# NOTES

# Identification of a Polyprenylphosphomannosyl Synthase Involved in the Synthesis of Mycobacterial Mannosides$^{\nabla}$†

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We report on the identification of a glycosyltransferase (GT) from *Mycobacterium tuberculosis* H37Rv, Rv3779, of the membranous GT-C superfamily responsible for the direct synthesis of polyprenyl-phospho-mannopyranose and thus indirectly for lipoarabinomannan, lipomannan, and the higher-order phosphatidyl-*myo*-inositol mannosides.

The mycobacterial cell envelope consists of a multilayered structure of covalently linked peptidoglycan, arabinogalactan, and mycolic acids (the mAGP complex) and, among other important constituents, various noncovalently bound glycosylated lipids, notably the phosphatidyl-*myo*-inositol mannosides (PIMs) and their more glycosylated end products lipomannan (LM) and lipoarabinomannan (LAM) (6, 8). These glycolipids and lipoglycans exhibit a broad range of immunomodulatory activities implicated in the pathogenesis of tuberculosis and leprosy (for recent reviews, see references 5, 8, and 10).

Many steps in the biosynthesis of these phosphoinositides have been defined (for recent reviews, see references 3 and 12). Mannosyltransferases (ManTs) responsible for the polymerization aspects of the synthesis of the higher-order extracytoplasmic PIMs ($PIM_4$ to $PIM_6$), LM and LAM, are of the glycosyltransferase C (GT-C) multi-transmembrane domain superfamily, whose members are dependent on polyprenyl-phospho-mannopyranose (polyprenyl-P-Man$p$), specifically $C_{35}$-P-Man or $C_{50}$-P-Man, as the Man$p$ donor, which is in contrast with what occurs in the early steps of the synthesis of the simpler PIMs, which directly utilize GDP-Man (3, 4, 12). However, the biosynthetic origins of $C_{35}/C_{50}$-P-Man have not been fully explored. In this report, we identify Rv3779, an unassigned GT-C, as a ManT directly responsible for the origins of polyprenyl-P-Man and indirectly for the synthesis of the more polar PIMs, LM and LAM.

A survey of the *Mycobacterium tuberculosis* H37Rv genome for genes with predicted (poly)saccharide-associated functions led to the identification of a cluster of 31 "cell wall biosynthetic genes" (2, 3), including not only arabinogalactan synthetic genes (*embCA* and *embCB*, *glf*, *glfT1*, and *glfT2*) but the genes for the putative ABC transporter proteins and mycolyl transferases and Rv3779, among five open reading frames, apparently encoding polyprenyl-P sugar-dependent GT-Cs (3). In particular, the integral membrane protein Rv3779 (666 amino acids) was identified as a putative GT-C, due to a conserved hallmark DXD motif and 12 to 14 predicted membrane-spanning domains (3). However, unlike with other GT-C enzymes, which typically carry the signature DXD motif on extracytoplasmic loops, the DLD motif of Rv3779 (at amino acid position 82) is predicted to map to the second loop and to be on the cytosolic side of the plasma membrane. This observation suggests that unlike other GT-C superfamily enzymes, Rv3779 utilizes a cytosolic sugar donor, presumably a nucleotide sugar. Rv3779 has orthologs in all slow-growing mycobacteria whose genome sequences are available but is not found in *Mycobacterium smegmatis* or in any fast-growing mycobacteria, with the notable exception of *Mycobacterium abscessus*. Orthologs of this gene are also not found in any other members of the suborder *Corynebacterineae*.

Since Rv3779 is not naturally present in *M. smegmatis*, *M. smegmatis* strain mc²155 was transformed with a multicopy plasmid (pVV16-*Rv3779*), allowing the expression of a recombinant C-terminal His₆-tagged Rv3779 protein under the control of the p*hsp60* promoter (13). A cell-free ManT assay using membranes or whole lysate, similar to the one described by Korduláková et al. (13), was then conducted to determine if Rv3779 is involved in some aspect of PIM/LM/LAM biosynthesis. Thin-layer chromatography (TLC) autoradiography demonstrated an approximately threefold increase in a time-dependent manner in the incorporation of [$^{14}$C]Man from GDP-[$^{14}$C]Man into $C_{35}$-P-Man and $C_{50}$-P-Man by mc²155/pVV16-*Rv3779* extracts compared with that of the control (Fig. 1A). The two accumulated Man-containing glycolipids were mildly alkali stable and mildly acid labile (data not shown), with TLC mobility properties confirming their identities as precursor glycolipids of the mycobacterial polyisoprenyl-P class (4). An important and substrate concentration-dependent
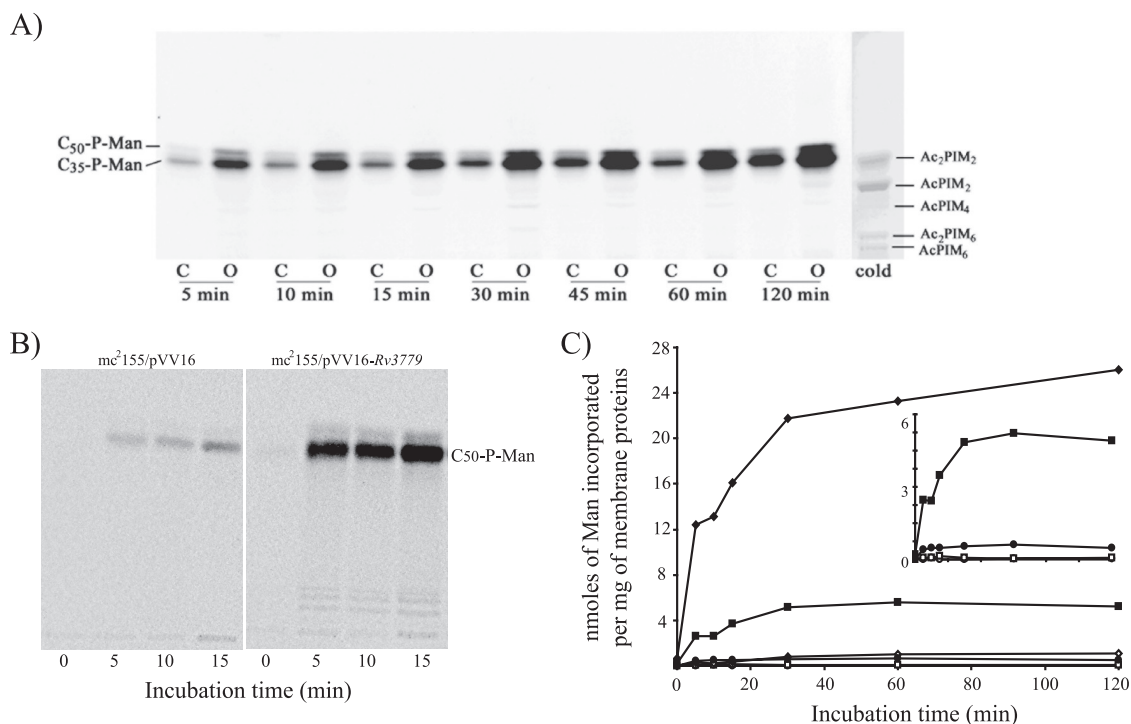
A)



B)



C)



FIG. 1. Effect of Rv3779 overexpression on the incorporation of $[^{14}C]$Man from GDP-$[^{14}C]$Man into mannolipids by cell extracts from *M. smegmatis*. (A) TLC analysis of an in vitro cell-free assay using GDP-$[^{14}C]$Man and crude cell lysates from mc$^2$155/pVV16 and mc$^2$155/pVV16-*Rv3779*. Lysates were incubated with GDP-$[^{14}C]$Man at 37°C for the indicated periods of time. The synthesized mannolipids were extracted with CHCl$_3$-CH$_3$OH (2:1, vol/vol), and a 10% aliquot of each sample was analyzed by TLC followed by autoradiography. The TLC plate was developed in CHCl$_3$-CH$_3$OH-H$_2$O-NH$_4$OH (65:25:4:0.5). Lanes: C, mc$^2$155/pVV16; O, mc$^2$155/pVV16-*Rv3779*. (B and C) Incorporation of $[^{14}C]$Man from GDP-$[^{14}C]$Man into exogenous decaprenyl-phosphate (C$_{50}$)-P using membrane extracts from mc$^2$155/pVV16 and mc$^2$155/pVV16-*Rv3779*. (B) TLC analysis of the first 15 min of the in vitro cell-free assays using 0.5 mM of (C$_{50}$)-P. (C) Quantification of the Man incorporated by the control (open symbols) and overexpressor (filled symbols) into C$_{50}$-P-Man over time. The concentrations of (C$_{50}$)-P used in the assays were 0 (circles), 0.005 (rectangles), and 0.05 (diamonds) mM. The inset shows a closeup of results of the assays using 0 and 0.005 mM (C$_{50}$)-P.

increase in mannosyl transfer following Rv3779 overexpression was also observed when C$_{50}$-P, the only form of polyprenylphosphate apparently produced by *M. tuberculosis* (7), was added as an acceptor substrate to the reaction mixture (Fig. 1B and C). This increase in activity over the control was about 27-fold during the first 30 min of the reaction when 0.05 mM C$_{50}$-P was used in the assay (Fig. 1C). The background polyprenyl-P-Man*p* syntase activity detected in the control strain most likely resulted from MSMEG_3859/MSMEG_3860 (Ppm1/Ppm2), an *M. smegmatis* ortholog of the Ppm1 synthase from *M. tuberculosis* (68% identity, as determined with a 781-amino-acid overlap) (1, 9, 11). Unfortunately but not unexpectedly in light of the reported difficulty of expressing polytopic membrane GTs in *Escherichia coli* (14), attempts to produce Rv3779 in an active form in *E. coli* using different expression systems, host strains, growth conditions, and induction protocols were unsuccessful.

Incubation of whole-cell lysate with GDP-$[^{14}C]$Man for more-extended periods (4 to 24 h) and extraction of products with more-polar solvents (e.g., hot phenol) provides a ready estimate of the degree of synthesis of at least the LM metabolic end product. A comparison of the products synthesized by mc$^2$155/pVV16 and mc$^2$155/pVV16-*Rv3779* (Fig. 2) showed little synthesis of $[^{14}C]$LM at 4 h but the production of some low-molecular-weight $[^{14}C]$LM at 24 h by the control lysate. In contrast, there was a distinct production of $[^{14}C]$LM popula-

tions by the lysate from mc$^2$155/pVV16-*Rv3779* at both times, indicative of a secondary stimulatory effect of Rv3779 overexpression on LM, and presumably LAM, synthesis.

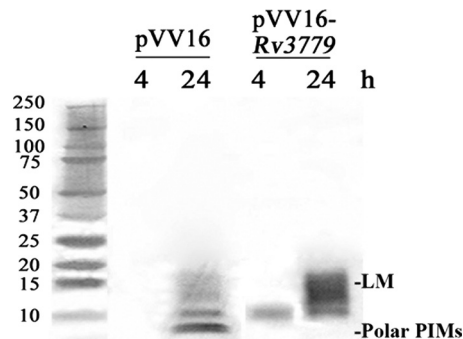To confirm these effects of Rv3779, an *M. tuberculosis*



FIG. 2. In vitro LM biosynthesis in *M. smegmatis* overexpressing Rv3779. Crude cell extracts (4 mg protein) of mc$^2$155/pVV16 and mc$^2$155/pVV16-*Rv3779* were incubated with 1.0 μCi of GDP-$[^{14}C]$Man (specific activity, 305 mCi/mmol) for 4 and 24 h. The reaction was stopped by adding CHCl$_3$-CH$_3$OH (2:1, vol/vol), and the LM/LAM contained in the cell pellet was extracted with hot phenol. LM/LAM separated on 10 to 20% Tricine gel and subsequently blotted to a nitrocellulose membrane was revealed by autoradiography. The leftmost column shows molecular mass markers (in kilodaltons).
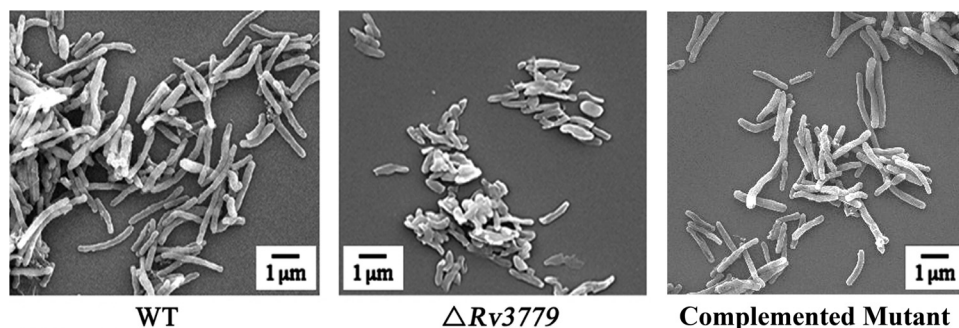
FIG. 3. Scanning electron micrographs of *M. tuberculosis* H37Rv (WT), the mutant H37RvΔRv3779, and the complemented mutant H37RvΔRv3779/pVV16-*Rv3779* cultured in 7H9-oleic acid-albumin-dextrose-catalase-Tween 80 broth at 37°C. The length of the H37RvΔRv3779 mutant cells was on average 1.2 ± 0.1 μm, compared to 2.3 ± 0.1 μm for the WT strain and 2.1 ± 0.3 μm for the complemented mutant.

H37Rv Rv3779 knockout mutant (H37RvΔRv3779) was generated by homologous recombination using standard procedures (15) (see Fig. S1 in the supplemental material). H37RvΔRv3779 grew at a much lower rate than WT H37Rv (see Fig. S2 in the supplemental material). Moreover, when



FIG. 4. Analysis of polar PIMs LM and LAM from WT *M. tuberculosis* H$_{37}$Rv, the H37RvΔRv3779 mutant, and H37RvΔRv3779/ pVV16-*Rv3779*. (A) Equal amounts of total cellular lipids from WT H$_{37}$Rv (lane 1), the Rv3779 mutant H37RvΔRv3779 (lane 2), the mutant carrying an empty plasmid, H37RvΔRv3779/pVV16 (lane 3), and the complemented mutant H37RvΔRv3779/pVV16-*Rv3779* (lane 4) were analyzed by TLC developed in $CHCl_3$-$CH_3OH$-$H_2O$-$NH_4OH$ (65:25:4:0.5). (B) LM and LAM extracted from equal-weight cells of WT H$_{37}$Rv (lane 1), the Rv3779 mutant H37RvΔRv3779 (lane 2), and the complemented mutant H37RvΔRv3779/pVV16-*Rv3779* (lane 3) were separated on a 10 to 20% Tricine gel and revealed by periodic acid-Schiff staining. The Western blot analyses were performed on the same samples using concanavalin A (ConA) reacting with the t-Man*p* residues of LM/LAM, as well as the CS-35 monoclonal antibody known to react with the arabinan segment of LAM.

examined by scanning electron microscopy, the mutant cells were found to be significantly shorter than their wild-type (WT) parent (Fig. 3). Normal growth rate and cell length were restored in the mutant upon complementation with a WT copy of Rv3779 (Fig. 3; see Fig. S2 in the supplemental material). TLC profiles of lipid extracts and matrix-assisted laser desorption ionization–time of flight mass spectrometry analyses showed a profound decrease in the amounts of acyl-PIM$_6$ (AcPIM$_6$) and Ac$_2$PIM$_6$ in H37RvΔRv3779 compared to those of the WT strain (Fig. 4A; see Fig. S3 in the supplemental material). Importantly, the synthesis of the simpler PIMs, those arising directly from GDP-Man, was not altered in the mutant (Fig. 4A; see Fig. S3 in the supplemental material). The examination by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting with the monoclonal antibody CS-35 and concanavalin A of the phenol-extracted LM/ LAM fraction from WT H37Rv and H37RvΔRv3779 also revealed considerably smaller amounts of both LM and LAM in the mutant (Fig. 4B). Complementation of H37RvΔRv3779 with a WT copy of Rv3779 restored both a normal polar PIM and a normal LM/LAM profile to the mutant.

From this evidence, we conclude that Rv3779 is involved in the synthesis of the polar PIMs LM and LAM through its primary role as a polyprenyl-P-Man synthase. The disruption of this gene, which results in important alterations in PIM, LM, and LAM profiles, also has profound effects on cell growth and shape. Rv3779 is the second polyprenyl-P-Man synthase involved in PIM/LM/LAM biosynthesis identified in *M. tuberculosis* (11).

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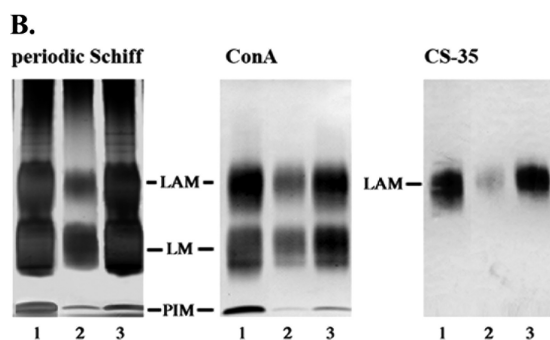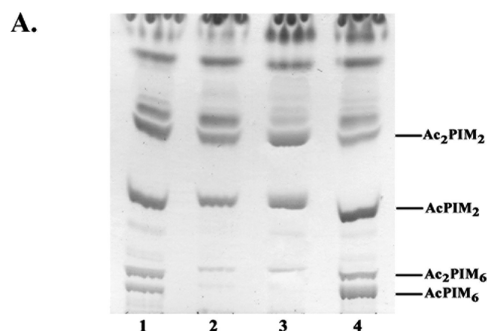4. **Besra, G. S., C. B. Morehouse, C. M. Rittner, C. J. Waechter, and P. J.**

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