Characterization of a putative α-mannosyltransferase involved in phosphatidylinositol trimannoside biosynthesis in *Mycobacterium tuberculosis*

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Phosphatidyl-*myo*-inositol mannosides (PIMs), lipomannan (LM) and lipoarabinomannan (LAM) are an important class of bacterial factors termed modulins that are found in tuberculosis and leprosy. Although their structures are well established, little is known with respect to the molecular aspects of the biosynthetic machinery involved in the synthesis of these glycolipids. On the basis of sequence similarity to other glycosyltransferases and our previous studies defining an α-mannosyltransferase from *Mycobacterium tuberculosis*, named PimB [Schaeffer, Khoo, Besra, Chatterjee, Brennan, Belisle and Inamine (1999) J. Biol. Chem. **274**, 31625–31631], which catalysed the formation of triacyl (Ac_3)-PIM_2 (i.e. the dimannoside), we have identified a related gene from *M. tuberculosis* CDC1551, now designated *pimC*. The use of a cell-free assay containing GDP-[^{14}C]mannose, amphomycin and membranes from *Myobacterium smegmatis* overexpressing PimC led to the synthesis of a new alkali-labile PIM product. Fast-atom-bombardment MS established the identity of the new enzymically synthesized product as Ac_3PIM_3 (i.e. the trimannoside). The results indicate that *pimC* encodes an α-mannosyltransferase involved in Ac_3PIM_3 biosynthesis. However, inactivation of *pimC* in *Myobacterium bovis* Bacille Calmette–Guérin (BCG) did not affect the production of higher PIMs, LM and LAM when compared with wild-type *M. bovis* BCG, suggesting the existence of redundant gene(s) or an alternate pathway that may compensate for this PimC deficiency. Further analyses, which compared the distribution of *pimC* in a panel of *M. tuberculosis* strains, revealed that *pimC* was present in only 22% of the clinical isolates examined.

Key words: cell-free, glycosyltransferases, GDP-mannose, lipomannan, lipoarabinomannan, mycobacteria.

INTRODUCTION

The emergence of multi-drug-resistant strains of *Mycobacterium tuberculosis* and the increased incidence of tuberculosis have made it clear that there is an urgent need for new anti-tubercular agents [1]. In this regard, the mycobacterial cell-wall core, the mycolyl–arabinogalactan–peptidoglycan complex, which represents the site of action of many of the current first-line agents used to treat tuberculosis, represents a very attractive target for new anti-mycobacterial agents [2]. The cell wall also contains a variety of non-covalently bound lipids that are presumed to be necessary for cell viability and survival within the host [3]. Lipoarabinomannan (LAM) is one such component that belongs to the class of bacterial factors named modulins, and operates by inducing synthesis of host cytokines, including tumour necrosis factor-α ('TNF-α') [4]. It also induces several early genes involved in macrophage activation [5], and stimulates production of nitric oxide synergistically with interferon-γ ('IFN-γ') [6]. *M. tuberculosis* LAM has also been shown to mediate phagocytosis of mycobacteria within phagocytic cells [7]. [Phosphatidyl-*myo*-inositol (PI)]mannosides (PIMs), known to be precursors of lipomannan (LM) and LAM, have recently been proposed to recruit natural killer (NK) T cells, which have a primary role in the granulomatous response [8,9]. Moreover, a role for surface-exposed PIMs as *M. tuberculosis* adhesins that mediate attachment to non-phagocytic cells has also been established [10,11].

Although the structure of LAM has been well documented [12–15], the genetics of the biosynthesis of this complex lipopolysaccharide remains a 'black box'. The recent availability of mycobacterial genome sequences [16] and the development of *in vitro* assays have provided a means to study detailed enzyme function [17,18]. The biosynthesis of LAM involves the addition of mannose (Man) residues to PI to produce both the short PIMs (2–5 Man residues) and LM, which is glycosylated further with arabinan to form LAM [12,14,19]. The biosynthetic relationship of PI → PIMs → LM → LAM has recently been supported by biochemical [14,18] and genetic studies [20], but the details of this pathway remain highly speculative.

The biosynthesis of PIMs is initiated by distinct α-mannosyltransferase activities that utilize GDP-Man as a sugar donor,

Abbreviations used: Ac, acylated; BCG, Bacille Calmette–Guérin; C_{35}/C_{50}-P-Man, polyprenol-monophosphorylmannose; FAB, fast atom bombardment; DIG, digoxigenin; GT1F, glycosyltransferase 1 family; hyg, hygromycin; LAM, lipoarabinomannan; LM, lipomannan; Man, mannose; NK, natural killer; ORF, open reading frame; *p*, pyranose; PI, phosphatidyl-*myo*-inositol; PIM, PI mannoside; TIGR, The Institute for Genomic Research; WT, wild-type.

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transferring initially a Man*p* (where *p* is pyranose) sugar residue to the 2-position of *myo*-inositol to form PI monomannoside (PIM_1), followed by a further addition to the 6-position of *myo*-inositol to generate the dimannoside (PIM_2) [14,18]. The latter GDP-Man-dependent α-mannosyltransferase has recently been shown to be catalysed by the *pimB* gene product from *M. tuberculosis* [20]. The addition of Man*p* residues to PIM_2 to form higher PIMs (PIM_3–PIM_5) also requires GDP-Man, according to the earlier studies of Brennan and Ballou [21]. Recently, using a cell-free assay and amphomycin, Besra et al. [18] demonstrated that triacylated-PIM_2 (Ac_3PIM_2, according to the nomenclature of Gilleron et al. [9], where 'Ac' is 'acylated') is specifically extended by the addition of Man*p* residues from the alkali-stable sugar donor, polyprenol-monophosphorylmannose (C_{35}/C_{50}-P-Man), which is generated from GDP-Man and the corresponding C_{35}/C_{50}-polyprenol phosphate, to form higher PIMs (at least Ac_3PIM_3 and Ac_3PIM_4) and linear LM possessing an $\alpha(1 \rightarrow 6)$-linked Man*p* backbone. The linear conformation of LM is then postulated to be mannosylated further, resulting in mature branched LM, which undergoes subsequent arabinosylation to form LAM.

PIMs, LM and LAM exist as discrete but heterogeneous populations with a Gaussian distribution that differ from each other in the extent and manner of mannosylation/arabinosylation, acylation and other subtle modifications [22]. It is speculated that numerous α-mannosyltransferases are required for the formation of PIMs and LM, dictated by their chemically distinct precursors and utilization of different sugar donors (GDP-Man or C_{35}/C_{50}-P-Man). In the present study, we have undertaken the identification of a gene encoding a putative α-mannosyltransferase (RvD2-*ORF1*, where 'ORF' is 'open reading frame') found within *M. tuberculosis* CDC1551. We have assigned the function of this protein as being implicated in the biosynthesis of PIMs, and have addressed the precise question of its role in PIM synthesis by inactivating the analogous gene in *Myobacterium bovis* Bacille Calmette–Guérin (BCG).

MATERIALS AND METHODS

Bacterial strains and growth conditions

All cloning steps were performed in either *Escherichia coli* XL1-Blue (Stratagene, La Jolla, CA, U.S.A.) or *E. coli* TOP-10 (Invitrogen, Carlsbad, CA, U.S.A.). *Mycobacterium smegmatis* mc²155 is an electroporation-efficient mutant of *M. smegmatis* mc²6 [23]. Large-scale cultures of *M. smegmatis* were grown to mid-exponential phase (D_{600} 0.6–0.7) in Luria–Bertani medium containing 0.05% (v/v) Tween 80 in the presence or absence of 25 μg/ml kanamycin (Sigma, St Louis, MO, U.S.A.), harvested, washed with PBS, and then stored at −20 °C until further use. Disruption of RvD2-*ORF1* was performed using the *M. bovis* BCG vaccine strain 1173P2 (World Health Organization, Stockholm, Sweden). *M. bovis* BCG strains were plated on to Middlebrook 7H10 agar containing oleic acid-albumin-dextrose-catalase enrichment (Difco, Detroit, MI, U.S.A.) and eventually supplemented with 50 μg/ml hygromycin (hyg) (Roche Molecular Biochemicals, Basel, Switzerland), whereas liquid cultures were grown at 37 °C in Sauton medium.

Plasmids and DNA manipulation

Plasmid pUC18 was purchased from New England Biolabs (Beverly, MA, U.S.A.). Expression of RvD2-ORF1 in mycobacteria was achieved using the *E. coli*–mycobacterial shuttle vector pMV261 containing the *hsp60* promoter [24]. Genomic DNA from *M. tuberculosis* CDC1551 was obtained from J. Belisle (Department of Microbiology, Colorado State University, Fort Collins, CO, U.S.A.). The cosmid library pYUB328 described by Balasubramanian et al. [25] was generously provided by W. R. Jacobs (Howard Hughes Medical Institute, Albert Einstein College of Medicine, Bronx, NY, U.S.A.). The plasmid pUC-HY [26] was kindly provided by R. W. Stokes (Division of Infectious and Immunological Diseases, University of British Columbia, Vancouver, Canada). Restriction enzymes and T4 DNA ligase were purchased from Roche Molecular Biochemicals and Vent DNA polymerase was purchased from New England Biolabs. All DNA manipulations were performed using standard protocols, as described by Sambrook et al. [27].

Bioinformatic methods

BLAST analysis was performed against the *M. tuberculosis* CDC1551 genome database at The Institute for Genomic Research (TIGR; http://www.tigr.org). Putative α-mannosyltransferases from *M. tuberculosis* H37Rv were identified at http://genolist.pasteur.fr/TubercuList/. The classification of glycosyltransferases is accessible at CAZy (http://afmb.cnrs-mrs.fr/CAZY/), whereas Pfam glycosyltransferase 1 family (GT1F) can be accessed at www.sanger.ac.uk/Software/Pfam/. Multiple sequence alignments were performed using the Clustal Method of DNA Star.

Overexpression of RvD2-ORF1 in *E. coli* and *M. smegmatis*

RvD2-*ORF1* from *M. tuberculosis* CDC1551 was cloned into the mycobacterial overexpression vector pMV261 as follows. PCR amplification was performed using the upstream primer M1 5′-GTG TTG TTC AGG TCG CAA ACT TCT ATG G-3′ and the downstream primer M2 5′-GCA GGATCC GGC CGC AAA CTT-3′ which contains a *Bam*HI restriction site (underlined). The 1169 bp PCR product was then digested with *Bam*HI and cloned into the *Mlu*NI/*Bam*HI-restricted pMV261, giving rise to pMV261-RvD2-*ORF1*. For expression in *E. coli*, the blunt-ended PCR fragment was cloned into a pUC8 plasmid cut by *Sma*I. Coding sequence of RvD2-*ORF1*, as well as its junctions with either the *hsp60* or the *Plac* promoter, were verified by DNA sequencing.

Mannosyltransferase assays

Membrane fractions prepared from *M. smegmatis* expressing recombinant RvD2-ORF1 were assayed and compared with membranes from *M. smegmatis* transformed with empty pMV261 for α-mannosyltransferase activity, using a method adapted from Besra et al. [18]. Reaction mixtures contained 500 μg of membranes (total protein), buffer A containing 0.1 mM dithiothreitol, 10 mM $CaCl_2$ and 2.5 μg of amphomycin (a lipopeptide antibiotic that specifically inhibits polyprenyl-P-requiring translocases and the synthesis of C_{35}/C_{50}-P-Man) in a total volume of 50 μl. Reactions were incubated for 10 min at 37 °C before the addition of 0.25 μCi of GDP-[^{14}C]Man (303 mCi/mmol; Amersham Biosciences, Uppsala, Sweden), and held at 37 °C for a further 30 min. The reactions were stopped by the addition of 4 ml of trichloromethane/methanol/water (10:10:3, by vol.) and incubated at room temperature for 30 min, followed by the addition of 1.75 ml of trichloromethane and 0.75 ml of water. The lower organic layer of the biphasic mixture was washed three times with 2 ml of trichloromethane/methanol/water (3:47:48, by vol.), dried under a stream of nitrogen and re-suspended in 200 μl of trichloromethane/methanol (2:1, v/v). The transfer of [^{14}C]Man from GDP-[^{14}C]Man to the PIMs was quantified by

scintillation counting, and the material was analysed by TLC autoradiography, as described previously [20].

Analysis of reaction products

Large-scale reaction mixtures containing unlabelled GDP-Man (80 mM) and the other components were prepared and processed as described above. The reaction products were dried, applied to preparative TLC plates in addition to radiolabelled material (50000 c.p.m.) to trace the cold enzymically synthesized products, and developed in trichloromethane/methanol/ammonium hydroxide/water (65:25:0.5:3.6, by vol.). Autoradiography was performed by exposing the TLC to X-ray film (Kodak X-Omat, Rochester, NY) for 24 h. The band corresponding to the new enzymically synthesized PIM product was recovered from the plates using 4 ml of trichloromethane/methanol/water (10:10:3, by vol.) at room temperature for 30 min, followed by the addition of 1.75 ml of trichloromethane and 0.75 ml of water. The lower organic layer of the biphasic mixture was washed three times with 2 ml of trichloromethane/methanol/water (3:47:48, by vol.), dried under a stream of nitrogen and per-O-acetylated before fast-atom-bombardment (FAB) MS analysis.

Chemical derivative-formation for FAB-MS analysis

The PIM product synthesized by RvD2-ORF1 was per-O-acetylated using 100 μl of pyridine/acetic anhydride (1:1, v/v) at 80 °C for 2 h, after which the reagents were removed under a stream of nitrogen and the samples were extracted into trichloromethane and washed several times with water, before drying under a stream of nitrogen. FAB-MS spectra were acquired in the positive-ion mode using a ZAB-2SE 2FPD mass spectrometer fitted with a caesium ion gun operated at 30 kV. Data acquisition and processing were performed using VG Analytical Opus software [28].

Screening of the *M. bovis* BCG genomic DNA library

The pYUB328 cosmid DNA library of *M. bovis* BCG [25] was used to transform *E. coli* XL1-Blue and screening was performed by colony-blot hybridization using RvD2-*ORF1* as a probe. The RvD2-*ORF1* probe corresponding to the entire RvD2-*ORF1* sequence was labelled with digoxigenin (DIG)-dUTP5′ using the PCR DIG Probe Synthesis Kit (Roche Molecular Biochemicals) with oligonucleotides M1 and M2. Pre-hybridization and hybridization were performed at 42 °C and 50 °C respectively, using the DIG Easy Hyb buffer (Roche Molecular Biochemicals). Post-hybridization washes were performed twice in 2 × SSC (where 1 × SSC is 0.15 M NaCl/0.015 M sodium citrate)/0.1 % (w/v) SDS for 5 min at room temperature, and twice in 0.1 × SSC, 0.1 % SDS for 15 min at 68 °C. The hybridized probe was detected using a DIG luminescence detection kit (Roche Molecular Biochemicals) according to the manufacturer's recommendations. Approx. 400 individual clones were screened and five positive clones were obtained. Cosmid DNA was prepared and analysed further by restriction digests and Southern-blot hybridization using the RvD2-*ORF1* probe. A 10.5 kb *Bam*HI restriction fragment bearing the RvD2-*ORF1* was subcloned from one of these cosmids into pUC18, creating pUC18-10.5.

Construction of a suicide vector for RvD2-*ORF1* gene disruption

Plasmid pUC18-10.5 was examined for restriction sites, which could facilitate RvD2-*ORF1* disruption. A unique *Sfu*I restriction site was found within RvD2-*ORF1*, and used for subsequent cloning of a *hyg*-resistance cassette, which was obtained by PCR amplification. PCR was performed using pUC-HY and the following primers: sense primer, *hyg1* 5′-ACC AAG CCC TCG GCG ACG TTC C-3′, and antisense primer, *hyg2* 5′-GAG GAT CCC CGG GTA CCG CCG TC-3′. The suicide vector designated pKO-RvD2-*ORF1* contained 3.5-kb- and 7-kb-homologous mycobacterial DNA segments flanking the *hyg* cassette. The plasmid genotype was confirmed by restriction analysis and PCR amplification.

Transformation of *M. bovis* BCG with pKO-RvD2-*ORF1*, and the screening of transformants

Construct pKO-RvD2-*ORF1* was linearized with *Hin*dIII and electroporated in *M. bovis* BCG-competent cells, as described previously [29]. Transformant colonies growing on Middlebrook 7H10 with 50 μg/ml hyg were screened by PCR using primers M3 (5′-CGC GCA CCG AAC GAC ACC TCC TAC-3′) and M4 (5′-GCC GCC GCG GGT GGA AGG TCT T-3′), as described by Flesselles et al. [30].

Genomic DNA preparation and Southern-blot hybridization

Genomic DNA from *M. bovis* BCG cultures was prepared as follows. D-Cycloserine (25 μg; Sigma) was added to a 10 ml saturated culture of *M. bovis* BCG, which was then incubated overnight at 37 °C. Cells were then pelleted and re-suspended in 250 μl of solution I [25 % sucrose/50 mM Tris/HCl (pH 8.0)/50 mM EDTA/500 μg/ml lysozyme] and incubated for 4 h at 37 °C. Solution II [100 mM Tris/HCl (pH 8.0)/1 % (w/v) SDS/400 μg/ml proteinase K] was added (250 μl), and the mixture was incubated for 3 h at 55 °C. The lysate was then extracted three times with phenol/chloroform/3-methylbutan-1-ol (25:24:1, by vol.), and DNA was concentrated by ethanol precipitation. Approx. 2 μg of genomic DNA was digested overnight with an excess of restriction enzyme (*Pst*I or *Asp*718). The fragments were then separated by electrophoresis using a 0.7 % (w/v) agarose gel and transferred on to a positively charged nylon membrane (Roche Molecular Biochemicals). DNA was then UV-cross-linked using a Stratalinker apparatus (Stratagene) incubated in DIG Easy Hyb buffer for pre-hybridization, and finally hybridized with different probes using the same conditions as for colony-blot hybridization. Detection was performed using the DIG luminescence detection kit (Roche Molecular Biochemicals), according to the manufacturer's recommendations.

Southern-blot hybridization analysis of clinical isolates was performed according to the standard protocol used for molecular fingerprinting [31]. Briefly, DNA from clinical isolates was digested with *Pvu*II and electrophoresed on 1 % (w/v) agarose gels. The products were transferred to nylon membranes, hybridized with a non-radioactively labelled IS*6110* probe, and then subsequently with the RvD2-*ORF1* probe. Detection was performed according to the standard protocol using the enhanced chemiluminescence direct nucleic acid labelling and detection kit (Amersham Biosciences) [31].

Biochemical analysis of PIM, LM and LAM from both *M. bovis* BCG and the *M. bovis* BCG-disrupted RvD2-*ORF1* mutant

Extraction and analysis of PIMs was performed as described by Besra [32]. Briefly, *M. bovis* BCG cultures were labelled by adding 1 μCi/ml [1,2-^{14}C]acetate (50–62 mCi/mmol; Amersham Biosciences) and the ^{14}C-labelled cells were harvested by centrifugation and washed with PBS. The bacterial pellet was extracted

using 2 ml of methanol/0.3 % (w/v) NaCl (100:10, v/v) and 1 ml of petroleum ether. Following centrifugation, the upper petroleum ether layer was removed, and the polar lipids were extracted further from the residue. The methanolic-saline fraction was heated at 65 °C for 5 min and mixed with 2.3 ml of trichloromethane/methanol/0.3 % NaCl (9:10:3, by vol.). After centrifugation, the supernatant was retained and the residue was extracted further by adding 0.75 ml of trichloromethane/methanol/0.3 % NaCl (5:10:4, by vol.). The solvent extracts were then combined with 1.3 ml of trichloromethane and 1.3 ml of 0.3 % NaCl. The lower organic phase obtained after centrifugation was evaporated to dryness and the polar lipids were resuspended in trichloromethane/methanol/water (10:10:3, by vol.). Equal amounts of material (50000 c.p.m.) were loaded on to silica gel plates (5735 silica gel $60F_{254}$; Merck, Darmstadt, Germany). Two-dimensional TLC was performed using trichloromethane/methanol/water (60:30:6, by vol.) in the first dimension, and trichloromethane/acetic acid/methanol/water (40:25:3:6, by vol.) in the second dimension. Autoradiograms were produced by exposure to Kodak X-Omat AR film after 4–5 days to reveal ^{14}C-labelled PIMs. LM and LAM were extracted as described previously [32], and analysed by SDS/PAGE using silver/periodic acid staining.

RESULTS

Identification of RvD2-*ORF1* in *M. tuberculosis* CDC1551 as a putative α-mannosyltransferase

Schaeffer et al. [20] recently identified *M. tuberculosis* H37Rv *pimB*, which encoded an α-mannosyltransferase involved in the formation of Ac_3PIM_2 from GDP-Man and Ac_3PIM_1. BLAST analysis of the *M. tuberculosis* CDC1551 genome sequence database at TIGR has revealed the presence of several proteins with similarity to PimB (Rv0557) (Figure 1). One of these, annotated as RvD2-ORF1 as described by Gordon et al. [33], or MT1800 according to TIGR, shares 33 % identity with PimB. RvD2-ORF1 (accession number AAK46073) encodes a putative α-glycosyltransferase found in Family 4 [34] of the CAZy classification of glycosyltransferases (http://afmb.cnrs-mrs.fr/CAZY/) and in glycosyltransferase 1 family (GT1F) in the Pfam database [34,35]. CAZy Family 4 consists of more than 300 proteins, whereas Pfam GT1F contains 397 proteins, including members of CAZy Family 4. RvD2-ORF1 and PimB, as well as six other putative α-mannosyltransferases found in the *M. tuberculosis* H37Rv, contain the conserved EX_7E motif (Glu-Xaa_7-Glu; amino acids 283–291). This motif is thought to be associated with the retention of C1 configuration in various retaining glycosyltransferases [36], such as the CAZy Family 4 α-mannosyltransferase AceA from *Acetobacter xylinum* [37]. It has also been suggested that the EX_7E motif from AceA is involved in sugar-nucleotide binding [37]. In addition, the sequence alignments presented in Figure 1 show the presence of a conserved lysine residue (Lys^{212}), which is present in all CAZy Family 4 glycosyltransferases [34,36]. Replacement of Lys^{211} with alanine in AceA results in a complete loss of activity, which suggests a possible role in binding of the diphosphate group of the sugar donor, GDP-Man [37]. Other significant conserved residues include Gly^{175}, Asp^{177} and Arg^{207}, which are highlighted in Figure 1, although no function has been assigned to these residues. Thus the similarity between RvD2-ORF1 and PimB with other retaining CAZy Family 4 glycosyltransferases suggests that RvD2-ORF1 is presumably a GDP-Man-dependent α-mannosyltransferase involved in PIM biosynthesis.

We therefore conducted a study first to determine whether RvD2-ORF1 encodes an α-mannosyltransferase involved in PIM biosynthesis, and secondly, to examine the consequence of

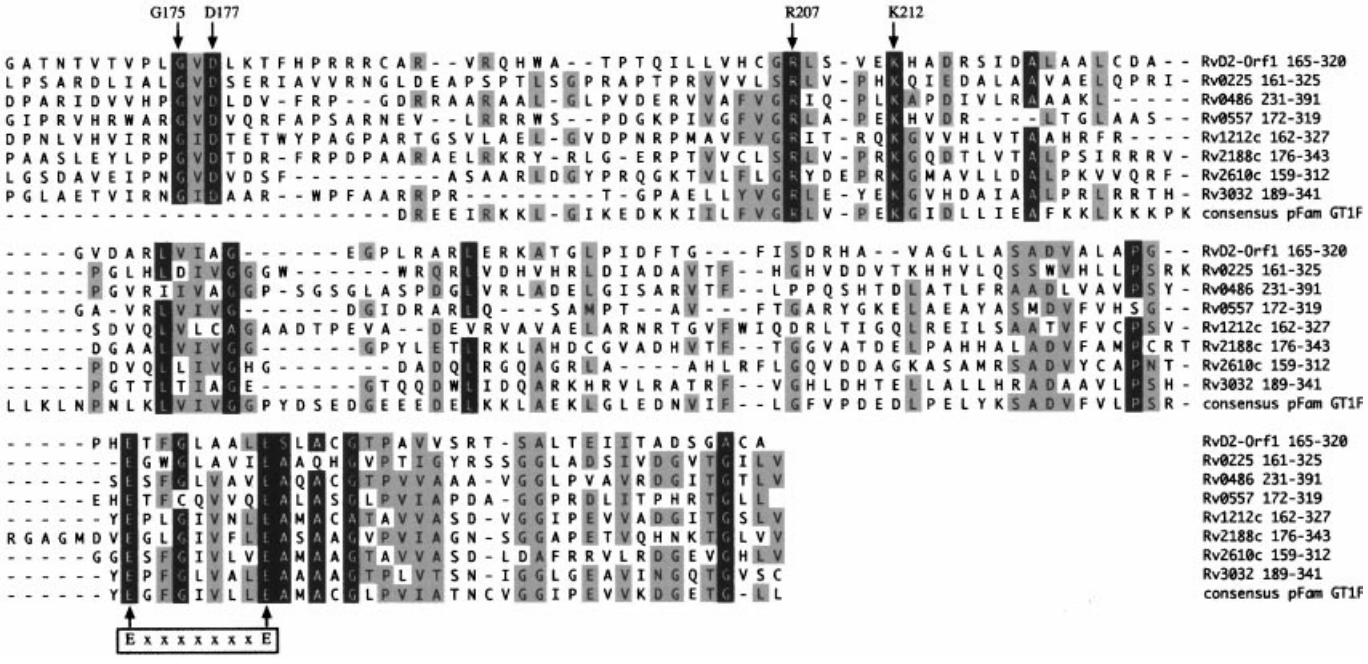


Figure 1 Alignment of RvD2-ORF1 with other putative α-mannosyltransferases identified in the genome of *M. tuberculosis* H37Rv

The alignment was generated using the Clustal algorithm of MegAlign (DNAStar). Identical amino acids are highlighted in reversed-out lettering on a black background, and similar residues are highlighted in grey. The last entry is the pFam consensus proposed for the GT1F family. The boxed consensus sequence delineates the conserved EX_7E motif, which, with Lys^{212}, is strictly conserved in all members of the CAZy Family 4 glycosyltransferases. Other strictly conserved residues, such as Gly^{175}, Asp^{177} and Arg^{207} are also highlighted by arrows.

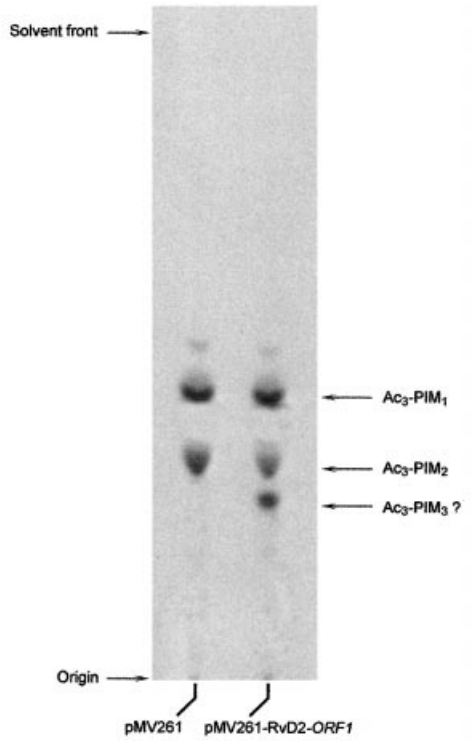


Figure 2 Comparison of α-mannosyltransferase activity in membrane extracts of *M. smegmatis* cells transformed with pMV261 and pMV261-RvD2-*ORF1* in *M. smegmatis*

Mannosyltransferase assays contained *M. smegmatis* membranes and GDP-[^{14}C]Man, as well as amphomycin. After incubation for 30 min at 37 °C, the reaction was stopped, and extraction of [^{14}C]PIMs was performed as described in the Materials and methods section. The reaction products (2000 c.p.m.) were applied to aluminium-backed thin-layer chromatograms and subjected to TLC using $CHCl_3/CH_3OH/H_2O$ (65:25:4, by vol.). Autoradiograms were obtained after exposure to a Kodak X-Omat film for 4–5 days. Assays were performed in triplicate using freshly prepared membranes, with the thin-layer chromatogram being a representative example of a number of samples from independent experiments.

targeted gene disruption in the context of PIM, LM and LAM biosynthesis.

Evidence that RvD2-ORF1 catalyses the formation of triacylated-PIM_3

RvD2-*ORF1* was first cloned into several expression vectors and transformed into *E. coli*. Overexpression of the gene in *E. coli* led to the production of a new protein; however, the observed level of expression was lower than normally obtained with these expression systems, and all of the recombinant protein was found in the cell-debris fraction (27000 ***g*** pellet) of cell homogenates (results not shown). In addition, no enzymic activity was detected in the soluble cytosolic portion (100000 ***g*** supernatant) or the membrane (100000 ***g*** pellet) of the cell homogenates (results not shown). Therefore expression studies were conducted in *M. smegmatis* by cloning the gene into pMV261, which drives expression from a native mycobacterial *hsp60* promoter.

M. smegmatis transformed either with pMV261-RvD2-*ORF1* or pMV261 were examined for GDP-Man-dependent α-mannosyltransferase activities associated with PIM biosynthesis using a cell-free assay [18,20]. Cell-free assays performed with membranes prepared from cells transformed with empty pMV261 produced only Ac_3PIM_1 and Ac_3PIM_2. The subsequent synthesis of higher PIMs (Ac_3PIM_3, etc.) and linear-LM was abrogated due to inhibition of the required C_{35}/C_{50}-P-Man sugar donor by amphomycin for these particular α-mannosyltransferases [18] (Figure 2). Under these experimental conditions, overproduction of RvD2-ORF1 in several independent experiments using membranes prepared with cells transformed with pMV261-RvD2-*ORF1* resulted in a consistent 4-fold increase in α-mannosyltransferase activity when compared with membranes prepared from cells transformed with pMV261 [*M. smegmatis* pMV261, 2361 ± 488 c.p.m.; and *M. smegmatis* pMV261-RvD2-*ORF1*, 9652 ± 588 c.p.m. (values given as means ± S.E.M.)]. Further TLC-autoradiographic analysis revealed that overexpression of RvD2-ORF1 influenced directly the *in vitro* PIM profile of *M. smegmatis* (Figure 2). In comparison with assays performed with membranes prepared from cells transformed with pMV261, membranes prepared from cells transformed with pMV261-RvD2-*ORF1* revealed a new product, which was

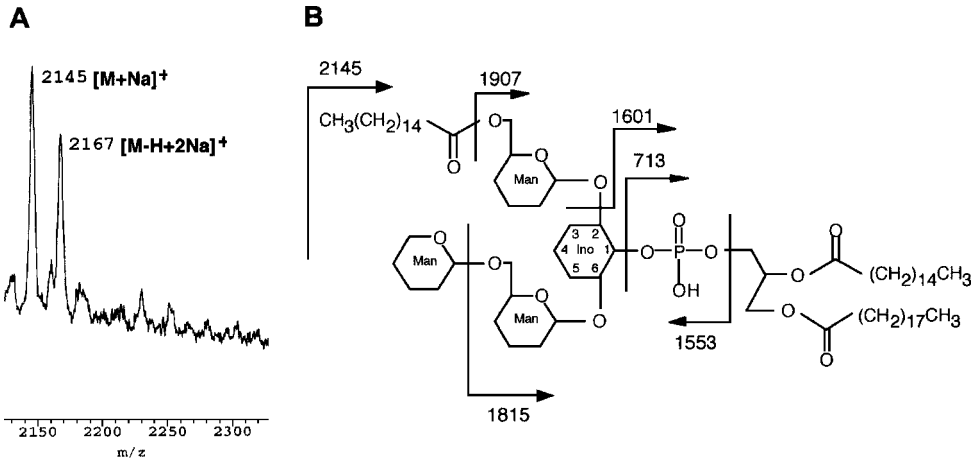


Figure 3 Identification and structural characterization of Ac_3PIM_3

(**A**) High-mass range using FAB MS analysis of the per-O-acetylated Ac_3PIM_3 in the positive-ion mode. The $[M+Na]^+$ ion at *m/z* 2145 corresponds to the schematic representation shown in (**B**). The signal at *m/z* 2167 carries an additional sodium atom, probably on the phosphate moiety. (**B**) Key fragment ions (low-mass results not shown) are indicated on the schematic drawing.

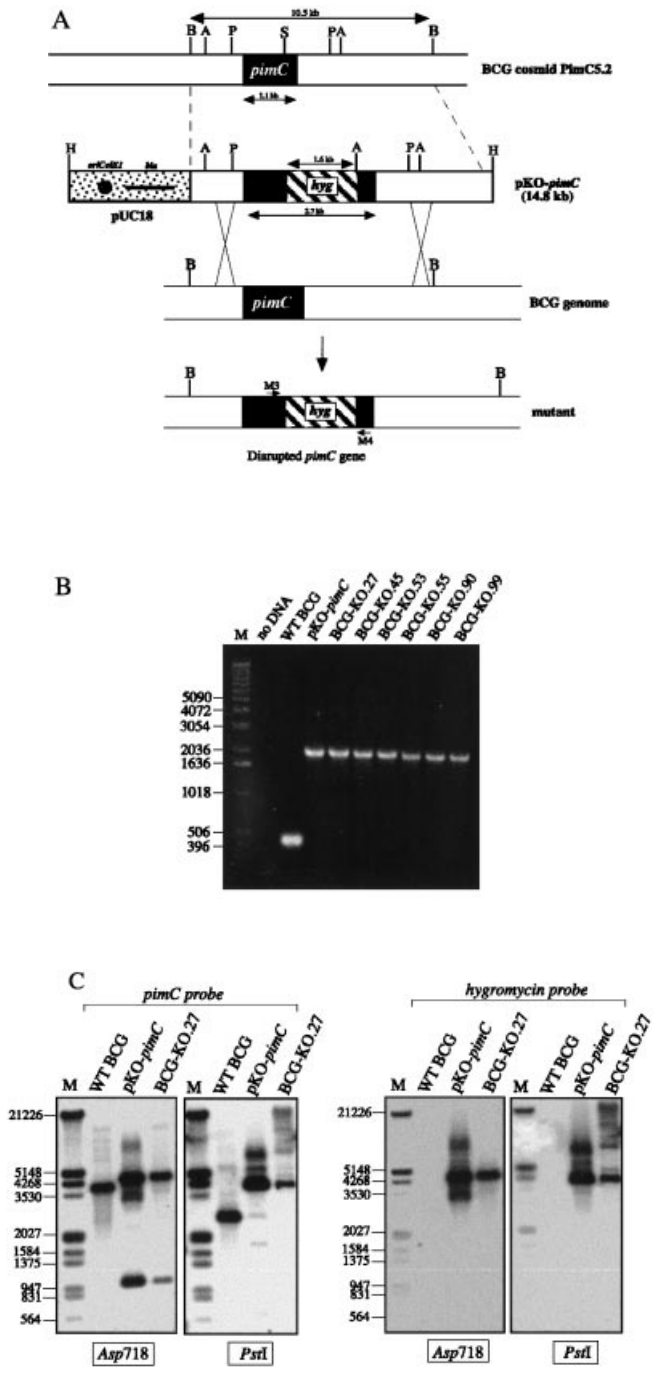


Figure 4 Inactivation of *pimC* in *M. bovis* BCG

(**A**) The strategy used to inactivate *pimC* in *M. bovis* BCG. A *M. bovis* BCG cosmid designated PimC5.2 was first isolated from a *M. bovis* BCG cosmid library. A *Bam*HI 10.5 kb fragment containing the entire *pimC* sequence was isolated from cosmid PimC5.2 and subcloned into pUC18. The *pimC* was then interrupted by cloning a *hyg*-resistance cassette within the unique *Sfu*I restriction site. This suicide vector, pKO-*pimC*, was linearized with *Hin*dIII and subsequently used to transform *M. bovis* BCG. Arrows indicate the position of the two primers M3 and M4 used for PCR screening. A, *Asp*718; B, *Bam*HI; P, *Pst*I; S, *Sfu*I; H, *Hin*dIII; *bla*, β-lactamase; *hyg*, hyg-resistance cassette. (**B**) Genomic DNA was prepared from WT *M. bovis* BCG, and from six individual *M. bovis* BCG clones that showed a disrupted genotype by PCR screening on colonies (BCG-KO.27 to BCG-KO.99). The first lane contains no DNA, as a negative control of PCR, whereas pKO-*pimC* (third lane) was added as a positive control. As expected, WT *M. bovis* BCG generated a 0.43 kb fragment, that was increased further by 1.6 kb (corresponding to the *hyg* cassette) for pKO-*pimC*, as well as for the six mutants. (**C**) Southern-blot hybridization was performed on pKO-*pimC* and on genomic DNA prepared from both WT *M. bovis* BCG and BCG-KO.27. DNA was restricted with either *Asp*718 or *Pst*I, and probed with either *pimC* (left panels) or *hyg* (right panels). M, DIG-labelled molecular-mass markers.

tentatively assigned as Ac_3PIM_3, on the basis of its relative retardation factor and our previous studies in relation to PimB and the identification of Ac_3PIM_1/Ac_3PIM_2 [20]. The synthesis of Ac_3PIM_1 and Ac_3PIM_2 was slightly diminished in assays performed with membranes prepared from cells transformed with pMV261-RvD2-*ORF1* in comparison with pMV261 (Figure 2). The fact that Ac_3PIM_3 cannot be isolated from membrane preparations of untransformed *M. smegmatis* may be explained by either the absence of the *pimC* gene in this mycobacterial species, or by a low expression level of PimC. On the other hand, Ac_3PIM_3 may be produced transiently due to a very rapid turnover. A BLAST search in the *M. smegmatis* genome database revealed the absence of the *pimC* gene (results not shown).

It was originally envisaged that RvD2-ORF1 would be involved in the synthesis of Ac_3PIM_1 via a GDP-Man-dependent α-mannosyltransferase (PimA) to PI, followed by a subsequent acyltransferase. As a consequence, the expected TLC phenotype would have presumably been an increase in the synthesis of Ac_3PIM_1, and possibly Ac_3PIM_2, with the absence of higher PIMs (Ac_3PIM_3, etc.) due to the inhibition of C_{35}/C_{50}-P-Man biosynthesis via amphomycin [18]. The results indicate that overexpression of RvD2-ORF1, now termed PimC, appears to catalyse the direct transfer of Man*p* from GDP-[^{14}C]Man to Ac_3PIM_2 to afford Ac_3PIM_3, since the synthesis of the C_{35}/C_{50}-P-[^{14}C]Man sugar donor is blocked by the inclusion of amphomycin [18]. The absence of higher PIMs (Ac_3PIM_4) would presumably be due to the lack of the appropriate sugar donor (C_{35}/C_{50}-P-Man) required for the synthesis of Ac_3PIM_4. The latter phenotype was clearly observed on TLC with assays performed with amphomycin and membranes prepared from cells transformed with pMV261-RvD2-ORF1 (Figure 2). An alternative, indirect explanation would be that overexpression of RvD2-ORF1 leads to the up-regulation and restoration of C_{35}/C_{50}-P-Man production in the presence of amphomycin, and thus the synthesis of higher PIMs. This possibility was ruled out, since membrane extracts prepared from *M. smegmatis* transformed with either pMV261-RvD2-*ORF1* or with pMV261 possessed similar levels of C_{35}/C_{50}-P-Man production. The newly synthesized product was shown to be mild alkali-labile, indicating that it was a member of the PIM family; TLC analysis of trifluoroacetic acid hydrolysates showed the presence of Man, in comparison with sugar standards (results not shown). The chemical identity of the tentatively assigned Ac_3PIM_3 was established by FAB MS. The high-mass region obtained by FAB MS analysis of the per-O-acetylated lipid (Figure 3A) shows two major molecular ion signals at m/z 2145 and 2167. The signal at m/z 2145 can be assigned as the sodiated molecular ion $[M+Na]^+$ of Ac_3PIM_3, as shown in Figure 3(B), with a total of three fatty acyl chains (two $C_{16:0}$, one $C_{19:0}$) and three hexose residues. The signal at m/z 2167 $[M-H+2Na]^+$ carries an additional sodium atom. In addition, informative fragment ions from the low-mass region of the FAB-MS spectrum (results not shown) are shown schematically in Figure 3(B), confirming the identity of the new lipid as Ac_3PIM_3. It should be noted that Ac_3PIM_3 was undetectable by FAB MS in control experiments performed with membrane extracts prepared from *M. smegmatis* pMV261 (results not shown).

Disruption of *pimC* by allelic exchange in *M. bovis* BCG

To establish further whether *pimC* encodes an α-mannosyltransferase involved in the biosynthesis of Ac_3PIM_3, presumably from the Ac_3PIM_2 precursor (the metabolic product of PimB [20]), the gene was disrupted in *M. bovis* BCG. To achieve allelic exchange, pKO-*pimC* was constructed, which contained a 10.5 kb *Bam*HI

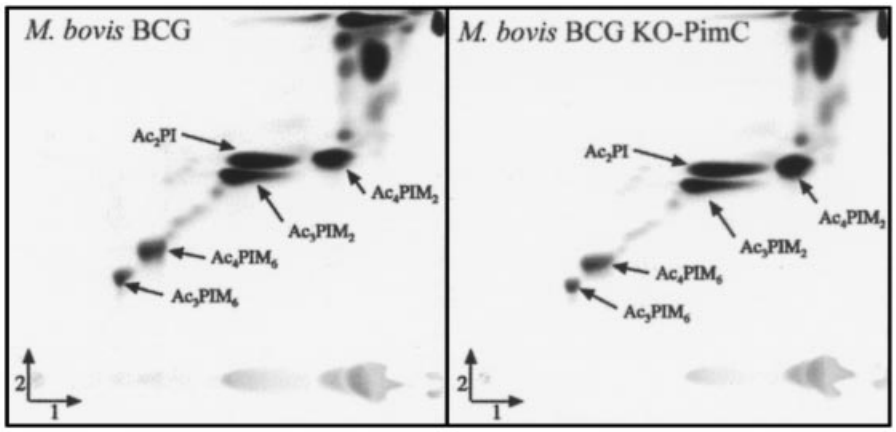


Figure 5 Analysis of the PIM content in *M. bovis* BCG and *M. bovis* BCG KO-PimC by two-dimensional TLC

PIMs were extracted from *M. bovis* BCG cultures following incubation in the presence of [1,2-^{14}C]acetate. Equal counts (50000 c.p.m.) were loaded on to a thin-layer chromatogram that was resolved using $CHCl_3/CH_3OH/H_2O$ (60:30:6, by vol.) in the first dimension, and $CHCl_3/CH_3COOH/CH_3OH/H_2O$ (40:25:3:6, by vol.) in the second dimension. ^{14}C-labelled PIMs were revealed by exposure to a Kodak X-Omat AR film after 4–5 days. The nomenclature (Ac_3PIM_2, etc.) is on the basis of the recent identification of the PIM family from *M. bovis* BCG by Gilleron et al. [9] using electrospray-ionization MS.

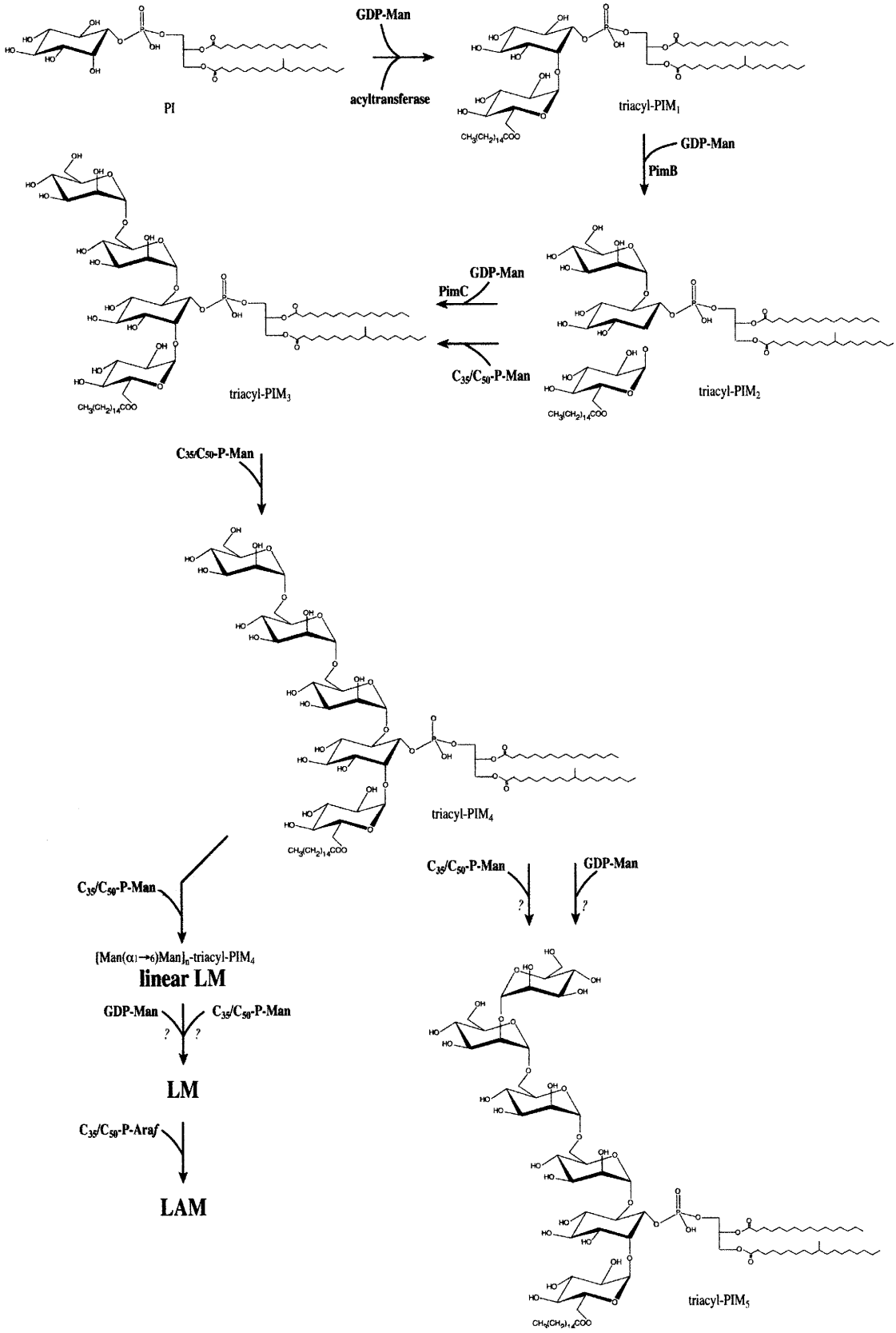
fragment of homologous DNA, including *pimC* (Figure 4A). In this DNA segment, *pimC* was interrupted with a *hyg*-resistance cassette. Using this suicide vector, allelic exchange is only possible by homologous recombination via a double crossover, which would insert the disrupted *pimC* into the mycobacterial chromosome. A hundred *hyg*-resistant *M. bovis* BCG transformants generated by electroporation of pKO-*pimC* were screened by PCR to amplify both sides of the inserted *hyg* gene. Six transformants, designated BCG-KO.27, BCG-KO.45, BCG-KO.53, BCG-KO.55, BCG-KO.90 and BCG-KO.99, yielded a 2 kb PCR product, as expected from homologous recombination via a double crossover. The wild-type (WT) product, indicative of spontaneous *hyg*-resistant clones, resulted in a 0.43 kb fragment. PCR amplification of WT *M. bovis* BCG and six transformants that had undergone allelic replacement were confirmed using purified genomic DNA from each strain (Figure 4B). To confirm the results of the PCR screen, the putative mutants were examined by Southern-blot hybridization for the presence of the mutated *pimC* allele using two different probes (Figure 4C). When *pimC* was used as a probe, digestion of genomic DNA from BCG-KO.27 with *Asp*718 resulted in an increase in size over the 3.8 kb parental fragment length and the presence of an additional band of 0.98 kb. When digested with *Pst*I, DNA from WT *M. bovis* BCG showed a single band of 2.5 kb, which was increased in size by 1.6 kb corresponding to the size of the *hyg* gene. As expected, no signal was observed when *hyg* was used to probe DNA from WT *M. bovis* BCG, regardless of the restriction enzyme used. However, single bands of the expected size confirmed again the disruption of *pimC* by allelic exchange. Identical restriction patterns were obtained for the other five *pimC*-inactivated *M. bovis* BCG clones (results not shown).

Characterization of the *M. bovis* BCG disrupted *pimC* strain

Analysis of PIM distribution in the *M. bovis* BCG and *pimC* mutant *M. bovis* BCG strains was achieved by labelling cells using [^{14}C]acetate, extracting the labelled PIMs and then resolving the different PIM species by two-dimensional TLC. As shown in Figure 5, no change in the PIM profile was observed in the mutant strain compared with the WT strain. Moreover, the mutant strain was still able to produce LM and LAM in similar proportions when compared with the WT *M. bovis* BCG strain (results not shown). Our earlier homology-based searches for GDP-Man-dependent α-mannosyltransferases centred around PimB, supported by the above biochemical findings, do suggest that PimC is involved in synthesis of Ac_3PIM_3. However, the results with the *pimC*-disrupted *M. bovis* BCG mutant suggest that additional α-mannosyltransferase(s) may be responsible for the synthesis of Ac_3PIM_3, implying the existence of compensatory gene(s) or an alternate pathway in the genome of *M. bovis* BCG and *M. tuberculosis* CDC1551 (see below), presumably using the C_{35}/C_{50}-P-Man sugar donor and Ac_3PIM_2, as suggested in our earlier studies *in vitro* ([18]; see Scheme 1).

Genetic distribution of *pimC* among *M. tuberculosis* clinical isolates

A comparative genomic analysis of *M. tuberculosis* CDC1551, *M. tuberculosis* H37Rv and *M. bovis* BCG revealed that *pimC* corresponds to ORF1 of the RvD2 deletion of the published H37Rv sequence [16,33]. A recent study aimed at identifying attenuation mutations in *M. tuberculosis* H37Ra has shown that the RvD2 locus was present in the *M. tuberculosis* H37Ra substrain, but not in *M. tuberculosis* H37Rv [38]. The *M. tuberculosis* H37Ra RvD2 locus contains three ORFs, with two flanking IS*6110* elements. The observation that the RvD2 deletion in *M. tuberculosis* H37Rv contains a copy of IS*6110* lacking direct repeats that flank the ends of the element indicates that this deletion was probably due to an IS*6110*-mediated mechanism, characterized by recombination between insertion sequences ('ISs'), with loss of the region between the elements. Gordon et al. [33] have also analysed the distribution of the RvD2 deletion among the *M. tuberculosis* complex, as well as in seven different *M. tuberculosis* clinical isolates. In order to extend this study, we have analysed by Southern blotting the distribution of *pimC* in over 80 representative *M. tuberculosis* clinical isolates (Figure 6). All clinical isolates were characterized previously by IS*6110* fingerprinting, and represent both clustered and unique isolates from a population-based study in the state of New Jersey [39]. Overall, *pimC* was found to be present in $< 22\%$ (17/80) of the



Scheme 1 Postulated pathways for the biosynthesis of the PIMs, LM and LAM in *M. tuberculosis*

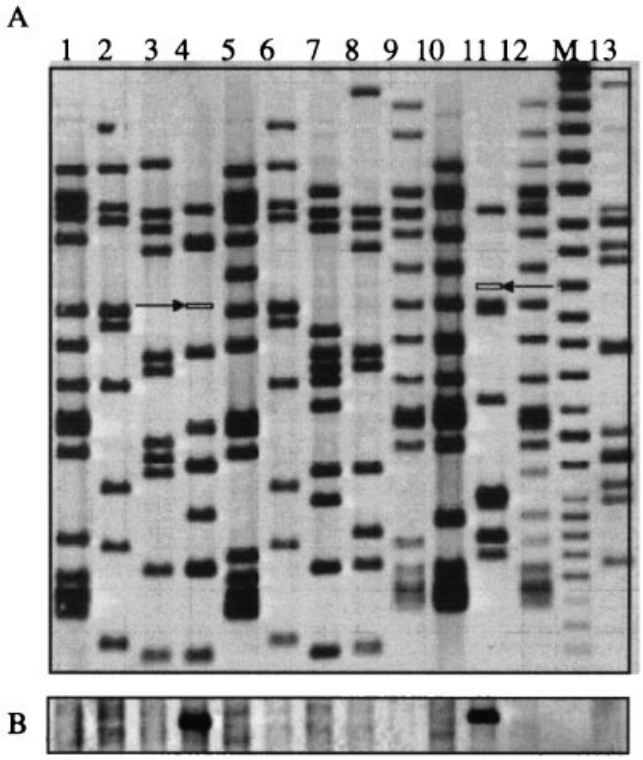


Figure 6 Genetic distribution of *pimC* in various *M. tuberculosis* clinical strains

Southern-blot hybridization of representative clinical isolates from a population-based study [39]. Hybridization was first performed using a IS*6110* probe (**A**), and then the *pimC* probe (**B**), according to standard protocols [31]. The IS*6110* pattern diversity revealed in (**A**) corresponds to the different *M. tuberculosis* isolates analysed. Only two strains (lanes 4 and 11) were found to contain the *pimC* insert, as depicted by rectangles in (**A**). Each lane depicts a different clinical isolate, whereas M indicates the DNA molecular marker.

isolates analysed. In addition, the RvD2 locus was found to be present in only two of the large clusters investigated, primarily in isolates known as the 'Harlem' family [40], which includes the Erdman strain and the multi-drug-resistant 'P' cluster from New York City [41] (results not shown).

DISCUSSION

The specific number of α-mannosyltransferases that are involved in PIM, LM and LAM biosynthesis is a matter of speculation, depending on how the $\alpha(1 \rightarrow 6)$-linked mannan backbone and $\alpha(1 \rightarrow 2)$-Man side chains are assembled. One can speculate that numerous enzymes may be required for the formation of various linkages. In addition, different acceptor and donor specificities may be necessary at some stages, and there is a possibility that certain Man residues may be added sequentially, whereas others may come from intermediates accumulated on lipid carriers [18]. In addition, there may be species-specific enzyme requirements beyond the structurally conserved PIMs, LM and LAMs. For instance, enzymes that are involved in Man-capping of LAM will differ from those that are necessary to build its mannan core [42]. Indeed, BLAST analysis of the *M. tuberculosis* H37Rv genome sequence database shows similarity between PimC and various predicted proteins. For instance, PimB (Rv0557) and also Rv0225, Rv0486, Rv1212c, Rv2188c, Rv2610c and Rv3032 all contain the EX_7E motif. The similarity of these predicted gene products to PimC suggests that they may also be α-mannosyltransferases involved in the biosynthesis of PIM, LM and LAM, although the EX_7E motif is present in a number of bacterial glycosyltransferases other than α-mannosyltransferases [36]. Further analysis of PimC predicts two transmembrane regions from amino acids 265–285 and 354–372, suggesting that PimC may be a membrane or a membrane-associated protein. This is consistent with the observed localization of GDP-Man-dependent α-mannosyltransferases in mycobacteria [18]. However, it is worth noting that hydrophobic helices may be buried into a globular structure, and that glycosyltransferases may be associated to membranes by different mechanisms [43,44].

Overexpression of *pimC* from *M. tuberculosis* CDC1551 in *M. smegmatis* led to an overall enhanced α-mannosyltransferase activity associated with PIMs. Qualitative analysis by TLC autoradiography of the reaction products shows an accumulation of a new PIM member migrating with a lower retardation factor than Ac_3PIM_2. The structural characterization of this product by FAB MS established that the compound was Ac_3PIM_3. As discussed, the overexpression of PimC would catalyse the transfer of Man*p* from GDP-Man to Ac_3PIM_2 to afford Ac_3PIM_3, with possibly a slight decrease in Ac_3PIM_2 and no subsequent 'chase' due to the absence of the C_{35}/C_{50}-P-Man sugar donor. Thus, *pimC* encodes a GDP-Man-dependent, α-D-Man-α-$(1 \rightarrow 6)$-PI dimannoside transferase that mediates the transfer of Man*p* from GDP-Man to Ac_3PIM_2 to form Ac_3PIM_3 (see Scheme 1).

In the present study, we also describe the inactivation of *pimC* by allelic exchange in *M. bovis* BCG. We observed that, first, disruption of the gene does not affect *in vivo* growth of the mutant strain, indicating that this particular gene is not essential for growth and, secondly, inactivation of *pimC* does not alter the abundance of PIMs, LM and LAM. This suggests the presence of compensatory mechanisms that would implicate inverting α-D-Man-α-$(1 \rightarrow 6)$-PI dimannoside transferases, presumably via the use of C_{35}/C_{50}-P-Man (Scheme 1). Interestingly, the use of alternate sugar donors, implying multiple glycosyltransferases, has been observed by Shimada et al. [45] during the *in vitro* glycosylation of lipoteichoic acid in several *Bacillus* strains. Membrane preparations obtained from *Bacillus* strains were shown to catalyse the transfer of [^{14}C]GlcNAc from [^{14}C] GlcNAcP-undecaprenol to endogenous polymerized lipoteichoic acid. The authors also demonstrated that the transfer from UDP-[^{14}C]GlcNAc could occur directly, and independently of the synthesis of the lipid-linked GlcNAcP-undecaprenol sugar donor, on the basis of enzyme kinetics and conditions that were unfavourable to the formation of GlcNAcP-undecaprenol [45].

This hypothesis is strengthened by the fact that *pimC* was originally isolated and cloned from *M. tuberculosis* CDC1551, and was absent from *M. tuberculosis* H37Rv [16], a strain producing PIMs, LM and LAM. Comparative genomics has recently revealed that *M. tuberculosis* H37Rv contains two deletions, RvD1 and RvD2, relative to the other members of the complex [33]. The RvD2 region was found to be present in *M. africanum*, *M. bovis*, *M. bovis* BCG, *M. microti* OV254 and *M. tuberculosis* CDC1551 [33], as well as in the attenuated *M. tuberculosis* H37Ra [38]. The RvD2 deletion locus contains three ORFs, designated RvD2-*ORF1*, RvD2-*ORF2* and RvD2-*ORF3*, with two flanking IS*6110* elements interrupted by two further ORFs [38]. We have extended this study by analysing the genetic distribution of *pimC* (formerly RvD2-*ORF1*) in over 80 clinical *M. tuberculosis* isolates characterized previously by IS*6110* fingerprinting [39], and found that the gene was present in only 22% of the isolates. This observation is in agreement with other reported investigations [46], which identify the RvD2 locus to be within a highly variable region of the chromosome. Analysis of

24 isolates revealed extensive genetic diversity, including 14 variants characterized by IS*6110* insertion and deletion events resulting from probable homologous recombination [46]. Alterations to this locus were also found within at least some of the H37Rv progenies of the laboratory strain H37Rv/Ra [38,46]. Searches in the mycobacterial genome databases also revealed the presence of *pimC* in *M. avium*, and the absence of this gene in *M. leprae* and *M. smegmatis* (results not shown).

The possible redundancy of genes that are involved in mycobacterial cell-wall biosynthesis seems not to be an uncommon issue in *M. tuberculosis*. It has been shown that the dominant exported proteins of *M. tuberculosis* (called the antigen 85 complex) consisting of Ag85A, Ag85B and Ag85C, are highly related gene products that all display mycolyltransferase activity involved in the final stages of mycobacterial cell-wall assembly [47]. The importance of the 85 antigen proteins was highlighted through an analysis of a mutant strain deficient in Ag85C. The Ag85C mutant possessed 40% less mycolic acids in the cell wall compared with WT bacilli [48]. Since mycolyltransferase activity was not completely abrogated in the Ag85C knock-out strain, it has been suggested that the other members of the complex may compensate, at least partially, the loss of mycolic acids in the cell wall. The possibility of redundancy is further supported by our own recent studies, which demonstrated that the GDP-Man-dependent α-mannosyltransferase involved in the synthesis of Ac_3PIM_2 catalysed by PimB [20] is also a dispensable gene, suggesting that other putative GDP-Man-dependent α-mannosyltransferases within the *M. tuberculosis* genome may compensate for this loss *in vivo* (L. DesJardin, G.S. Besra and L. Schlesinger, unpublished work).

An increasing body of evidence has shown that LAM has a key role in the pathogenesis of mycobacterial diseases, so agents that disrupt its biosynthesis will undoubtedly affect the ability of *M. tuberculosis* to survive within the host. Drugs that specifically inhibit unique steps involved in PIM biosynthesis will also affect the ability of the organism to produce LM and LAM, thus altering cell wall structure and integrity. Therefore α-mannosyltransferases that are involved in the biosynthesis of PIM and LM are prospective targets for the design of novel chemotherapeutic agents against pathogenic mycobacteria. The present report demonstrates that *pimC* is a dispensable gene due to the presence of compensatory mechanisms, and therefore does not represent an attractive target for the development of new anti-mycobacterial agents. However, it does shed valuable information on understanding the biochemical and molecular basis of PIM, LM and LAM biosynthesis in the genomics era, and will hopefully lead to the further development of the PIM-biosynthetic pathway in terms of the identification of other α-mannosyltransferases that are utilized in the subsequent addition of Man*p* residues to form PIMs, and ultimately LM and LAM.

Finally, the availability of the recombinant *M. smegmatis* strain overexpressing PimC now allows us to produce and purify Ac_3PIM_3, a glycolipid undetectable in crude mycobacterial lysates. This will be particularly useful in investigating the biological functions associated with Ac_3PIM_3, such as its capacity to mediate attachment to cells [10,11], to recruit NK T cells or to participate in the granulomatous response [8,9].

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