Biosynthesis of the Galactan Component of the Mycobacterial Cell Wall*

The infrastructure, or core, of the cell wall of *Mycobacterium tuberculosis* is composed of a covalently linked complex of mycolic acids, arabinan, and galactan attached to peptidoglycan by a -Rhap(1→3)-GlcNAc-P- linker unit (LU)\(^3\), the mycolyl-AG-LU-peptidoglycan complex. Most of the primary structure has been elucidated (5), and new evidence supports the concept of a dynamic, asymmetric, lipid bilayer in which the mycolic acid monolayer, interspersed with porin-like proteins and perpendicular to the arabinogalactan-peptidoglycan complex, is complemented by an assortment of phospholipids and glycolipids to provide a relatively impermeable lipid barrier (6).

The initial steps in the assembly of the complex have been recently defined (7). Isolated membranes and cell walls from *Mycobacterium smegmatis* catalyze the transfer of GlcNAc-1-P and Rha from their respective nucleotide donors to endogenous polypropenyl-P (probably C\(_{50}\)-P), giving rise to polypropenyl-P-P-GlcNAc-Rha\(^3\). We now establish that the enzymes responsible for the conversion of UDP-Galp to UDP-Galp mutase (the *glf* gene product) and is catalyzed by a reference to the Galp donor. The polyprenyl-P-P-GLU-galactan-arabinan has been partially characterized in terms of its heterogeneity, size, and composition. Biosynthesis of the major components of mycobacterial cell walls is proving to be extremely complex. However, partial definition of arabinogalactan synthesis, the site of action of several major anti-tuberculosis drugs, facilitates the present day thrust for new drugs to counteract multiple drug-resistant tuberculosis.

The cell wall of *Mycobacterium* spp. is required for growth and survival (1), and its formation has become the focus of the search for essential targets in the development of new drugs against tuberculosis (2). Some of the constituents of the widely applied anti-tuberculosis four-drug regimen DOTS (directly observed therapy, short course) (isoniazid, ethionamide, and rifampicin) is extremely complex. However, partial definition of arabinogalactan synthesis, the site of action of several major anti-tuberculosis drugs, facilitates the present day thrust for new drugs to counteract multiple drug-resistant tuberculosis.

**The structural core of the cell walls of *Mycobacterium* spp. consists of peptidoglycan bound by a linker unit (-α-L-Rhap(1→3)-D-GlcNAc-P-) to a galactofuran, which in turn is attached to arabinofuranosyl and mycolic acids. The sequence of reactions leading to the biogenesis of this complex starts with the formation of the linker unit on a polypropenyl-P to produce polypropenyl-P-P-GlcNAc-Rha (Mikušová, K., Mikuš, M., Besra, G. S., Hancock, I., and Brennan, P. J. (1996) *J. Biol. Chem.* 271, 7820–7828). We now establish that formation of the galactofuran takes place on this intermediate with UDP-Galp as the Galp donor presented in the form of UDP-Galp and UDP-Galp mutase (the *glf* gene product) and is catalyzed by galactofuranosyl transferases, one of which, the *Mycobacterium tuberculosis* H37Rv3808c gene product, has been identified. Evidence is also presented for the growth of the arabinofuran on this polypropenyl-P-P-linker unit-galactan intermediate catalyzed by unidentified arabinofuranoyl transferases, with decaprenyl-P-Ara or P-ribosyl-PP as the Araf donor. The product of these steps, the lipid-linked-LU-galactan-arabinan has been partially characterized in terms of its heterogeneity, size, and composition. Biosynthesis of the major components of mycobacterial cell walls is proving to be extremely complex. However, partial definition of arabinogalactan synthesis, the site of action of several major anti-tuberculosis drugs, facilitates the present day thrust for new drugs to counteract multiple drug-resistant tuberculosis.**

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**Experimental Procedures**

**Preparation of UDP-GalpMutase—*Escherichia coli* BL21 (DE3) (Stratagene, Cedar Creek, TX) was transformed with plasmid pORF6 containing Rv3809c (glf) as described (9). UDP-Galp mutase was prepared and assayed as described (10). The enzyme preparation (33 mg/ml) was stable at \(-20^\circ C\).**

**Preparation of dTDP-Rha and dTDP-[\(^{14}\)C]Rha—The synthesis of dTDP-Rha relied on the presence of the full array of the Rha synthetic
enzymes and endogenous cofactors in M. smegmatis (11). dTDP-Glc (sodium salt; Sigma; 12 nmol) was incubated at 37 °C for 1 h with 25 μl of the 100,000 × g supernatant of disrupted M. smegmatis (70 μg of cytosolic protein), followed by the addition of 100 μl of cold ethanol. After 20 min on ice, the sample was centrifuged at 14,000 × g, and the supernatant was discarded. Membranes were resuspended in 25 μl of deionized water and used as a source of dTDP-Rha. Conversion of dTDP-Glc to dTDP-Rha under these conditions was about 95%, as revealed by analytical HPLC on a Partisil 10 SAX column as described (7).

dTDP-[14C]Rha was prepared from [U-14C]sucrose by conversion of the glucosamine moiety of the sucrose-1-phosphate-[14C]glucose-1-phosphate followed by further conversion to dTDP-[14C]Glc by α-glucose-1-phosphate thymidylyl transferase (RmlA) and to dTDP-[14C]Rha by RmlB-D. The α-glucose-1-phosphate thymidylyl transferase was prepared from M. tuberculosis rmA (11) expressed in E. coli (the remaining Rml enzymes were merely those found in the expression strain of E. coli) and in E. coli B (ATCC, Manassas, VA). The dTDP-[14C]Rha-generating mixture was as follows: 50 μl (113 nmol of 442 mCi/mmol) of [U-14C]sucrose (PerkinElmer Life Sciences) was dried in a tube followed by the addition of 16 μl of 1 M KH2PO4, pH 7.0, 80 μl (0.5 μmol) of sucrose phosphorase (Sigma), 10 μl of 40 mM TTP, 4 μl (2 units) of inorganic pyrophosphatase (Sigma), 200 μl of lysate (−5 mg/ml protein) of E. coli BL21(DES) expressing M. tuberculosis RmA (11), 10 mM NADPH, 55 μl of 50 mM HEPES buffer containing 10 mM MgCl2 (pH 7.0), in a total volume 400 μl. After 1 h of incubation at 37 °C, additional RmlB-D (1 mg of protein of crude E. coli B lysate) and NADPH (35 μl of 10 mM solution) were added to fully convert the dTDP-[14C]Glc to dTDP-[14C]Rha. Then 700 μl of absolute ethanol were added, and the precipitated protein was removed by centrifugation at 14,000 × g for 5 min. The bulk of the ethanol was removed by evaporation, and the dTDP-[14C]Rha was purified by HPLC as described (12).

Preparation of Enzymatically Active Membranes and Cell Envelope—M. smegmatis mc2155 and M. smegmatis pJ77–3880c were grown as described (7). Enzymatically active membranes and cell envelope (wall and membrane) were prepared as follows. Cells (10 g) suspended in 50 ml MOPS buffer, pH 7.9, containing 5 mM 2-mercaptoethanol and 10 mM MgCl2 (buffer A), subjected to probe sonication (7), and centrifuged at 23,000 × g for 20 min at 4 °C. The pellet was resuspended in buffer A, and Percoll (Amersham Pharmacia Biotech) was added to achieve a 60% suspension, which was centrifuged at 10,000 × g for 75 min at 4 °C. The supernatant was discarded, and the pellet was further extracted with 5 ml each of 60% CH3OH in H2O containing 0.1% NH4OH. The lipid-linked polymer-containing fractions (10,000 cpm) were loaded on the column, which was developed with 5 ml of 0.02 N HCl at 60 °C for 30 min (18) and then neutralized with 10 μl of 0.2 N NaOH. Products released by mild acid methylation of the [14C]Gal-labeled lipid-linked polymer was accomplished by the NaOH method (19). The per-O-methylated [14C]Gal-labeled lipid-linked polymer was hydrolyzed in 2 M CF3COOH for 1 h at 120 °C. Hydrolysates were analyzed by TLC on silica gel plates (Merck) developed twice in pyridine/ethyl acetate/glacial acetic acid/water (5:5:1:5). TLC plates were subjected to autoradiography using Kodak NEF X-OMAT MR film. Cold sugars and standards were visualized by charring with 10% cupric sulfate in 8% phosphoric acid.

Methylation of the [14C]Gal-labeled lipid-linked polymer was accomplished by the NaOH method (19). The per-O-methylated [14C]Gal-labeled lipid-linked polymer was hydrolyzed in 2 M CF3COOH at 110 °C for 2 h, the acid was evaporated, and sugars were reduced with NaBH4 and per-O-acetylated. Radiolabeled O-methylated alditol acetates were then analyzed on a 0.1 × 1-fused silica column (Scientific, Rancho Cordova, CA) in a Hewlett-Packard 5890 Series II Plus gas chromatograph, coupled to the Lablogic GC-RAM radioactivity counter (INUS Systems, Tampa, FL) (7). SDS-PAGE was performed on 15% polyacrylamide gels or on commercial 10–20% gradient Tricine SDS-polyacrylamide gels obtained from Novex (San Diego, CA), under conditions recommended by the manufacturer. Blotting to nitrocellulose was performed at 50 V for 1 h.
RESULTS

Requirement for UDP-Galf for Galactan Synthesis—Previously, we had demonstrated the biosynthesis of polypropenyl-P-P-GlcNAc (GL-1) from endogenous polypropenyl-P and UDP-GlcNAc followed by synthesis of propenyl-P-P-GlcNAc-Rha (GL-2) with the addition of TDP-Rha (7). Further propenyl-P-P-GlcNAc-Rha-Gal (GL-3) and propenyl-P-P-GlcNAc-Rha-Gal-Gal (GL-4) were formed from the newly synthesized propenyl-P-P-GlcNAc-propenyl-P-P-GlcNAc-Rha (GL-1/GL-2) in the presence of added UDP-Galp, mycobacterial membranes, and cell envelope fraction. In E. coli K12 (9), Klebsiella pneumoniae (20), and M. smegmatis (8), UDP-Galp is formed by a one-step transformation of UDP-Galp catalyzed by UDP-Galp mutase (EC 5.4.99.9), the product of the glf gene. To differentiate the role of membranes from cell envelope in the biosynthesis of GL-1 to -4 and to examine the role of UDP-Galp mutase, experiments were conducted as described in Fig. 1. Thoroughly washed membranes alone produce GL-1 to -4 if UDP-Galp mutase is included in the reaction (Fig. 1, A and B). Membranes in the absence of endogenous UDP-Galp mutase showed only slight incorporation (about 10%) of [14C]Gal from UDP-[14C]Galp into the glycolipid fraction. The results also demonstrate that the presence of DTDP-Glc stimulated the formation of GL-2 and that TDP-Rha, which is not commercially available, can be replaced by DTDP-Glc and cytosol followed by inactivation of the cytosolic enzymes to avoid catabolism of nucleotide sugars. The results confirm the role of cytoplasmic RmlB (DTDP-Glc 4,6-dehydratase), RmlC (DTDP-6-deoxy-4-ketoglucone epimerase), and RmlD (DTDP-Rha synthase) and membrane rhansosyltransferase in synthesis of the Rha of the LU, in accordance with the presence of the corresponding genes (rmlB to -D and wbbL) in the M. tuberculosis genome (21). The presence of tunicamycin in reaction mixtures drastically inhibited synthesis of the [14C]Gal-labeled GLs (Fig. 1C), confirming the role of polypropenyl-P in this synthesis.

Incorporation of [14C]Gal from UDP-[14C]Galp into Galactofuran—With the realization that the apparent [14C]Gal-containing intermediates of galactan synthesis were lipid-linked, solvents developed for solubilization of dolichyl-P-P-linked oligosaccharides (13) and phytosphingosylglycolipids (14) were applied in search of more polymerized galactan intermediates. After extraction with CHCl₃/CH₃OH (2:1) and subsequent washing, the pellet was extracted with CHCl₃/CH₃OH/H₂O (10:10:3), followed by water/ethanol/diethyl ether/pyridine/NH₄OH (“E-soak”) (Table I). About 20% of the applied radioactivity was incorporated into [14C]Gal-labeled products, of which about 8% was in CHCl₃/CH₃OH (2:1), 40% in CHCl₃/CH₃OH/H₂O (10:10:3), 50% in E-soak fractions, and about 2% in the final pellet; the distribution of the counts between the two polar solvents varied from experiment to experiment. A surprising outcome of this approach was the paucity of radioactivity in the insoluble peptidoglycan-bound galactan and the preponderance in lipid-soluble material. TLC of the CHCl₃/CH₃OH-soluble products in CHCl₃, CH₃OH, NH₄OH, 1 M ammonium acetate, H₂O (180:140:9:9:23) showed the presence of a ladder of GLs of increasing polarity, indicative of sequential glycosylation of GL-1/GL-2, apparently 1 Galp unit at a time, but apparently to a finite length of about 4 Galp units. The products all demonstrated mild acid sensitivity and mild alkali resistance (7), in accordance with polypropenyl-P linkage. The more highly glycosylated lipid-linked polymers in the CHCl₃/CH₃OH/H₂O and E-soak solvents did not migrate under these conditions (Fig. 2).

The relative contributions of the cell envelope and membrane fractions to incorporation of [14C]Galp into these extracts were examined (Table I). The absence of the cell envelope enzyme fraction had a profound effect on the incorporation of [14C]Galp into the more polar, presumably more glycosylated CHCl₃/CH₃OH/H₂O (10:10:3) and E-soak-soluble products. The omission of membranes from the reaction mixture still allowed significant synthesis of [14C]Gal-labeled polymer, indicating that the cell envelope fraction contained all of the enzymes involved in biosynthesis of these products. Inclusion of tunicamycin, which is known to inhibit transfer of GlcNAc-1-P from UDP-GlcNAc to dolichyl-P-polypropenyl-P (22–24), dramatically inhibited production of the [14C]Gal-labeled polymer (Table I), confirming that these reactions are polypropenyl-P-based and probably serve as the initiation point for galactan biosynthesis. The relatively smaller inhibition by tunicamycin of incorporation of radioactivity into the CHCl₃/CH₂O/H₂O (60%) compared with the CHCl₃/CH₃OH/H₂O (94%) and E-soak (88%) extracts was probably due to synthesis of galactolipids (the glycosyl diacylglycerides) other than GL-3 to -5 (see Fig. 1C).
mers were chromatographed in CHCl₃, CH₃OH, NH₄OH, 1 M ammonium acetate, H₂O-, and E-soak-soluble fractions. The signal/response ratio of the terminal, nonreducing end Gal was determined by the column coupled to the Lablogic GC-RAM radioactive counter. The products recovered through extraction (Fig. 4) lipid-linked polymers demonstrated the heterogeneity of these products (Fig. 4B).

**Evidence for the Presence of Ara: Composition of the Lipid-linked Polymers—** The products recovered through extraction with CHCl₃/CH₃OH/H₂O (10:10:3) and E-soak (−500,000 cpm) were dried thoroughly, per-O-methylated, hydrolyzed, reduced, per-O-acetylated, and analyzed on a fused silica Durabond-1 column coupled to the Lablogic GC-RAM radioactive counter. The signal/response ratio of the terminal, nonreducing end Gal (i.e. 1-O-Ac-2,3,5,6-tetra-O-Me-galactitol) to the combined 5- and 6-linked Gal (i.e. 1,5-di-O-Ac-2,3,6-tri-O-Me-galactitol and 1,6-di-O-Ac-2,3,5-tri-O-Me-galactitol) in the case of the E-soak-soluble material (Fig. 5) was about 1:6. Importantly, there was clear evidence for 5,6-linked Gal (i.e. 1,5,6-tri-O-Ac-2,3-di-O-Me-galactitol), which can only be attributed to the attachment of Ara residue(s) to the galactan (5).

To provide further evidence for the presence of Ara in the lipid-linked polymer, the enzymatically active membranes and column, the approximate mass of the larger polymer is ~10.8 kDa, and the smaller polymer is about the same mass as the mannan from lipomannan, i.e. ~8 kDa. The size of the mature AG released from the mycobacterial cell wall is of the order of 15 kDa (5), and thus the polyprenyl-P-P-linked polymer generated by the in vitro system is apparently not fully glycosylated. The acidic nature of the population of lipid-linked polymer macromolecules was confirmed by chromatography on DEAE-cellulose; about 85% were recovered by elution with 50 mM ammonium formate in 60% CH₃OH in H₂O with 0.1% NH₄OH.

Despite their sizes, the lipid-linked polymers migrated on a cellulose; about 85% were recovered by elution with 50 mM ammonium formate in 60% CH₃OH in H₂O with 0.1% NH₄OH. Hydrolysis were also visualized by this means. SDS-PAGE of these products (Fig. 4) was performed, and the CHCl₃-CH₃OH (2:1)-soluble lipids were dried, suspended in 50 mM sodium acetate, H₂O (180:140:9:9:23) and exposed to x-ray film for 10 days at ~70 °C.
Gal-labeled E-soak-soluble lipid-linked polymer. The incubation, extraction, and mild acid hydrolysis conditions were described in the legend to Fig. 3. A, 500 cpn of native (lane 1) and acid-hydrolyzed (lane 2) [14C]Gal-labeled E-soak-soluble products were applied to a silica gel TLC plate and developed in 60% methanol in water, containing 0.025% ammonium hydroxide. The plate was exposed to Kodak X-Omat AR film at 70 °C for 7 days. B, 5000 cpn of [14C]Gal-labeled E-soak-soluble material (lane 1) and 5000 cpn of the CHCl3/CH3OH/H2O (10:10:3)-soluble material (lane 2) were dried, immediately dissolved in 10 µl of SDS-sample buffer, boiled for 3 min, and loaded to a 15% SDS-polyacrylamide gel along with radiolabeled protein molecular weight markers provided as a gauge of relative mobility (lane 3). Blotting to nitrocellulose membrane was performed at 50 V for 1 h. The membrane was exposed to Kodak X-OMat AR film at 70 °C for 2 days.

Fig. 5. Linkage analysis of per-O-methylated, per-O-acetylated [14C]Gal-labeled E-soak-soluble lipid-linked polymer. About 500000 cpn of [14C]Gal-labeled E-soak material were dried in vacuo and subjected to NaOH methylation as described (19). Per-O-methylated oligosaccharide alditol acetates were prepared, and loaded to a 15% SDS-polyacrylamide gel along with radiolabeled protein molecular weight markers provided as a gauge of relative mobility (lane 3). Blotting to nitrocellulose membrane was performed at 50 V for 1 h. The membrane was exposed to Kodak X-OMat AR film at 70 °C for 2 days.

Cell envelope fraction were incubated with [14C]-D-Araf-Cmp. About 2% of the input radioactivity was incorporated into combined CHCl3/CH2OH/H2O (10:10:3) and E-soak-soluble polymer and insoluble residue. Incorporation into E-soak-soluble lipid-linked polymer was twice that into CHCl3/CH2OH/H2O-soluble polymer, suggesting that the two families of lipid-linked polymers differed in the degree of arabinosylation. The nature of the E-soak-soluble [14C]Ara-labeled polymer was examined after mild acid hydrolysis by gel filtration on Bio-Gel P-100 and was shown to be similar in size to [14C]Gal-labeled E-soak-soluble products.

The composition of the lipid-linked polymer was further examined by incorporation of [14C] from the individual sugar nucleotides, UDP-[14C]GlcNAc, dTDP-[14C]Rha, and the Ara precursor, 5-P-[14C]riboisyl-PP, into the CHCl3/CH2OH/H2O (10:10:3) and the E-soak extracts (−1500 cpn) in 2 µl of CF2COOH for 1 h at 120 °C. Hydrolysates (500 cpn) were chromatographed on silica gel plates in pyridine/ethyl acetate/glacial acetic acid/water (5:5:1:3) and developed twice. TLC plates were exposed to Kodak BioMax MR film at −70 °C for 14 days. Lanes 1–4, CHCl3/CH2OH/H2O-soluble lipid-linked polymer labeled in [14C]GlcNAc (lane 1; 800 cpn), [14C]Rha (lane 2; 600 cpn), [14C]Ara (lane 3; 800 cpn), [14C]Gal (lane 4; 18000 cpn). Lanes 5–8, E-soak-soluble lipid-linked polymer labeled in [14C]GlcNAc (lane 6; 400 cpn), [14C]Rha (lane 7; 600 cpn), [14C]Ara (lane 7; 600 cpn), [14C]Gal (lane 8; 8000 cpn). In the image, lanes 4 and 8 from this autoradiogram were replaced with the same lanes from an autoradiogram that was exposed to film for 1 day. B, complete acid hydrolysis was conducted on the CHCl3/CH2OH/H2O (10:10:3) and the E-soak extracts (−1500 cpn) in 2 µl of CF2COOH for 1 h at 120 °C. Hydrolysates (500 cpn) were chromatographed on silica gel plates in pyridine/ethyl acetate/glacial acetic acid/water (5:5:1:3) and developed twice. TLC plates were exposed to Kodak BioMax MR film at −70 °C for 8 days. C, cold sugar standards were visualized by charring with 10% cupric sulfate in 8% phosphoric acid. The plate shown is from E-soak-soluble hydrolyzed polymer; however, the results from hydrolysis of the CHCl3/CH2OH/H2O (10:10:3)-soluble polymer were identical.

Fig. 6. Composition of lipid-linked polymer. The basic reaction mixture containing UDP-[14C]GlcNAc, dTDP-Rha, UDP-Gal and mutants, cell envelope fraction (1 mg), and buffer A was modified as follows: (i) cold UDP-[14C]GlcNAc was replaced with 1 µCi of UDP-[14C]GlcNAc; (ii) cold dTDP-Rha was replaced with 0.5 µCi of dTDP-[14C]Rha; (iii) 5-phospho-[14C]ribosylpyrophosphate (0.5 µCi) was added; (iv) cold UDP-Gal was replaced with 1 µCi of UDP-[14C]Gal. A, SDS-PAGE, followed by blotting to nitrocellulose was performed on the products of the above reactions. Aliquots representing 10% of the CHCl3/CH2OH/H2O and 5% of the E-soak extracts were dried, immediately dissolved in 10 µl of SDS-sample buffer, boiled for 2 min, and loaded to a 10–20% Tricine SDS-polyacrylamide gel along with radiolabeled protein molecular weight markers. The blot was exposed to Kodak BioMax MR film and subjected to NaOH methylation as described (19). Per-O-methylated, peracetylated acid solubilized arabinogalactan from M. bovis C50-C70, cold sugar standards were visualized by charring with 10% cupric sulfate in 8% phosphoric acid. The plate shown is from E-soak-soluble hydrolyzed polymer; however, the results from hydrolysis of the CHCl3/CH2OH/H2O (10:10:3)-soluble polymer were identical.

GL-1/11 GL-2 Are Precursors of the Lipid-linked Polymer—The products from a reaction mixture containing 60,000 cpn of the
purified GL-1/GL-2 mixture as the radioactive precursors were extracted with CHCl₃/CH₃OH/H₂O and E-soak. Over 12% of the input radioactivity was incorporated into the final macromolecules, most of which was in the E-soak-extractable material. That these were the lipid-linked polymer was confirmed by DEAE-cellulose chromatography and mild acid hydrolysis followed by gel filtration. In another innovative approach to address the relationship between the simpler glycolipid and the lipid-linker polymers, a time course experiment was conducted using isolated membranes that had been prelabeled with UDP-[14C]GlcNAc and then chased with cold UDP-Gal in the presence of the UDP-Gal mutase. A comparison of total radioactivity in the extracted fractions showed a decrease in the amount of CHCl₃/CH₃OH (2:1)-soluble precursors (i.e. [14C] GlcNAc-labeled GL-1/GL-2) accompanied by an increase in radioactivity in the more polar, more glycosylated CHCl₃/CH₃OH/H₂O and E-soak-soluble products (Fig. 7). Although counts lost did not equate fully with counts gained, which may be attributed to partial decomposition of the substrates, it is clear that conversion of the radiolabeled GL-1/GL-2 precursor to lipid-linked polymer occurred, probably due to endogenous glycosyl transferases present in the membrane and cell envelope fractions.

Cloning and Enzymatic Activity of the Galactosyltransferase Gene M. tuberculosis Rv3808c—Analysis of the genomic data base of M. tuberculosis H37Rv (21) revealed the gene Rv3808c downstream from the UDP-Gal mutase glf gene (Rv3809c). The first four nucleotides of Rv3808c and the last four of Rv3809c overlap. In light of this four-nucleotide overlap between RV3808c and glf, it seemed likely that Rv3808c comprises an operon with the glf (Rv3809c) gene, although both genes have possible ribosome binding sites. (This operon might extend up to Rv3805c, because Rv3807c, Rv3806c, and Rv3805c apparently do not have their own promoters.) Furthermore, hydrophobic cluster analysis of Rv3808c demonstrated a conserved β-glycosyltransferase domain (26). Thus, it seemed possible that Rv3808c was a galactosyltransferase transcriptionally coupled to glf. Rv3808c was cloned into the pET29b and pJJV7 expression vectors and transformed into E. coli and M. smegmatis cells. Overexpression of the gene in E. coli led to production of a new protein with an apparent molecular mass of 68 kDa, as determined by SDS-PAGE (data not shown) and as predicted. However, the observed level of expression was lower than normally obtained with this expression system, and all of the recombinant protein was found in the insoluble fraction of cell homogenates; no enzymatic activity was detected in the soluble portion of the homogenates. The mycobacterial expression plasmid pJJV7 differs in that expression is driven from a native, mycobacterial groEL promoter. M. smegmatis transformed with pJJV7–3808c plasmid or empty plasmid was examined for galactosyl transferase activity in the basic cell-free assay. A time course experiment showed almost no differences in the incorporation of [14C]Gal from UDP-[14C]Gal into the CHCl₃/CH₃OH (2:1) extracts; however, there was a substantial increase in radioactivity incorporated into CHCl₃/CH₃OH/H₂O (10:10:3) and E-soak (Fig. 8). The former lipid-linked polymer was subjected to mild acid hydrolysis followed by gel filtration chromatography. The radioactive profile revealed that the products from the control strain and the strain with the cloned gene had the same size, and thus the increase in the incorporation of the [14C]Gal into the products is not due to further extension of the galactan chain but rather due to greater production of the same material. Methylation analysis of the lipid-linked polymer confirmed that true galactofuran was synthesized in the reaction. Thus, Rv3808c encodes a galactosyl transferase responsible for the synthesis of bulk 5- and 6-linked galactofuran.

DISCUSSION

To date, the only polypropenyl-P implicated in aspects of mycobacterial cell wall biosynthesis are decaprenyl-P and heptaprenyl-P (27, 28). The addition of a cell wall-membrane enzyme preparation and UDP-[14C]Gal to reaction mixtures capable of synthesizing polypropenyl-P-P-GlcNac (GL-1) and polypropenyl-P-P-GlcNac-Rha (GL-2) resulted in the synthesis of Galf-labeled more polar glycolipids, GL-3 and GL-4, indicating stepwise
growth of the initial segments of the galactan chain on the polyprenyl-P-P-GlcNAc-Rha unit, 1 Gal unit at a time (7). Present evidence shows that thoroughly washed membrane preparations are not able to synthesize GL-3 and GL-4, which, however, could be achieved by the addition of UDP-Galp mutase encoded by the glf gene (11), demonstrating a requirement for UDP-Galp as donor. Moreover, analysis of the CHCl₃/CH₃OH (2:1)-soluble lipids in polar solvent demonstrated a hierarchial array of galactolipids, with all of the evidence for polyprenyl-P linkage, again pointing to sequential addition of single Galp units. Calling on the approaches that led to the solubilization of the oligosaccharide-P-P-dolichol intermediates of glycoprotein synthesis (13) and to the extraction of phosphophingolipids from yeast (14) and the lipophosphoglycan of Leishmania donovani (29), we successfully solubilized the newly synthesized galactofuran. Surprisingly, two distinct populations exist, differentially transferred by the two solvents. As in the case of the dolichyl-bound oligosaccharides, the identification of a polyprenol-P linkage was based on mild acid lability, mild alkali stability, solubility in extremely polar organic solvents, and exclusion from Bio-Gel P-100, all suggesting a highly polymerized lipid-linked version of GL-1–4. De novo synthesis of the lipid-linked polymer is also sensitive to tunicamycin, and evidence is presented for the incorporation of GL-1/GL-2 into the lipid-linked polymer. Glycosyl linkage analysis of the polymer produced t-Galf, 5-linked Galp, 6-linked Galp, and 5,6-linked Galp, indicating that there is substitution of one or more of the linear Galp residues, presumably with arabinian. Moreover, ¹⁴C Araf, donated by synthetic C₅₀-P⁴-C¹⁴C Araf, or formed from 5-phospho-D-ribofuranose was incorporated into this same polymer as characterized by solubility in polar lipid solvents, SDS-PAGE mobility, mild acid lability, and hence lipid linkage. The combined evidence points to the pathway shown in Fig. 9 for the synthesis of the AG component of the mycobacterial cell wall core.

The elucidation of the basic elements of synthesis of the cell wall core of mycobacteria should substantially enhance current tuberculosis drug discovery efforts, in that aspects of cell wall synthesis are the targets of many of the current front-line anti-tuberculosis drugs (2), and the pathways and their end products are distinctly xenogeneic.

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FIG. 9. Pathway for the early steps in the synthesis of the arabino-galactan heteropolysaccharide of mycobacterial cell wall core. The genes encoding the galactosyltransferases have not yet been defined, except for the putative galactosyl transferase, M. tuberculosis Rv3808c gene product, identified in this study. The arabino-galactosyltransferases may be encoded by the ethambutol resistance genes, embA to -C (34). The values for m, n, x, and y are not known.
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