular α-D-glucan produced by this bacterium but did not appreciably affect its MGLP and glycogen contents (23). However, capsular α-D-glucan production in the *Rv1212c* mutant was restored upon overexpression of *Rv3032*, confirming a partial redundancy of the two enzymes even though, under physiological conditions, Rv3032 seems to be involved primarily in the production of MGLP and glycogen and Rv1212c in the elongation of the capsular α-D-glucan (23). Failure to disrupt both the *Rv3032* and *Rv1212c* genes in the same *M. tuberculosis* H37Rv strain further indicated that bacterial growth requires at least one of these two genes to be functional (23). In this regard, it is interesting to note that although not all mycobacterial species display functional orthologs of *Rv1212c* and *Rv3032*, they all have retained at least one of the two genes. For instance, *M. leprae*, which produces MGLPs (5), carries an ortholog of *Rv3032* in its genome, but *Rv1212c* is a pseudogene (supplemental Material 2). Conversely, a frameshift mutation at the 5′-end of the *Rv3032* gene of *M. smegmatis* apparently abolishes the activity of this gene.[4] *M. smegmatis* does, however, carry an ortholog of *Rv1212c*, which most likely accounts for the production of MGLPs in this species.

As is the case for *Rv3032*, *Rv1212c* is located in a cluster of genes encoding putative sugar-modifying enzymes (Fig. 1*C*). We have undertaken the characterization of some of the genes from this cluster and found that disruption of the putative ADP-glucose pyrophosphorylase gene *glgC* in *M. tuberculosis* H37Rv led to a 40–50% reduction in glycogen and capsular glucan contents (23). The product of *Rv1208* was predicted to be a CAZy family 2 sugar-nucleotide-utilizing GT of unknown function. Weak sequence similarities to the glucosyl-3-phosphoglycerate synthase (GpgS) from *Persephonella marina* (~24% amino acid identity) suggested, however, that this enzyme might be responsible for the transfer of the first Glc*p* residue onto D-3-phosphoglycerate or D-glycerate (24) to form the glucosyl-(1→2)-glycerate found at the reducing end of MGLP (Fig. 1*A*). Supporting this hypothesis, recombinant forms of the *M. smegmatis* and *M. bovis* BCG Rv1208 enzymes produced in *Escherichia coli* were shown to display GpgS activity *in vitro* (25). Moreover, our recent evidence indicated that the disruption of the ortholog of *Rv1208* in *M. smegmatis* (*MSMEG_5084*) reduced by ~80% the *de novo* production of MGLP in this species.[3] The ability of the mutant to synthesize residual amounts of the lipopolysaccharides was apparently attributable to the existence of compensatory GpgS activity in *M. smegmatis*. The crystal structure of the ortholog of Rv1208 in *Mycobacterium avium* spp. *paratuberculosis* (MAP2569c) has been solved (26, 27), and diffraction-quality crystals of Rv1208 from *M. tuberculosis* were obtained.[5]

[3] D. Kaur *et al.*, unpublished data.
[4] G. Stadthagen and M. Jackson, unpublished data.
[5] Gest, P., Kaur, D., Pham, H. T., van der Woerd, M., Hansen, E., Brennan, P. J., Jackson, M., and Guerin, M. E. (2008) *Acta Crystallogr. F Struct. Biol. Crystalliz. Comm.*, in press.

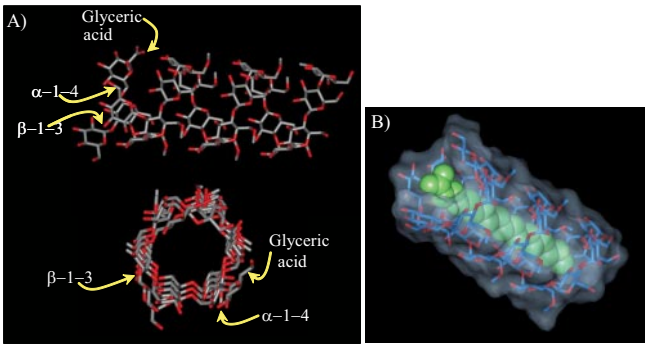

FIGURE 2. *A*, top and side views of a structural model of MGP containing 19 glucosyl units of which 12 are *O*-methylated ($MGP_{19,12}$); *B*, structural model of stearic acid (in *green*) bound to $MGP_{20,12}$.

Recent studies thus revealed the participation of at least two clusters of genes in the biosynthesis of MGLPs and other α-(1→4)-glucans in *M. tuberculosis* and a partial redundancy in the α-(1→4)-glucosyltransferase activities responsible for their elongation. The gene(s) encoding the GT(s) responsible for the glucosylation of position 3 of the second and fourth α-D-Glc*p* residues closest to the reducing end of MGLPs are not known.

Biological Activities

Postulated Roles of PMPS in Regulation of Fatty Acid Metabolism—Studies from the Ballou and Bloch laboratories have highlighted the unique regulatory roles of MMPs and MGLPs in mycobacterial fatty acid synthase I (FAS-I) in cell-free assays. *In vitro*, both PMPS raise the overall rate of synthesis of fatty acids by FAS-I and shift the *neo*-synthesized chain length pattern from long (C_{20}–C_{24}) to short (C_{14}–C_{18}) (28). These effects are thought to result from the ability of the (lipo)polysaccharides to form complexes with fatty acyl chains and acyl-CoAs. In sequestering the products of FAS-I, PMPS are thought to facilitate the release of the *neo*-synthesized chains from the enzyme, thereby not only reopening active sites essential for enzyme turnover but also terminating their elongation. In addition, in complexing the acyl-CoAs released in the reaction mixture, MGLPs and MMPs are thought to relieve product inhibition of the synthetase. With an intracellular concentration of long-chain acyl-CoAs in *M. smegmatis* of ~0.3 mM, the concentration of PMPS approaching 1 mM, and the dissociation constant of the polysaccharide-lipid complex estimated to 0.1 μM, all of the long-chain fatty acids of the cytosol may form complexes with PMPS (29). Under these circumstances, PMPS could be regarded as general fatty acyl carriers in mycobacteria, protecting long-chain fatty acyl-CoAs from attack by degradative enzymes and regulating their further processing for the synthesis of more complex fatty acids and lipids, including mycolates (28–30).

Evidence for the formation of complexes between PMPS and fatty acyl/fatty acyl-CoAs was supported by multiple experimental studies using a variety of techniques (29, 31–35). The fact that both MMPs and MGLPs are composed of hexose units predominantly or exclusively in α-(1→4)-linkage confers on these molecules a proclivity to assume the helical conformation characteristic of amylose (Fig. 2*A*). Within the complexes, the fatty acyl chain is included in the nonpolar cavity of the coiled polysaccharide chain (Fig. 2*B*). NMR studies have shown that MMP, a random coil in its free form, undergoes a major conformational transition upon enclosing long-chain acyl-CoAs. This polysaccharide, probably in helical conformation in the complexed form, interacts with both the paraffinic chain and the CoA moieties of the included fatty acyl thioester (29, 33). Similar conclusions apply to MGLPs (34). However, the presence of acetate, propionate, isobutyrate, octanoate, and succinate groups acylating the polysaccharide backbone and glyceric acid of MGP may confer upon the native MGLP a coiled conformation even in the absence of added long-chain fatty acyls. It was proposed that the acylation of MGP may alter its fatty acid-binding specificity and serve to fine-tune its regulatory activity in the cell (34). As expected, the number of sugar units of PMPS was shown to greatly impact their fatty acid-binding properties (32).

Thus far, physiological evidence for a role of PMPS in the regulation of mycobacterial lipid metabolism has been lacking. In the only published study on the role of PMPS in mycobacterial fatty acid metabolism *in vivo* (36), a *M. smegmatis* spontaneous mutant was described that contained only ~50 and 7% of the wild-type levels of MGLPs and MMPs, respectively. Although this mutant accumulated slightly more short-chain and less long-chain unsaturated fatty acids than the wild-type strain, fatty acid synthesis was not dramatically altered. Further doubts regarding the prevailing hypothesis that MGLPs are involved in the regulation of fatty acid metabolism were cast by the observation that *Rv3032* and *MSMEG_5084* knock-out mutants of *M. tuberculosis* H37Rv and *M. smegmatis*, known to be impaired in MGLP synthesis, displayed wild-type fatty acid contents (14).[3] The question of the regulatory roles of these unique PMPS in fatty acid metabolism thus remains open, as is the reason for the existence of two types of PMPS with apparent redundant functions in some *Mycobacterium* spp.

Other Possible Functions—Although the main environmental factor(s) governing the synthesis of MGLPs remain to be identified, studies from Ballou's laboratory (15, 20) and our own observations suggest that the MGLP content of mycobacteria greatly varies with the culture medium and/or oxygen tension. We found that the greatest amounts of these compounds were recovered from *M. tuberculosis* or *M. smegmatis* grown as surface pellicles in minimal Sauton's medium.[4] Observations made on the growth characteristics of *Rv3032* or *Rv3030* mutants of *M. tuberculosis* and *M. smegmatis* displaying reduced MGLP contents indicated that a diminution in the concentration of these polysaccharides dramatically affected growth at high temperatures (14). Finally, the occurrence of glucosylglycerate at the reducing end of MGLPs may suggest a role for these lipopolysaccharides in osmoprotection (24).

Are PMPS Essential for Mycobacterial Growth?—Our results indicate that at least one of the two α-1,4-glucosyltransferase genes of *M. tuberculosis*, *Rv1212c* or *Rv3032*, must be functional for sustained growth *in vitro* (23), implying that the production of glycogen, glucan, and/or MGLPs is an essential physiological requirement under axenic conditions. Whether this requirement for α-1,4-glucosyltransferase activity is a consequence of the essentiality of MGLPs will have to await the con-

struction of defined (conditional) mutants totally deficient in the production of these molecules. *In vivo*, partial loss of MGLPs in the *M. tuberculosis Rv3032* mutant did not compromise the growth and pathogenicity of this strain in BALB/c mice (23).

Conclusions

Despite a wealth of *in vitro* and *in vivo* data pointing to a role of MGLPs and MMPs in fatty acid metabolism, the questions of the essentiality, regulation, and physiological role(s) of these molecules remain open. Answers to these questions will require the construction of mutants totally deficient in their synthesis. In the case of MGLPs, this goal is likely to be achievable in *M. tuberculosis* through the individual or combined inactivation of the glucosyl-3-phosphoglycerate synthase, branching enzyme, α-(1→4)-glucosyltransferase, methyltransferase, and the acetyltransferase genes (*Rv1208*, *Rv3032*, *Rv3031*, *Rv3030*, *Rv3037c*, and *Rv3034c*). In contrast, the construction of knock-out mutants deficient in the production of MMPs will have to await the identification of the genes involved in their synthesis in fast-growing mycobacteria. The construction of defined mutants, together with the development of cell-free assays for each identified enzyme, will also shed light on the sequential reactions leading to the formation of these unique polysaccharides.

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