Biosynthetic Origin of the Galactosamine Substituent of Arabinogalactan in Mycobacterium tuberculosis*§

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The arabinogalactan (AG) of slow growing pathogenic Mycobacterium spp. is characterized by the presence of galactosamine (GalN) modifying some of the interior branched arabinosyl residues. The biosynthetic origin of this substituent and its role(s) in the physiology and/or pathogenicity of mycobacteria are not known. We report on the discovery of a polyprenyl-phospho-N-acetylgalactosaminyl synthase (PpgS) and the glycosyltransferase Rv3779 from Mycobacterium tuberculosis required, respectively, for providing and transferring the GalN substituent. The availability of a ppgS knock-out mutant of M. tuberculosis provides unique opportunities to investigate the physiological function of the GalN substituent and the potential impact it may have on host-pathogen interactions.

Galactosamine (D-GalN) was recognized as a minor covalently bound amino sugar component of the cell wall of slow growing mycobacteria (Mycobacterium tuberculosis H37Rv, Mycobacterium leprae, Mycobacterium microti) as early as in the 1970s (1–3). Advances in mass spectrometry later coupled with the use of an arabinanase isolated from Mycobacterium smegmatis allowed the GalN substituent to be mapped to the arabinogalactan (AG)7 component of the cell wall (3), and more specifically to the C2 position of a portion of the internal 3,5-branched d-Araf residues in the AG of M. tuberculosis (4, 5). d-GalN was estimated to occur at the level of about one residue per entire AG molecule (3–5). Interestingly, a similar sugar residue was found to substitute the AG of Mycobacterium avium, Mycobacterium kansasi, Mycobacterium bovis BCG (3) and Mycobacterium leprae,8 but not that of M. smegmatis, M. neoaurum, and M. phlei (3–5), suggesting that fast growing Mycobacterium spp. are devoid of GalN substituent.

The function and biosynthetic origin of this substituent are not known. Non-N-acetylated hexosamine residues, particularly GalN residues, are rather uncommon in prokaryotes. Thus far, they essentially have been described as components of the lipopolysaccharide (LPS) of some Gram-negative bacteria (6–9). In Francisella tularensis and subsp. novicida for instance, GalN covalently modifies the lipid A moiety of LPS neutralizing its negative charge and enhancing the virulence of the bacterium in mice (8, 9). In Gram-positive bacteria, GalN and glucosamine (GlcN) have been reported in the glycosylation motif of a glycoprotein toxin from Bacillus thuringiensis subsp. israelensis and proposed to be important to the

7 The abbreviations used are: AG, arabinogalactan; APTS, 8-aminopyrene-1,3,6-trisulfonate; CE, capillary electrophoresis; CE-LIF, CE monitored by laser-induced fluorescence; GT, glycosyltransferase; kan, kanamycin; PpgS, polyprenyl-phospho-N-acetylgalactosaminyl synthase.
8 S. Bhamidi, unpublished observation.
biological activities of the toxin (10). GalN was also identified as a possible component of a galactose-containing polysaccharide from *Streptococcus mutans* (11).

Recent studies have partially elucidated the biosynthetic origin of the ω-GalN unit modifying the lipid A of *Francisella* (8, 9, 12). It was shown to originate in the lipid-linked sugar donor, undecaprenyl phosphate-β-D-N-acetylagalactosamine, which, upon deacetylation by a specialized deacetylase, yields undecaprenyl phosphate-β-D-galactosamine. This is then used as the ω-GalN donor in a glycosyl transfer reaction onto lipid A catalyzed by the membrane-associated glycosyltransferase FlmK (8, 9, 12). This process is reminiscent of that involved in the addition of aminoarabinose to lipid A in enteric bacteria where an enzyme (termed ArnC in *Escherichia coli*) catalyzes the synthesis of undecaprenyl-phospho-β-N-formylaminoarabinoside from UDP-N-formyl-aminoarabinosine, and a glycosyltransferase (ArnT in *E. coli*) catalyzes the transfer of the deformylated aminoarabinose residue from this lipid donor onto lipid A (13).

Numerous glycosylation events in the biosynthesis of *M. tuberculosis* cell wall and other glycoconjugates are known to occur on the periplasmic face of the inner membrane, catalyzed by membrane-associated glycosyltransferases of the GT-C superfamily dependent on decaprenyl-phosphate-linked sugar donors rather than nucleotide-sugar donors (for a review, see Refs. 14–16). However, to date, the known polyprenyl-based sugar donors of *Mycobacterium* spp. only include β-D-glucosyl-1-monophosphoryl-decaprenol, β-D-mannosyl-1-monophosphoryl-decaprenol, β-D-ribofuranosyl-1-monophosphoryl-decaprenol, and β-D-arabinofuranosyl-1-monophosphoryl-decaprenol (17–19). To the best of our knowledge, polyprenyl-linked galactosamine or N-acetylagalactosamine has never been reported in mycobacteria. This study reports on the discovery of the biosynthetic pathway for the synthesis of lipid-linked-ω-GalNAc in mycobacteria and subsequent ω-GalNAc or ω-GalN transfer onto AG.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions—** *M. tuberculosis* H37Rv ATCC 25618 and H37Rv TMC102 were grown in Sauton’s and Modified 7H9 medium. *M. smegmatis* mc²155 was grown in 7H9 or LB medium. Kanamycin (kan) and hygromycin were added to final concentrations of 20 μg/ml and 50 μg/ml, respectively.

*M. tuberculosis Knock-out Mutants—* The Ts/sacB method was used to achieve allelic replacement at the *Rv3631* (ppgs) locus of *M. tuberculosis* H37Rv (20). The ppgs gene and flanking regions was PCR-amplified from *M. tuberculosis* H37Rv genomic DNA with primers Rv3631.5 (5′-ttggatctcagctccgacccgctggctc-3′) and Rv3631.6 (5′-ttgatctcagctccgacccgctggctc-3′) and a disrupted allele, ppgs::kan, was obtained by inserting the *Tn903* kanamycin resistance cassette at the FseI restriction site of ppgs. ppgs::kan was then cloned into the XbaI-cut and blunt-ended pPR27-xyIE (20), yielding pPR27ppgsXXK. The Rv3779 knock-out mutant of *M. tuberculosis* H37Rv TMC102 and complemented mutant strains were described elsewhere (21). The *mshB* knock-out mutant of *M. tuberculosis* Erdman (22) was kindly provided by Drs. Fahey and New-ton (University of California San Diego), and the *mca* transposon mutant of *M. tuberculosis* CDC1551 was obtained from CSU TB Vaccine Testing and Research Materials Contract (National Institutes of Health, NIAID N01-AI-40091).

**Expression of ppgs and Rv3632 in M. smegmatis—** The entire coding sequences of ppgs and Rv3632 were PCR-amplified from *M. tuberculosis* H37Rv genomic DNA and cloned into the expression vector pVVP26 and pVVRv3632, respectively. pVVPpVppgs-Rv3632 was generated by amplifying the ppgs and Rv3632 ORFs together and cloning the resulting PCR fragment into the same expression plasmid. These constructs allow the constitutive expression of ppgs, Rv3632, or both genes under control of the *hsp60* promoter. Primer sequences are available upon request.

**Enzyme Assays—** Membranes from *M. smegmatis* mc²155 were prepared as described (24) and resuspended in 50 mM MOPS buffer (pH 7.9) (buffer A). The typical reaction mixture for the production of polyprenyl-P-β-D-[3H]GalNAc from UDP-α-L-[3H]GalNAc by *M. smegmatis* membranes contained 0.5 μCi of UDP-α-D-[3H]GalNAc (specific activity 20 Ci mmol⁻¹; American Radiolabeled Chemicals, Inc., St. Louis, MO), 5 mM 2-mercaptoethanol, 10 mM MgCl₂, 0.1 mM ATP, 0.1 mM NADH, membrane fraction (0.5 mg of proteins), and buffer A in a final volume of 80 μl; thus, the membranes were the source of the endogenous polyprenyl-P acceptors. After incubation at 37 °C, the reactions were stopped by addition of 1.2 ml of CHCl₃/CH₃OH/NH₄OH (2:1:0.05, v/v). The upper aqueous phase was discarded, and the bottom organic phase was backwashed with CHCl₃/CH₃OH/H₂O (3:47:48). The dried organic phase was dissolved in 50 μl of CHCl₃/CH₃OH (2:1) and the oligosaccharides liberated were then analyzed by capillary electrophoresis monitored by laser-induced fluorescence (CE-LIF). Separations were performed using an uncoated fused-silica capillary column (Sigma) of 50-μm internal diameter and 40-cm effective length (47-cm total length). Analyses were carried out at a temperature of 25 °C, in the reverse mode, with an applied voltage of 20 kV using 1% acetic acid (w/v)-30 mM triethylammonium acetate (APTS) as described earlier (25). Labeled oligosaccharides were then analyzed by capillary electrophoresis monitored by laser-induced fluorescence (CE-LIF) on a P/ACE 5000 instrument (Beckman Instruments, Inc.). Separations were performed using an uncoated fused-silica capillary column (Sigma) of 50-μm internal diameter and 40-cm effective length (47-cm total length). Analyses were carried out at a temperature of 25 °C, in the reverse mode, with an applied voltage of 20 kV using 1% acetic acid (w/v)-30 mM triethylammonium, pH 3.5, as a running electrolyte (25). Picopreparative CE was performed as described previously (26).

**MALDI-TOF/MS and MS/MS—** Analyses were performed on a 4700 Proteomics Analyzer (with TOF/TOF optics, Applied Biosystems, Voyager DE-STR) in the reflectron mode. The matrix used was 2,5-dihydroxybenzoic acid (10 μg ml⁻¹ in a mixture of ethanol/water (1:1, v/v)). 0.3 μl of the CE-collected oligosaccharides resuspended in 10 μl of water or TLC-extracted lipids resuspended in 10 μl CHCl₃/CH₃OH (2:1) was mixed with 0.3 μl of the matrix solution. Mass spectra were recorded in the negative mode. The collision-induced
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dissociation gas type for MS/MS experiments was atmosphere, and the gas pressure was set to medium.

RESULTS

Disruption of ppgS in M. tuberculosis H37Rv—Rv3631 (thereafter renamed ppgS) is predicted to encode a GT-A-fold glycosyltransferase (GT) (14, 15) and, according to the Carbohydrate-Active enZymes classification of glycosyltransferases (27), to belong to the GT-2 family of inverting GTs dependent on NDP-sugars as donor substrates. Orthologs of ppgS are found in the genomes of slow growing mycobacteria including M. bovis, M. bovis BCG, M. leprae, Mycobacterium ulcerans, Mycobacterium marinum, M. avium, and Mycobacterium paratuberculosis, as well as in Mycobacterium abscessus, but not in the genomes of other rapidly growing Mycobacterium species such as M. smegmatis (supplemental Fig. S1). Interestingly, in all of the genomes analyzed, ppgS and orthologs map in close vicinity to galE1, a gene thought to encode the UDP-glucose 4-epimerase responsible for the conversion of UDP-β-Glcp to UDP-β-Galp in AG synthesis (16). ppgS and orthologs appear to be co-transcribed with a gene potentially encoding a small membrane protein (13 kDa) of unknown function (Rv3632 in M. tuberculosis H37Rv) (supplemental Fig. S1). PpgS shares significant sequence similarities with polyprenylmonophosphomannose synthases from various prokaryotic sources, including Ppm1 (Rv2051c) from M. tuberculosis H37Rv (24% identity, 39% similarity on a 228-amino acid overlap).

To investigate the putative involvement of this GT in AG synthesis, the ppgS gene from M. tuberculosis H37Rv was disrupted by homologous recombination using the Ts/sacB method (20). Allelic replacement at the ppgS locus was confirmed by Southern hybridization (supplemental Fig. S2A). The mutant grew similarly to its wild-type (WT) H37Rv parent in 7H9-ADC-Tween 80 broth at 37 °C (supplemental Fig. S2B).

Phenotypic Characterization of the ppgS Mutant Strain—Soluble AG was prepared from the WT and mutant strains as described previously (5), and the Ara/Gal ratios were determined by CE or GC upon total acid hydrolysis. No significant differences between WT and mutant strains were observed, indicating that PpgS was probably not involved in the polymerization of the arabinan or galactan domains. Because the distribution of ppgS within the Mycobacterium genus seemed to correlate with that of β-GalN on AG, we decided to probe the mutant for the presence of this substituent. To this end, a fast and sensitive CE-based methodology was first developed to facilitate the detection of this rare substituent. By analogy to the procedure that we previously developed to analyze the mannose caps of lipoarabinomannan (25, 28), we postulated that a controlled mild acid hydrolysis of AG would cleave the arabinofuranosidic bonds of the arabinan domain while preserving the pyranosidic linkage of β-GalN onto the relevant Araf unit, thereby releasing an oligosaccharide that could be detected by CE-LIF upon tagging with an APTS fluorescent probe. A typical electrophoretogram of M. tuberculosis H37Rv AG resulting from this analysis is shown in Fig. 1A. The monosaccharide region is dominated by two main peaks attributed to β-Araf-APTS (I) and β-Galp-APTS (II) arising from the hydrolysis of the arabinan and galactan domains. A third peak (III) of lower intensity was detected with a migration time matching that of a maltoheptaose-APTS, tentatively attributed to an oligosaccharide-APTS containing β-GalpN
and \(\alpha-D-Araf\) units. This assumption was confirmed by collecting peak III in a picopreparative CE step (26) and subjecting it to MALDI/MS analysis (Fig. 1B). The negative MALDI mass spectrum was dominated by a peak at \(m/z\) 882.9 attributed to deprotonated molecular ions [M – H] \(^{-}\) that could be assigned to an APTS derivative containing two pentosyl and one hexosaminyl units. The identity of these units as two arabinosyl and one galactosaminyl residues was revealed by CE analysis upon acid hydrolysis and labeling with APTS or fluorescein-succinimidyl ester. The exact trisaccharide sequence, \(\alpha-D-GalN-(1→2)-\alpha-D-Araf-(1→5)-\alpha-D-Araf\), was revealed by MS/MS analysis (Fig. 1C) showing fragment ions at \(m/z\) 721.8 (y2) and \(m/z\) 589.7 (y1) arising from the loss of anhydro-\(\alpha-D-GalNP\) and anhydro-\(\alpha-D-GalNP-Araf\), and based on the described structure of AG (5) (see also Fig. 6). Peak III was thus further used as a reporter peak for the presence of \(\alpha-D-Araf\) on AG.

Suggestive of an involvement of PpgS in the formation of the GalNAc substituent of AG, peak III was noticeably absent from the electrophoretogram of H37Rv\(\Delta ppgS::kan\) but restored in the complemented mutant strain H37Rv\(\Delta ppgS::kan/pVVpppS\). Corroborating this observation, peak III was also detected in the AG of several ppgS-expressing Mycobacterium species (\(M. bovis\) BCG, \(M. marinum\), and \(M. abscessus\)) but, as expected, was missing from that of \(M. smegmatis\) (supplemental Fig. S3). Although PpgS shares significant sequence similarities with polyrenol-monophosphomannose synthases, no significant differences were found between the mannolipid and lipoglycan (lipomannan and lipoarabinomannan) contents of the WT and mutant strains (supplemental Fig. S4).

Effect of Overexpressing \(ppgS\) on the Synthesis of Lipid-linked Sugars and AG in \(M. smegmatis\)—The absence of a \(ppgS\) ortholog in \(M. smegmatis\) provided a convenient mycobacterial host for the heterologous expression and functional characterization of this gene. Transformation of mc\(^{155}\) with pVV\(ppgS\), the plasmid used for complementation studies in \(M. tuberculosis\), resulted in the production of a C-terminal His\(_{\alpha}\)-tagged recombinant protein of the expected size (~26 kDa) (Fig. 2, A and B). The recombinant protein was almost exclusively found in the membrane and P60 fractions of the bacterium, suggestive of its association with membranes (supplemental Fig. S5). To optimize the chances of producing an active form of PpgS in \(M. smegmatis\), a recombinant strain of \(M. smegmatis\) expressing \(ppgS\) in combination with \(Rv3632\) and a control strain expressing \(Rv3632\) alone were also constructed. In all cases, recombinant strains grew similarly to the control in liquid broth and the production of recombinant proteins of the expected sizes was confirmed (Fig. 2B).

Analysis of the AG of the various recombinant strains as described above failed to reveal the presence of \(\alpha-D-GalNP\) (or \(N\)-acetyl-\(\alpha-D-GalNP\)) substituent on the AG of mc\(^{155}/pVV16\), mc\(^{155}/pVVpppS\), mc\(^{155}/pVV\(Rv3632\), and mc\(^{155}/pVV\(ppgS-Rv3632\) (supplemental Fig. S6). It thus appeared that the expression of \(ppgS\) alone or in combination with \(Rv3632\) was not sufficient for the formation of this substituent in \(M. smegmatis\).

Cell-free assays using membranes from the recombinant \(M. smegmatis\) strains were then conducted to determine whether PpgS is involved in some aspects of lipid-linked sugar biosynthesis. TLC autoradiographs clearly revealed the incorporation of \(\alpha-D-[\text{\(^{3}H\)}]GalNAc\) from UDP-\(\alpha-D-[\text{\(^{3}H\)}]GalNAc\) into two major glycolipids (A and B) by mc\(^{155}/pVVpppS\) membranes that were not detected in the control strain mc\(^{155}/pVV16\) or in the strain expressing the only \(Rv3632\) gene, mc\(^{155}/pVV\(Rv3632\) (Fig. 3A). Strikingly, this enzymatic activity was increased 40–50-fold in the strain co-expressing \(ppgS\) and \(Rv3632\) (Fig. 3A, lane 4). The accumulated \(\alpha-D-[\text{\(^{3}H\)}]GalNAc\) (or \(\alpha-D-[\text{\(^{3}H\)}]Gal\)) containing glycolipids were mild alkali-stable and mild acid-labile (supplemental Fig. 7S) with TLC mobility properties suggestive of lipid-linked sugars of the mycobacterial polysorosyl-P class (19, 21, 29). Further assays aimed at testing the effects of time and protein or acceptor substrate concentration on \(\alpha-D-[\text{\(^{3}H\)}]GalNAc\) transfer by mc\(^{155}/pVVpppS\(Rv3632\) membranes showed clear enzyme and substrate concentration-dependent increases in activity (Fig. 3, B and C). Increases in activity were similar whether decaprenyl (C\(_{20}\))-P or the shorter heptaprenyl (C\(_{35}\))-P were used as acceptor substrates (Fig. 3D) but inexistent when C\(_{10}\)-P served as the acceptor (supplemental Fig. S7B) indicative of PpgS preference for longer lipid carriers. Suggestive of PpgS specificity for the sugar transferred, assays performed under similar conditions using other sugar donors including GDP-\(\alpha-D-[\text{\(^{14}C\)}]Man\), UDP-\(\alpha-D-[\text{\(^{14}C\)}]GlcNAc\), ADP-\(\alpha-D-[\text{\(^{14}C\)}]Glc\), and UDP-\(\alpha-D-[\text{\(^{14}C\)}]Gal\) failed to reveal any effect of overexpressing \(ppgS\) on the incorporation of these radiolabeled sugars into glycolipids (data not shown).

Structural Characterization of Glycolipids A and B—To establish the nature of the products formed in the assay using mc\(^{155}/pVVpppS\(Rv3632\) membranes and UDP-\(\alpha-D-[\text{\(^{3}H\)}]GalNAc\), a nonradioactive assay was up-scaled, and the products of the reaction, purified by preparative TLC, were submitted to MALDI/MS analyses. The negative MALDI-TOF mass spectra of purified products A and B were dominated by peaks at \(m/z\) 980.5 and 784.4, respectively, attributed
to deprotonated molecular ions [$M–H]− that could be assigned to C$_{50}$-P-β-GalNAc and C$_{35}$-P-β-GalNAc (Fig. 4, A and B). This assignment was confirmed by MS/MS analyses showing for compound A fragment ions at m/z 300.5 and 778.2, arising from the loss of the decaprenyl and N-acetylgalactosaminyl moieties, respectively (Fig. 4C). The negative MALDI-TOF/TOF mass spectrum of purified compound B exhibited the same reporter fragment ions at m/z 300.5 and 581.1 (Fig. 4D). Altogether, our results thus indicate that ppgS encodes a polyprenyl-phospho-N-acetylgalactosaminyl synthase required for the synthesis of the GalN substituent of AG.

Identification of the Glycosyltransferase Responsible for GalN Transfer onto AG—Because the involvement of a lipid-linked D-GalN(Ac) donor suggested that the transfer of the amino sugar onto AG occurred on the periplasmic side of the plasma membrane, we thought to search for the responsible GT among the membrane-associated GT-C enzymes of M. tuberculosis (14). Of the 17 proposed GT-Cs of strain H37Rv, only 2 are missing an ortholog in M. smegmatis mc²155 but have orthologs in M. leprae, M. abscessus, and other species harboring a d-GalNp substituent on AG, namely Rv1635c and Rv3779. Rv1635c was demonstrated earlier to catalyze the addition of the first mannose residue in the Man-capping of mannosylated lipoarabinomannan (30). Rv3779 clusters on the chromosome of M. tuberculosis with genes involved in the biosynthesis of the cell wall core and was recently found to participate in the synthesis of polyprenylphosphomannose (21). Analysis of the AG of mutants deficient in the expression of each of these genes (21, 30) revealed that although the M. tuberculosis CDC1551 Rv1635c knock-out strain still carried the D-GalNp substituent (supplemental Fig. S8A), the AG of M. tuberculosis H37Rv Rv3779 was totally devoid of it (Fig. 5). Production of galactosaminylated AG was restored in the Rv3779 mutant upon complementation with a WT copy of Rv3779 expressed from a replicative plasmid (Fig. 5).

DISCUSSION

The presence of a covalently bound GalN residue typifies the AG of slow growing mycobacteria. We have identified the
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The identification of the PPM synthase *Rv3779* (21) as the galactosaminyltransferase (or \(N\)-acetylgalactosaminyltransferase) of the system indicates that this GT-C enzyme is endowed with at least two distinct enzymatic activities and highlights the complexity of the GT-C enzymes responsible for much of the biosynthesis of mycobacterial cell wall polysaccharides. Consistent with this finding, in addition to the cytoplasmic GT-C signature motif (DLD at position 86) which we proposed earlier to be involved in the transfer of a mannose residue from GDP-\(\alpha-D\)-Manp to polypropenyl phosphate (14, 21), a second putative GT-C motif predicted to be located in the last extracytoplasmic loop of the protein (TMHMM 2.0) and susceptible of catalyzing the transfer of \(\alpha\)-GalNAc was identified (DLD at position 536). This motif and associated downstream acidic, aromatic, and proline residues (YDYP at position 578) (14) are conserved among the *Mycobacterium* species reported to display a GalN-substituted AG. The pre-
dicted spatial distribution of each GT-C motif, which is in line with that of their respective donor and acceptor substrates (cytosolic GDP-Man and periplasmic arabinan chains of AG in particular), would thus govern the dual catalytic activities of Rv3779 (Fig. 6). Further experiments are under way to validate this hypothesis.

Missing biosynthetic components required for producing the GalN substituent of AG include the deacetylase responsible for deacetylating polyprenyl-P-D-GalNAc to the corresponding D-GalN derivative and the translocase required for translocating polyprenyl-P-D-GalN/polyprrenyl-P-D-GalNAc from the inner to the outer leaflet of the plasma membrane (Fig. 6). A search for the missing deacetylase of the system in the genome of \textit{M. tuberculosis} \textit{H37Rv} led to our interest in Rv1170 (\textit{mshB}) and Rv1082 (\textit{mca}). MshB participates in the deacetylation of 1-D-myo-inositol-2-acetamido-2-deoxy-\alpha-D-glucopyranoside in the biosynthesis of mycothiol and, \textit{in vitro}, displays deacetylase activity on various other acetylated substrates. The mycothiol S-conjugate amidase Mca shares with MshB sequence similarities and deacetylase activity \textit{in vitro}, although its primary function in the bacterium is the cleavage of various S-conjugates of mycothiol (22, 31, 32). Analysis of AG in the \textit{M. tuberculosis} \textit{mshB} and \textit{mca} knock-out strains, however, revealed WT profiles in both mutants (supplemental Fig. S8B), suggesting either that these enzymes are not involved in the formation of the \textit{d}-GalNp substituent or that compensatory enzymatic activities exist in the cell.

With regard to the translocase of the system, studies are in progress in our laboratories to investigate whether the small integral membrane protein Rv3632 may assume this function, thereby explaining its tight functional association with PpgS. Alternatively, the role of Rv3632 may be to target PpgS to the plasma membrane as shown for GT’s involved in \textit{O}-polysaccharide synthesis in Gram-negative bacteria (33) or to stabilize PpgS in the membrane as suggested for \textit{M. smegmatis} polyprenyl phosphomannose synthase Ppm1 and associated transmembrane protein Ppm2 (fused as one single polypeptide, Rv2051c, in \textit{M. tuberculosis} \textit{H37Rv}) (34).

The availability of a ppgS knock-out mutant of \textit{M. tuberculosis} now provides a unique opportunity to elucidate the function(s) of the \textit{d}-GalNp substituent of AG. Preliminary studies indicate that the mutant does not display any defects in growth or susceptibility to drugs under optimal laboratory growth conditions. Thus, it is unlikely that \textit{d}-GalNp serves a critical role in stabilizing the structure of the envelope as suggested earlier (3). Instead, the relative restriction of this substituent to pathogenic slow growing mycobacterial species (and emerging nosocomial pathogen, \textit{M. abscessus}) together with the reported role of the \textit{d}-GalN substituent of lipid A in the pathogenicity of \textit{F. tularensis} (8) make it tempting to speculate that the \textit{d}-GalNp substituent of AG serves a function during host infection. \textit{In vivo} studies have been undertaken to verify this hypothesis.
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