Definition of the first mannosylation step in phosphatidylinositol mannoside synthesis:

PimA is essential for growth of Mycobacteria

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Running title: Phosphatidylinositol mannoside synthesis in mycobacteria

Abbreviations: ORF, open reading frame; LB, Luria Bertani culture medium, TLC, thin-layer chromatography; PE, phosphatidylethanolamine; CL, cardiolipin; PI, phosphatidyl-myoinositol; PIM, phosphatidyl-myoinositol mannosides; LM, lipomannan; LAM, lipoarabinomannan; PCR, polymerase chain reaction; kb, kilobase; Km, kanamycin; Str, streptomycin; Hyg, hygromycin; Suc, sucrose; KmR, kanamycin-resistant; StrR, streptomycin-resistant; HygR, hygromycin-resistant; SucR, sucrose-resistant; MIC, minimal inhibitory

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concentration; IPTG, isopropyl-β-D-thiogalactopyranoside; MWM, molecular weight marker; Man, mannose; myo-Ins, myo-inositol; Ara, arabinose; HABA, 2-(4-hydroxy-phenylazo)-benzoic acid; MALDI, Matrix-Assisted Laser desorption/ionization; Tof, time of flight; C_{16}, palmitate; C_{19}, tuberculostearate (10-methyloctadecanoate);

PIM is used to describe the global family of PIM that carries one to four fatty acids and one to six mannose residues. In Ac_{x}PIM_{y}, x refers to the total number of acyl groups, including those attached to the glycerol (the diacylglycerol substituent), y refers to the number of mannose residues; e.g. Ac_{2}PIM_{1} corresponds to the di-acylated form of the phosphatidylinositol mono-mannoside PIM_{1}. This nomenclature requires careful enunciation since inherent in the abbreviation for phosphatidyl-my-o-inositol, PI, is the diacylglycerol unit.

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SUMMARY

We examined the function of the pimA (Rv2610c) gene, located in the vicinity of the phosphatidylinositol synthase gene in the genomes of Mycobacterium tuberculosis and Mycobacterium smegmatis, which encodes a putative mannosyltransferase involved in the early steps of phosphatidylinositol mannosides synthesis. A cell-free assay was developed in which membranes from M. smegmatis overexpressing the pimA gene incorporate mannose from GDP-[\(^{14}\)C]Man into di- and tri-acylated phosphatidylinositol mono-mannosides. Moreover, crude extracts from Escherichia coli producing a recombinant PimA protein synthesized diacylated phosphatidylinositol mono-mannoside from GDP-[\(^{14}\)C]Man and bovine phosphatidylinositol.

In order to determine if PimA is an essential enzyme of mycobacteria, we constructed a pimA conditional mutant of M. smegmatis. The ability of this mutant to synthesize the PimA mannosyltransferase was dependent on the presence of a functional copy of the pimA gene carried on a temperature-sensitive rescue plasmid. We demonstrate here that the pimA mutant is unable to grow at the higher temperature at which the rescue plasmid is lost. Thus, the synthesis of phosphatidylinositol mono-mannosides and derived higher PIM in Mycobacterium smegmatis appears to be dependent on PimA and essential for growth. This work provides the first direct evidence of the essentiality of phosphatidylinositol mannosides for the growth of mycobacteria.
Phosphatidylinositol (PI) and phosphatidylinositol mannosides (PIM) are the prominent and most distinguishable phospholipids of mycobacteria. They also provide the lipid anchor of two lipoglycans, lipomannan (LM) and lipoarabinomannan (LAM), the latter being an important modulator of the immune response in the course of tuberculosis and leprosy (1-3) as well as a key ligand in the interactions between *M. tuberculosis* and phagocytic cells (4-7).

In *M. bovis* BCG, PI and PIM represent as much as 56% of all phospholipids in the cell wall and 37% of those in the cytoplasmic membrane and are thus regarded as important structural components acting as “cementing substances” for the cell wall skeleton (8). In support of this assumption, the synthesis of PI was recently shown to be essential for growth of *M. smegmatis* (9).

Although little is known about the biosynthesis of PIM, LM and LAM, structural similarities based on a conserved GPI-anchor point to a metabolic relationship (10-14). Studies begun over thirty years ago provide evidence that the early steps of PIM synthesis start with the transfer of a mannose residue from GDP-Man to the 2-position of the *myo*-inositol ring of PI to form phosphatidylinositol monomannosides (PIM<sub>1</sub>). This step is followed by the transfer of another mannose residue (Man) to the 6-position of *myo*-inositol (*myo*-Ins) to form phosphatidylinositol dimannosides (PIM<sub>2</sub>) (15-17). From PIM<sub>2</sub>, it is proposed that the Man residue in the 6-position of *myo*-Ins is further glycosylated with Man and then with Ara to form the higher forms of PIM (PIM<sub>3</sub>-PIM<sub>6</sub>) and the highly branched lipoglycans LM and
LAM through reactions probably involving many different mannosyl- and arabinosyl-
transferases as well as nucleoside diphosphate- and polyrenyl-phosphate-based sugar donors
(14). Recently, the pimB gene of M. tuberculosis was characterized as encoding a α-D-
mannose- α(1→6)-phosphatidyl-myoinositol-monomannoside transferase that mediates the
transfer of Man from GDP-Man to tri-acylated PIM\textsubscript{1} (Ac\textsubscript{3}PIM\textsubscript{1}) to form tri-acylated PIM\textsubscript{2}
(Ac\textsubscript{3}PIM\textsubscript{2}) (18). The amino acid sequence of PimB contains the motif EXF(G/C)XXXXE
found in bacterial retaining α-mannosyltransferases that catalyze the formation of glycosidic
bonds using GDP-Man as the sugar donor (19). This motif, which is proposed to be involved
in the binding of GDP-Man, is found in four other M. tuberculosis predicted proteins (20)
among which is Rv2610c (amino acid residues 274-282). Interestingly, Rv2610c is the fourth
gene of a cluster of five ORFs (20) potentially organized as a single transcriptional unit (9)
and likely to be involved in the synthesis of PIM. The first ORF of this cluster (Rv2613c)
encodes a protein of unknown function. The second ORF encodes the phosphatidylinositol
synthase PgsA characterized earlier (9). The third ORF (Rv2611c) encodes a protein with
similarities to bacterial acyltransferases, and the fourth and fifth ORF encode, respectively,
the putative α-mannosyltransferase Rv2610c, and a putative GDP-Mannose hydrolase
(Rv2609c) carrying a MutT domain signature (PS00893) (21). This genetic organization
suggested that Rv2610c might encode a α-mannosyltransferase involved in the very early
steps of PIM synthesis (9). Rv2610c shares sequence similarity to many bacterial
glycosyltransferases, is present in all the mycobacterial genomes sequenced so far and has a
homolog in *Streptomyces coelicolor* (51% identity on a 375 amino acid overlap), an actinomycete that shares with mycobacteria the ability to synthesize PIM (22).

In this report, evidence is provided that Rv2610c is the $\alpha$-D-mannose- $\alpha$(1$\rightarrow$2)-phosphatidylmyo-inositol transferase responsible for the formation of Ac$_3$PIM$_1$ from GDP-Man and PI. Based on the name recently given to the mannosyltransferase responsible for the synthesis of Ac$_3$PIM$_2$ from Ac$_3$PIM$_1$ (PimB) (18), we name the Rv2610c enzyme PimA. Through the construction and analysis of a *pimA* conditional mutant of *M. smegmatis*, we demonstrate that PimA is essential for mycobacterial growth.

**EXPERIMENTAL PROCEDURES**

*Bacterial strains and growth conditions*

*E. coli* XL1-blue, the strain used in this study for cloning experiments, and *E. coli* BL21(DE3)pLysS were routinely propagated in Luria Bertani (LB) broth (Difco, Detroit, MI) at 37°C. *Mycobacterium smegmatis* strain mc$^2$155 (23) was routinely grown at 30°C, 37°C or 42°C in LB broth supplemented with 0.05% Tween 80. LB medium was used as the solid medium for all bacteria. Antibiotics were added at the following concentrations: ampicillin, 100 $\mu$g/ml; chloramphenicol, 34 $\mu$g/ml; kanamycin, 20 $\mu$g/ml; hygromycin, 50 $\mu$g/ml; streptomycin, 20 $\mu$g/ml. When required, 10% sucrose was added to the solid medium.
Cloning procedures, mycobacterial genomic DNA extraction and Southern analysis

For preparation of electrocompetent cells, *E. coli* XL1-blue cells grown in LB broth were washed two times with distilled water, one time in 10% glycerol and finally resuspended in 10% glycerol. The same procedure was used for the preparation of *M. smegmatis* electrocompetent cells except that 0.05% Tween80 was added to all washing solutions. Aliquots of electrocompetent cells were transformed using a Gene Pulser unit (Bio-Rad Laboratories, Richmond, CA) with a single pulse (2.5 kV; 25 µF; 200 ohms). Purification of DNA restriction fragments and PCR fragments were performed using the QIAquick Gel Extraction Kit and QIAquick PCR Purification Kit (QIAGEN, CA). Plasmids were isolated from *E. coli* XL1-blue using the QIAprep Miniprep kit (QIAGEN). Mycobacterial genomic DNA was isolated as follows: cells from a 5 ml overnight culture were pelleted by centrifugation at 3500 rpm for 15 minutes. The pellet was resuspended in 250 µl of solution I (25% sucrose, 50 mM Tris-HCl pH 8.0, 50 mM EDTA, 500 µg/ml lysozyme) and incubated overnight at 37°C under agitation. The next day, 250 µl of solution II (100 mM Tris-HCl pH 8.0, 1% SDS, 400 µg/ml proteinase K) were added and the reaction mixture was incubated at 55°C for 4 hours. DNA was extracted twice with phenol and chloroform and concentrated by ethanol precipitation. Molecular cloning and restriction endonuclease digestions were performed by standard techniques according to the manufacturer’s recommendations. Labeling of DNA probes with α-32P[dCTP] and Southern blot analyses were performed as described (24). The *M. smegmatis* genomic DNA sequences used in this study were obtained
from the TIGR Center (http://www.tigr.org/). Sequences were processed using the DNA Strider program (CEA, Gif-sur-Yvette, France).

Overexpression of the M. smegmatis pimA gene in M. smegmatis

Standard PCR strategies with Taq DNA polymerase (Applied Biosystems, Roche) were used to amplify the M. smegmatis pimA gene. PCR amplification consisted of one denaturation cycle (95°C, 6 min) followed by 40 cycles of denaturation (95°C, 1 min), annealing (60°C, 1 min) and primer extension (72°C, 1.5 min), and a final extension at 72°C for 10 minutes. The plasmid pCGpisB (see below) was used as the DNA template and the primers were ManT1 (5'- ccaccaacatatgcgtatcgggatggtctgccc -3') and ManT2 (5'- cccaagcttgaccgattctccggccgtctcg -3'). The primers were designed to generate a PCR product corresponding to the entire pimA gene devoid of its stop codon and harboring NdeI and HindIII restriction sites (underlined in the primers’ sequences) enabling direct cloning into the pVV16 expression vector (9). pVV16 harbors a kanamycin and a hygromycin resistance marker, and allows genes to be constitutively expressed under the control of the hsp60 transcription and translation signals. Recombinant proteins produced with this system carry a six-histidine tag at their carboxyl terminus. M. smegmatis mc²155 was transformed with the resulting expression vector, pVVpimA, and transformants were selected on LB-Km-Hyg plates. The production of recombinant PimA protein in M. smegmatis was analyzed by SDS-PAGE on a 12% gel, followed by immunoblotting using a nitrocellulose transfer membrane Hybond C (Amersham Pharmacia Biotech) with a mouse monoclonal anti-His antibody (Penta-His antibody,
QIAGEN) diluted 1:1,000. The secondary antibody was a horseradish peroxidase-conjugated sheep anti-mouse IgG antibody (Amersham Pharmacia Biotech) and was used at a 1:10,000 dilution. Bound antibodies were detected using the ECL system (Amersham Pharmacia Biotech). *M. smegmatis* crude extracts were prepared by harvesting cultures of the recombinant strains (A600 nm = 1.5), suspending them in PBS, subjecting them to probe sonication at 4°C for 5 min in the form of 5 x 60 s pulses with 60 s cooling intervals between pulses, and removing the unbroken cells and bacterial debris by centrifugation of the sonicate at 10,000x g for 15 min.

**Whole cell radiolabeling experiment**

Radiolabeling of whole *M. smegmatis* mc²155/pVV16 and mc²155/pVVpimA cells with myo-[2-³H]Inositol (specific activity 17.0 Ci/mmol, Amersham Pharmacia Biotech) was performed in LB medium supplemented with 0.05% Tween 80 and kanamycin. 100 µCi myo-[2-³H]Inositol was added to 10 ml mid-log cultures, which were incubated at 37°C under agitation for a further 24 hours.

**Drug sensitivity assays**

LB-agar medium containing isoniazid (INH), chloramphenicol or ampicillin in dilution series was added to the wells of six-well plates. These plates were inoculated with appropriate dilutions of mc²155 or mc²155/pVVpimA cultures and incubated for 3-4 days at 37°C. 99% inhibition of the bacterial growth was determined as the MIC of the drug.
**Expression of the *M. smegmatis* pimA gene in *E. coli***

Recombinant PimA protein was produced in *E. coli* BL21(DE3)pLysS using the pET14b expression system (Novagen, Madison, WI). The *M. smegmatis* pimA gene was amplified using Taq DNA polymerase (Applied Biosystems, Roche), pCGpisB (see below) as the DNA template and the primers Man1 (5’- cggcgggcatatgcgtatcgggatggtctgc –3’) and Man2 (5’-cccggaacctgcacgatttccgctcgcgt –3’). PCR amplification consisted of one denaturation cycle (95°C, 6 min) followed by 40 cycles of denaturation (95°C, 1 min), annealing (64°C, 1 min) and primer extension (72°C, 1.5 min), and a final extension at 72°C for 10 minutes. The primers were designed to generate a PCR product corresponding to the entire pimA gene and harboring *Nde*I and *BamHI* restriction sites (underlined in the primers’ sequences) enabling direct cloning into the pET14b expression vector (Novagen, Madison, WI). The resulting expression vector was named pETpimA. It allows for the expression of the pimA gene under control of the strong bacteriophage T7 transcription and translation signals and for the production of a N-terminal six-histidine tagged PimA recombinant protein. The production of recombinant PimA protein in *E. coli* BL21/pETpimA transformants was induced by the addition of 0.4 mM IPTG to the culture medium with incubation at 37°C for three hours. The production of PimA protein was then analyzed by immunoblotting with a mouse monoclonal anti-His antibody (Penta-His antibody, QIAGEN) as described for the recombinant PimA protein produced in *M. smegmatis*. 
Cell-free assay for mannophospholipid synthesis in M. smegmatis using GDP-[\(^{14}\)C]Man

*M. smegmatis* mc²155/pVV16 and mc²155/pVVPimA cells (8 g wet weight) were washed and resuspended in 10 ml of buffer A (20 mM Tris-HCl buffer, pH 7.45) prior to probe sonication for 10 min at 4°C in the form of 10 x 60 s pulses with 90 s cooling intervals between pulses. The unbroken cells and bacterial debris were removed by centrifugation of the sonicate at 22,500 x g for 20 min at 4°C. The membrane fraction was prepared by centrifugation of the 22,500 x g supernatant at 100,000 x g for 2 h at 4°C. The concentration of proteins in the supernatant from this centrifugation representing the cytosol fraction was established to be 20 mg/ml. The pellet containing the membranes was suspended in about 800 µl of the buffer A to a concentration of 15 mg of protein/ml.

The reaction mixtures for incorporation of GDP-[\(^{14}\)C]Man into mannophospholipids contained 0.25 µCi GDP-[\(^{14}\)C]Man (specific activity 305 mCi/mmol, Amersham Pharmacia Biotech), 10 mM MgCl₂, 62.5 µM ATP, membrane fraction (1 mg of proteins) or cytosol fraction (1 mg of proteins) or their combination, and buffer A in a final volume of 1 ml. After one hour of incubation at 37°C, the reactions were stopped by addition of 6 ml of CHCl₃/CH₃OH (2:1). The samples were left rocking for 1 h at 37°C and then centrifuged at 2,000 x g for 10 min. The upper aqueous phase was discarded and the bottom organic phase was washed with CHCl₃/CH₃OH/ H₂O (1:47:48, v/v). The organic phase was finally dried under a stream of nitrogen and dissolved in 100 µl CHCl₃/CH₃OH (2:1, v/v) prior to scintillation counting and TLC analysis. All experiments were repeated at least three times and representative results are shown. For structural analyses of the reaction products, GDP-[\(^{14}\)C]Man was replaced with
unlabeled GDP-Man. The reaction mixtures contained 4 mg of membrane proteins from mc3155/pVV16 or mc3155/pVVpimA, 20μM GDP-Man (Sigma), 10 mM MgCl₂, 62.5 μM ATP and buffer A (pH 7.45) in the final volume of 1 ml. The total lipids from 6 reactions were extracted as described above, combined and analyzed by MALDI-MS. For the individual characterization of mannolipid products, the non-radioactive mannolipids were isolated by preparative TLC using the radiolabeled mannolipids as markers. Following autoradiography, the relevant regions of the TLC were scraped off, extracted with CHCl₃/CH₃OH (2:1) and subjected to MALDI-MS analysis. The same amounts of mannolipids were produced by membrane fractions of mc3155/pVV16 and mc3155/pVVpimA in the absence or presence of ATP in the reaction mixture, suggesting that ATP is not required in the assay.

Cell-free assay for mannophospholipid synthesis in E. coli

E. coli BL21/pET14b and E. coli BL21/pETpimA (4 g wet weight) were suspended in 4 ml of buffer A (pH 7.45) and subjected to probe sonication for 3 min 20 s in the form of 20 x 10 s pulses with 60 s cooling intervals between pulses. The sonicate was centrifuged for 20 min at 10,000 x g to remove the unbroken cells and bacterial debris and the resulting supernatant (E. coli crude extract) was kept frozen in small aliquots at - 20°C and then used directly in cell-free assays as the sole source of enzymes. Protein concentrations in the E. coli BL21/pET14b and E. coli BL21/pETpimA extracts were 20 and 55 mg/ml, respectively.
Reaction mixtures contained 0.25 µCi GDP-[\(^{14}\text{C}\)]Man (specific activity 305 mCi/mmol, Amersham Pharmacia Biotech), 40 µM bovine PI (Sigma), 10 mM MgCl\(_2\), 62.5 µM ATP, \textit{E. coli} BL21/pET14b or \textit{E. coli} BL21/pETpimA crude extracts (3 mg of proteins) and buffer A (pH 7.45) in the final volume of 1 ml. The reaction mixtures were incubated for 2 h at 37°C and the reactions were stopped by adding 6 ml of CHCl\(_3\)/CH\(_3\)OH (2:1). The lipids were extracted as described above and analyzed by TLC. For structural analyses of the reaction product, GDP-[\(^{14}\text{C}\)]Man was replaced with cold GDP-Man (10 µM) (Sigma). The total lipids extracted from 20 reactions carried out with crude extracts from \textit{E. coli} BL21/pET14b or \textit{E. coli} BL21/pETpimA were combined and subjected to MALDI-MS analysis.

\textit{Analytical procedures}

Lipids from labeled and non-labeled cells were extracted by two consecutive overnight extractions in CHCl\(_3\)/CH\(_3\)OH (2:1) followed by one overnight extraction in CHCl\(_3\)/CH\(_3\)OH (1:2). The CHCl\(_3\)/CH\(_3\)OH extracts were combined, Folch washed (CHCl\(_3\)/CH\(_3\)OH/H\(_2\)O (4:2:1)) and dried before reconstituting in CHCl\(_3\)/CH\(_3\)OH (2:1) for analysis by TLC. Characterization of the various PIM followed earlier work (9,15,18) and was based on one- and two-dimensional thin-layer chromatographic patterns, co-migration of labeled PIM products with authentic PIM standards and mass spectrometry analysis of total or purified lipids. The fatty acids of the PIM are primarily C\(_{16}\) (palmitate) and C\(_{19}\) (tuberculostearate) (15). TLC was conducted on aluminum-backed plates of silica gel 60 F\(_{254}\) (E. Merck, Darmstadt, Germany). The solvent system used was CHCl\(_3\)/CH\(_3\)OH/NH\(_2\)OH/H\(_2\)O.
(65:25:0.5:4, v/v). Two-dimensional TLCs were performed using solvent CHCl₃/CH₂OH/H₂O (60:30:6, v/v) in the first dimension and solvent CHCl₃/CH₂COOH/CH₂OH/H₂O (40:25:3:6, v/v) in the second dimension. A α-naphtol spray (1% α-naphtol in ethanol) and a cupric sulfate spray (10% CuSO₄ in a 8% phosphoric acid solution) were used to detect carbohydrate containing lipids and all organic compounds, respectively. Autoradiograms were obtained by exposing chromatograms to Kodak BIOMAX MR films at -70°C for 1 to 7 days. Relevant spots were scraped off for scintillation counting. For MALDI-MS analysis of the phospholipids containing fraction, total lipid extracts were suspended in CHCl₃ and washed twice with an equal volume of water. The organic phase was then brought to dryness, solubilized in acetone and allowed to precipitate overnight at 4°C. The suspension was then centrifuged at 4°C for 15 min (3,000 x g). The supernatant was removed and the precipitate was resuspended in CHCl₃ and analyzed in MALDI-Tof-MS.

Sample Preparation and MALDI-TOF Mass Spectrometry

Analysis by MALDI-Tof-MS was carried out on a Voyager DE-STR (PerSeptive Biosystems, Framingham, MA) using the reflectron mode. Ionization was effected by irradiation with pulsed UV light (337 nm) from a N₂ laser. PIM were analyzed by the instrument operating at 20 kV in the negative ion mode using an extraction delay time set at 200 ns. Typically, spectra from 100 to 250 laser shots were summed to obtain the final spectrum. All of the samples were prepared for MALDI analysis using the on-probe sample cleanup procedure with cation-exchange resin. The HABA matrix was used at a concentration of ~10 mg/ml in ethanol/water.
(1:1 v/v). Typically, 0.5 µl of PIM sample (10 µg) in a CHCl₃-CH₃OH-H₂O solution and 0.5 µl of the matrix solution, containing ~5 to 10 cation-exchange beads, were deposited on the target, mixed with a micropipet, and dried under a gentle stream of warm air. The measurements were externally calibrated at two points with PIM.

Construction of the M. smegmatis pimA conditional mutant

The essentiality of the *pimA* gene in *M. smegmatis* was investigated using a two-step homologous recombination procedure. The method relies upon the use of a suicide vector harboring the counterselectable marker *sacB* and a kanamycin cassette-disrupted copy of the gene of interest. In the first step of the experiment, a single crossover strain is isolated. This strain contains the *sacB* gene and should be sensitive to sucrose. In the second step of the experiment, a culture of the single crossover strain is plated onto sucrose-Km plates to select for clones that underwent a second intrachromosomal crossover leading to the excision of the body of the vector and to allelic replacement. In the case the gene of interest is essential, allelic replacement at the second step of the experiment should be achievable only in the presence of a rescue copy of this gene provided on a replicative or integrative vector.

The *M. smegmatis pimA* gene and flanking regions were excised from the plasmid pUCpgsA.Sm (9) on a 3.7 kb *BamH*I restriction fragment and inserted at the *BamH*I site of pACYC184 (New England Biolabs, Inc.), yielding plasmid pACYCpimA. A disrupted allele of the *pimA* gene, *pimA::Km*, was then constructed by cloning the kanamycin resistance cassette from pUC4K (Amersham Pharmacia Biotech) carried on a 1.2 kb *Hinc*II restriction
fragment into the KpnI-cut and blunt-ended pACYCpimA. pimA::Km was then excised from the resulting plasmid on a 4.9 kb BamHI restriction fragment, blunt-ended and inserted at the SmaI site of pXYL4 (a pBlueScript derivative carrying the xylE reporter gene) (25) yielding plasmid pX4pimAK. Finally, pJQpimA, the construct used for allelic replacement, was obtained by transferring a 5.9 kb BamHI fragment from pX4pimAK containing pimA::Km and xylE, into the BamHI-cut pJQ200, an E. coli cloning vector carrying the counterselectable marker sacB (26).

pCG76, a Mycobacterium/E. coli shuttle plasmid harboring a mycobacterial temperature-sensitive origin of replication and a streptomycin resistance cassette (27), was used as the rescue plasmid to carry a functional copy of the pimA gene in the M. smegmatis pimA conditional mutant. pCGpisB, one of the rescue plasmids used in this study, was constructed by inserting the 3.7 kb BamHI restriction fragment from pUCpgsA.Sm (9) at the BamHI site of pCG76. The 3.7 kb BamHI insert carries full-length wild type copies of the M. smegmatis pgsA, Rv2611c and pimA (Rv2610c) genes.

RESULTS AND DISCUSSION

Overexpression of the pimA gene in M. smegmatis

Sub-cellular localization of the PimA protein
The Rv2610c gene from \textit{M. tuberculosis} which encodes a putative mannosyltransferase involved in the early steps of PIM synthesis was renamed \textit{pimA}. The \textit{pimA} gene of \textit{M. smegmatis} was PCR-amplified and placed under control of the \textit{phsp60} promoter in the mycobacterial expression vector pVV16, yielding plasmid pVVpimA. Upon transformation of \textit{M. smegmatis} mc²155 with this construct, colonies of mc²155/pVVpimA were obtained which exhibited an unusual glossy morphology instead of the dry morphology of wild type mc²155 or mc²155/pVV16 colonies. The growth rates of the mc²155/pVVpimA and mc²155/pVV16 recombinant strains were identical in LB-Km-Hyg-Tween80 broth at 37°C although the strain overexpressing \textit{pimA} showed an increased tendency to clump (data not shown). Production of recombinant PimA protein in mc²155/pVVpimA was checked by Western blot using a mouse monoclonal anti-His antibody. Large quantities of PimA recombinant protein of the expected size (approximately 40 KDa) were detected in crude extracts of mc²155/pVVpimA, and found to be associated to the membrane and cytosol fractions (data not shown). The association of PimA with the membrane fraction is consistent with the prediction of at least one putative transmembrane segment from the amino acids 275 to 298, by the Tmpred (http://www.ch.embnet.org/) and DAS (http://www.sbc.su.se/~miklos/DAS/) transmembrane prediction programs. The uneven distribution of basic amino acid residues responsible for a high predicted pI of the N-terminal half of the protein (theoretical pI = 9.55 from residues 1 to 202) as compared to a theoretical pI of 5.10 for the C-terminal half of the protein (residues 203 to 378), may also reflect the ability of some N-terminal domains of PimA to interact with anionic phospholipids of the membrane. Finally, the association of PimA with the membrane
fraction is consistent with the observed co-localization of the mannosyltransferase activity with the membrane fraction, as shown below. The important amounts of non-active recombinant PimA protein in the cytosol fraction of mc2155/pVVpimA are probably the results of overexpression.

Overexpression of *pimA* alters the PIM composition of *M. smegmatis*

The phosphatidylinositol mannosides (PIM) composition of the mc2155/pVV16 and mc2155/pVVpimA strains was analyzed by metabolic labeling with *myo*-2-H]inositol. Overexpression of *pimA* in *M. smegmatis* resulted in an increased production of PIM2, particularly Ac3PIM2, relative to phosphatidylinositol (PI) (Fig. 1a). The spots corresponding to PI, Ac1PIM2 and Ac3PIM2 (Fig. 1a) were scraped off and counted for radioactivity. The ratio of PI to the two forms of PIM2 combined was 1:2.2 in the control strain mc2155/pVV16, and 1:13.6 in the overproducing strain mc2155/pVVpimA. Changes in the PI to PIM2 ratio were further confirmed by MALDI-MS analysis in the negative ion mode of the phospholipid-containing fraction obtained after chloroform/water partition and acetone precipitation (Fig. 1b). All the peaks observed in the mass spectra were assigned to deprotonated molecular ions [M-H]. The mass spectra show a clear increase in the PIM2 content (m/z = 1175.6 for Ac2PIM2 with C16/C19; m/z = 1413.8 for Ac3PIM2 with 2C16/C19; m/z = 1652.1 and 1694.2 for Ac3PIM2 with 3C16/C19 and 2C16/2C19, respectively) and PIM6 content (at m/z = 2062.1 for Ac3PIM6 with 2C16/C19; at m/z = 2462.4 and 2504.4 for Ac3PIM6 with 3C16/C19 and 2C16/2C19, respectively) relative to PI (m/z = 851.6 for Ac2PI with C16/C19) of the strain expressing the
recombinant PimA protein. These results suggested that PimA is involved in the metabolic pathway producing PIM$_2$ and PIM$_6$.

**Overexpression of pimA alters M. smegmatis sensitivity to ampicillin**

The low permeability of the mycobacterial cell envelope and, subsequently, the high intrinsic resistance of mycobacteria to chemotherapeutic agents are believed to be a result of the unusual structure and composition of the cell envelope (28). Since PIMs represent major components of the cell envelope, we investigated whether alterations in the PIM composition of mc$^2$155/pVVpimA affected its sensitivity to drugs. The MICs of three antibiotics against mc$^2$155/pVVpimA and mc$^2$155/pVV16 were measured. Both strains exhibited identical resistance to INH (3 µg/ml) and chloramphenicol (30 µg/ml) (hydrophilic and hydrophobic drugs, respectively), but mc$^2$155/pVVpimA showed higher resistance to ampicillin, a hydrophilic β-lactam (MIC = 300-400 µg/ml as compared to 100 µg/ml for the control strain). This result is in good agreement with the observation by Parish and collaborators (29) that a *M. smegmatis* mutant with decreased amounts of PIM$_2$ in its envelope was more sensitive to ampicillin.

**Overproduction of PimA in M. smegmatis stimulates the formation of Ac$_2$PIM$_1$ and Ac$_3$PIM$_1$ in cell-free assays**
An enzymatic system associated to the membrane fraction of *M. phlei* and capable of forming PIM₁ from GDP-Man was initially described by Hill and Ballou (16). GDP-Man was shown to be the only effective mannose donor in this system. In the present study, the mannosyltransferase activities of mc²155/pVV16 and mc²155/pVVpimA were compared in a cell-free assay based on that described for *M. phlei*. In this assay, a whole array of mannose-containing lipids, including polyphrenol-based mannolipids and PIM are synthesized. Changes in the lipid profiles of the PimA overproducing strain as compared to the control strain would suggest an involvement of PimA in a metabolic pathway leading to mannose-containing lipids. Since the recombinant PimA protein was found in the cytosol and membranes of the overproducing strain mc²155/pVVpimA, we first tested these two fractions, independently or in combination, for mannosyltransferase activity. The radioactive profiles of the lipids obtained after incubation of membranes with GDP-[¹⁴C]Man showed a clear accumulation of two mannolipids (mannolipids 1 and 2) migrating in the PIM region of the TLC in the case of mc²155/pVVpimA (Fig. 2, lane 2). The reactions performed with the cytosol of the control and overproducing strains yielded identical radioactive lipid profiles (Fig. 2, lanes 3, 4). Almost no mannolipid 1 and no mannolipid 2 could be detected when using the cytosol fraction, even when mycobacterial PI (approximately 15 µM) was added to the reaction mixture (data not shown). The formation of small amounts of polyphrenyl-phosphate mannose and Ac₃PIM₂ in the cytosol fraction is likely the result of a contamination of this fraction with traces of membrane. Combining the membrane and cytosol fractions did not increase significantly the synthesis of mannolipid 1, although slightly higher amounts of mannolipid 2
were detected in both the control and overexpressing strain (Fig. 2, lanes 5, 6). Thus, despite of the fact that PimA is present in the cytosol of *M. smegmatis* mc²155/pVVpimA, the mannosyltransferase activity of this protein is associated only with the membrane fraction. Initial characterization of the two accumulated mannolipids confirmed that they are mild-acid stable and mild-alkali labile, indicating that they are members of the PIM family (data not shown). Further analysis by MALDI-MS of the total lipids from reaction mixtures in which radioactive GDP-[¹⁴C]Man was replaced by cold GDP-Man revealed the presence of two compounds corresponding to Ac₂PIM₁ ([M-H]⁻, m/z = 1013.6) and Ac₃PIM₁ ([M-H]⁻, m/z = 1251.9) in mc²155/pVVpimA which were not detected in mc²155/pVV16 (data not shown). In order to characterize the mannolipids 1 and 2, non-radioactive products from cold reactions were partially purified by preparative TLC and subjected to MALDI-MS analysis. The mass spectrum for the mannolipid 1-containing sample shows presence of the major product at m/z = 1013.6, corresponding to Ac₂PIM₁ with C₁₆/C₁₉ (Fig. 3a), contaminated by traces of co-purified PIM (Ac₂PIM₂, Ac₃PIM₂ and Ac₃PIM₃). Mannolipid 2-containing sample afforded predominant deprotonated molecular ions at m/z = 1251.9, corresponding to Ac₃PIM₁ with 2C₁₆/C₁₉ (Fig. 3b).

Therefore, the major radiolabeled product of the reaction catalyzed by PimA in *M. smegmatis* is Ac₂PIM₁. Part of this product is presumably acylated into Ac₃PIM₁.

**PimA catalyzes the formation of Ac₂PIM₁ in E. coli cell-free assays**
The function of the PimA protein was further studied in crude extracts of *E. coli* BL21/pETpimA overexpressing *pimA*. Since *E. coli* does not produce any PI, no background mannosyltransferase and acyltransferase activities on PI or derived products were expected in these extracts. In the reaction mixture, commercial bovine PI (carrying primarily stearate (C18:0) and arachidonate (C20:4) fatty acyl chains) and GDP-[14C]Man served as the substrates for the predicted reaction PI + GDP-[14C]Man $\rightarrow$ Ac$_2$PI[14C]M$_1$ + GDP catalyzed by the recombinant PimA protein. In the presence of crude extract from *E. coli* BL21/pETpimA but not in that of crude extract from BL21/pET14b, a single product was formed (Fig. 4, lanes 3-4). Its synthesis was strictly dependent upon the addition of PI to the reaction mixture (Fig. 4, lanes 1-4). This product migrated with a similar Rf to Ac$_2$PIM$_1$ produced in the *M. smegmatis* cell-free assays (Fig. 4, lanes 5, 6). A slight shift in TLC mobility of this product can be attributed to the difference in acyl groups of mycobacterial PI (containing C$_{16}$/C$_{19}$) compared to commercial bovine PI. MALDI-MS analysis of the total lipids from reaction mixtures in which radioactive GDP-[14C]Man was replaced by cold GDP-Man showed that the only detected PIM corresponded to Ac$_2$PIM$_1$ (with peaks at $m/z$ =1025.7 and $m/z$ =1049.7) in BL21/pETpimA (Fig. 5), which were not detected in BL21/pET14b. It is obvious that these compounds arose from PI ($m/z$ =863.6; $m/z$ =887.6) by addition of a single hexose (i. e., mannose). Duplicity of the peaks on the spectrum is due to heterogeneity of commercial PI, which is claimed to contain primarily, but not exclusively stearate and arachidonate fatty acyl chains.
Altogether, the results obtained from the *M. smegmatis* and *E. coli* cell-free assays provide evidence that the PimA enzyme of *M. smegmatis* catalyzes the transfer of the first Man residue from GDP-Man to the myo-Ins residue of PI, yielding Ac₃PIM₁. Since earlier work (16, 17) clearly showed that the very first transfer of a Man residue onto PI in PIM synthesis occurred at the 2-position of myo-Ins, we therefore conclude that PimA is an α-D-mannose-α(1→2)-phosphatidyl-myoinositol transferase.

**pimA is an essential gene of M. smegmatis**

**Construction of a pimA conditional mutant of M. smegmatis**

In order to address the question of the essentiality of the mannosyltransferase PimA in mycobacteria we constructed a *pimA* conditional mutant of *M. smegmatis*. Essentially the same strategy was used to construct this mutant as was used to construct a *pgsA* conditional mutant (9). It uses a two-step homologous recombination procedure to achieve allelic exchange at the *pimA* locus (30) and a mycobacterial temperature-sensitive rescue plasmid to perform complementation experiments (27). A kanamycin-disrupted copy of the *pimA* gene, *pimA::Km*, and the *xylE* reporter gene were inserted into the *sacB* suicide vector pJQ200, yielding pJQpimA. pJQpimA was introduced into the wild type *M. smegmatis* mc²155 by electroporation and kanamycin resistant transformants were selected on LB-Km plates at 37°C. A Southern blot analysis performed on the DNA of eighteen of these transformants indicated that fourteen of them resulted from a single homologous recombination event at the
pimA locus (data not shown). Two of them, mc^2pJQpimA.1 and mc^2pJQpimA.2, were selected for the subsequent steps of the experiment. The other four transformants analyzed by Southern blotting arose from illegitimate recombination. mc^2pJQpimA.1 and mc^2pJQpimA.2 were grown in LB-Km broth and then plated onto LB-Km-Suc to select for clones that had undergone a second intra-chromosomal cross-over leading to the excision of the body of vector and to allelic replacement. Allelic exchange mutants are expected to carry the disrupted allele pimA::Km and to have lost the sacB and xylE genes carried by pJQpimA. Therefore, allelic exchange mutants should be resistant to kanamycin and to sucrose and remain white upon spraying with catechol (i.e. XylE negative). Spraying of thousands of kanamycin-sucrose resistant colonies with catechol revealed that none of them exhibited the expected phenotype for allelic exchange mutants. Instead, these clones had probably undergone some mutations in the sacB gene that conferred upon them resistance to sucrose. In order to investigate whether the failure to disrupt the pimA gene in this first experiment could be due to the essentiality of that gene, we next proceeded to the construction of a pimA conditional mutant.

For this purpose, the single crossover strain mc^2pJQpimA.1 was transformed with the temperature-sensitive rescue plasmid pCGpisB or with the empty pCG76 vector. pCGpisB carries functional copies of the M. smegmatis pgsA, Rv2611c and pimA genes. Transformants were selected on LB-Km-Str plates, grown in LB-Km-Str broth at permissive temperature (30°C) and finally plated onto LB-Km-Str-Suc plates at 30°C to select for allelic exchange mutants as described previously. The XylE phenotype of approximately one thousand KmR-
Suc\textsuperscript{R}-Str\textsuperscript{R} colonies was tested for both types of transformants plated. No allelic exchange mutant (Km\textsuperscript{R}-Suc\textsuperscript{R}-Str\textsuperscript{R}-XylE\textsuperscript{+} colony) was found when mc\textsuperscript{2}pJQpimA.1/pCG76 was plated, confirming our previous result obtained at 37°C with the non-transformed mc\textsuperscript{2}pJQpimA.1 strain. In contrast, plating of mc\textsuperscript{2}pJQpimA.1/pCGpisB yielded a majority (69\%) of Km\textsuperscript{R}-Suc\textsuperscript{R}-Str\textsuperscript{R} colonies with a XylE-negative phenotype. Further analysis by Southern blot of a Km\textsuperscript{R}-Suc\textsuperscript{R}-Str\textsuperscript{R}-XylE\textsuperscript{+} colony revealed that it had undergone gene replacement at the pimA locus (Fig. 6a). pimA conditional mutants thus carry a non-functional pimA::Km gene on their chromosome and a functional pimA gene on a conditionally replicative vector.

In conclusion, allelic replacement at the chromosomal pimA locus of M. smegmatis was achievable only in the presence of a rescue plasmid carrying a functional copy of the pimA gene. To conclusively provide evidence that the inability to achieve gene inactivation at the pimA locus is attributable to the pimA gene alone and not to polar effects of the mutation affecting the expression of adjacent genes (and which could be complemented by the pCGpisB plasmid), another allelic replacement experiment was designed in which the single crossover strain mc\textsuperscript{2}pJQpimA.1 transformed with either pVV16 or pVVpimA was plated onto LB-Km-Hyg-Suc plates at 37°C. Because of the presence of a kanamycin resistance marker on the pVV16 and pVVpimA vectors, both allelic exchange mutants and revertants were selected at this step. To distinguish between allelic replacement and reversion, Km\textsuperscript{R}-Hyg\textsuperscript{R}-Suc\textsuperscript{R}-XylE\textsuperscript{+} colonies were analyzed by PCR or Southern blot. In the case of mc\textsuperscript{2}pJQpimA.1 transformed with the pVVpimA, five out of the fourteen clones (35.7 \%) tested were allelic exchange mutants (Fig. 6b). The remaining clones were revertants carrying a wild type allele.
of \textit{pimA} on their chromosome. In the case of mc\textsuperscript{2}pJQpimA.1 transformed with the empty pVV16 vector, the ten clones tested were revertants (data not shown). Therefore, the expression of the only \textit{pimA} gene from pVV\textit{pimA} is sufficient to rescue a \textit{M. smegmatis} \textit{pimA} knock-out mutant.

These results strongly suggest that the \textit{pimA} gene is essential to mycobacteria.

**Growth characteristics of the \textit{pimA} conditional mutant under permissive and non-permissive conditions**

To conclusively provide evidence that the \textit{pimA} gene is essential to \textit{M. smegmatis}, we investigated the ability of a pCGpisB-complemented \textit{pimA} mutant of \textit{M. smegmatis} (named strain MYC1572) to survive at 42°C, a temperature at which the pCGpisB vector is unable to replicate. The growth characteristics of MYC1572 and wild type mc\textsuperscript{2}155 strain at 30°C and 42°C are presented on figures 7a and 7b, respectively. As expected, at 30°C, the temperature-sensitive pCGpisB vector replicates and MYC1572 exhibited the same growth characteristics as the control strain mc\textsuperscript{2}155. After a shift of temperature from 30°C to 42°C, while the control strain continued to grow exponentially, the OD\textsubscript{600} of the MYC1572 culture started to decline after 10 h paralleling the loss of the temperature-sensitive rescue plasmid (figure 7b). Therefore, the \textit{pimA} gene appears to be essential for mycobacterial growth. These data suggest that PimA has a unique function that cannot be compensated by any other mannosyltransferase in the bacteria under the different conditions tested.
Conclusions

This study provides evidence that the pimA gene encodes the α-mannosyltransferase involved in the transfer of the very first mannose residue from GDP-Man to the 2-position of the inositol moiety of phosphatidylinositol leading to the synthesis of Ac₂PIM₁, the biosynthetic precursor of higher PIM, lipomannan and lipoarabinomannan. The characterization of the pimA gene, located in the vicinity of the phosphatidylinositol synthase gene (pgsA), confirms the existence of a cluster of genes dedicated to the early steps of the synthesis of PIM in all mycobacterial genomes sequenced so far (9). The remarkable conservation of this cluster of genes among Mycobacterium spp. is to be related to its essentiality for mycobacterial growth. Indeed, we had previously demonstrated that phosphatidylinositol is an essential component of the mycobacterial cell envelope (9), we now provide the first demonstration that PIM are also essential for the growth of mycobacteria. The role of PIM and derived lipoglycans (LM and LAM) in the envelope may be structural as suggested earlier (8). The PIM composition of the envelope may also have a profound impact on its permeability as suggested by the increased resistance of a M. smegmatis strain overproducing phosphatidylinositol dimannosides (mc²155/pVVpimA) to the hydrophilic drug ampicillin. Finally, the essentiality of the PimA mannosyltransferase and its involvement in a biosynthetic pathway that is confined to Mycobacterium spp. and to a few other actinomycetes (22) makes it an attractive drug target for anti-tuberculosis therapy.
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REFERENCES


LEGENDS TO FIGURES

Fig. 1: Effect of overexpressing *pimA* on the phosphatidylinositol mannosides composition of *M. smegmatis*

(a) 2D-TLC autoradiograph of the *myo-[2-3H]*inositol-labeled lipids from mc²155/pVV16 and mc²155/pVVpimA. Lipid extracts (200 000 dpm) were loaded to TLC plates and developed in two dimensions as described under Experimental Procedures. (b) MALDI-MS analysis of the total lipids from mc²155/pVV16 and mc²155/pVVpimA. Cold lipids from mc²155/pVV16 and mc²155/pVVpimA were extracted and subjected to MALDI-MS analysis in the negative ion mode as described under Experimental Procedures. Peaks observed are \( m/z \) 851.6, Ac₂PI with \( C_{16}/C_{19} \); \( m/z \) 1175.6, Ac₂PIM₂ with \( C_{16}/C_{19} \); \( m/z \) 1413.8, Ac₃PIM₂ with \( 2C_{16}/C_{19} \); \( m/z \) 1652.1 and 1694.2, Ac₄PIM₂ with \( 3C_{16}/C_{19} \) and \( 2C_{16}/2C_{19} \), respectively; \( m/z \) 2062.1, Ac₅PIM₆ with \( 2C_{16}/C_{19} \); \( m/z \) 2462.4 and 2504.4, Ac₆PIM₆ with \( 3C_{16}/C_{19} \) and \( 2C_{16}/2C_{19} \), respectively.

Fig. 2: Effect of overexpressing *pimA* on the *in vitro* incorporation of GDP-[\(^{14}\)C]Man into mannolipids.

TLC autoradiograph of the incorporation of GDP-[\(^{14}\)C]Man into mannolipids by membrane and cytosol fractions prepared from mc²155/pVV16 (lanes 1, 3, 5) and mc²155/pVVpimA (lanes 2, 4, 6). *In vitro* enzymatic reactions were carried out as described under Experimental Procedures. Membrane (M), and cytosol (C) fractions of mc²155/pVV16 and mc²155/pVVpimA or the combination of these two fractions (M+C) were used as the source.
of enzyme. Lipids were extracted and 5% of the labeled material from each reaction was applied to TLC plates and developed with CHCl₃/CH₃OH/NH₄OH/H₂O (65:25:0.5:4).

**Fig. 3:** MALDI-MS analysis of mannolipid 1 (a) and mannolipid 2 (b) produced *in vitro* by membranes of mc²155/pVVpimA. Cold cell-free assays were performed as described under Experimental Procedures. Incubation lasted for 32 hours. The lipids from six reactions were extracted, combined and mannolipids 1 and 2 were isolated by preparative TLC using the radiolabeled mannolipids as markers. MALDI-MS was operated in the negative ion mode. Peaks observed are m/z 1013.6, Ac₂PIM₁ with C₁₆/C₁₉; m/z 1133.6 and 1175.6, Ac₂PIM₂ with 2C₁₆ and C₁₆/C₁₉, respectively; m/z 1251.9, Ac₃PIM₁ with 2C₁₆/C₁₉; m/z 1413.8, Ac₃PIM₂ with 2C₁₆/C₁₉; m/z 1576.0, Ac₃PIM₃ with 2C₁₆/C₁₉; m/z 1652.1 and 1694.2, Ac₃PIM₄ with 3C₁₆/C₁₉ and 2C₁₆/2C₁₉, respectively.

**Fig. 4:** Ac₃PIM₁ synthesis by a recombinant PimA protein produced in *E. coli*. TLC autoradiograph of reactions performed with crude extracts of *E. coli* BL21/pET14b or *E. coli* BL21/pETpimA in the absence (lanes 1, 2) or presence (lanes 3, 4) of bovine PI. The lipids from the cell-free reactions were extracted and 10% of the samples was analyzed by TLC in the solvent system CHCl₃/CH₃OH/NH₄OH/H₂O (65:25:0.5:4). For comparison of migration, reaction products of the cell free assay performed with membranes from mc²155/pVV16 (lane 5) and mc²155/pVVpimA (lane 6) are included.
**Fig. 5:** MALDI-MS analysis of mannolipids synthesized by crude extracts from *E. coli* BL21/pETpimA. Cold cell-free assays using crude extracts from *E. coli* BL21/pETpimA were performed as described under Experimental Procedures. The lipids from twenty reactions were extracted, combined and subjected to MALDI-MS analysis in the negative ion mode. Only the major species of each family are marked. Peak at m/z 885.6 is attributed to Ac₂PI with C₁₈/C₂₀:₄. Peaks at m/z 887.6 could then be tentatively attributed to Ac₂PI with C₁₈/C₂₀:₃ and m/z 861.6 and 863.6 to Ac₂PI with 2C₁₈:₁ and C₁₈/C₁₈:₁ respectively.

**Fig. 6:** Allelic replacement at the *M. smegmatis* pimA locus.

**a):** Southern blot analysis and expected hybridization profiles of the conditional mutant MYC1572 (lane 1) and mc²155 (lane 2). Chromosomal DNA was digested with *BamHI*. The probe used corresponds to a 2.7 kb *BamHI*-StuI fragment from pCGpisB encompassing the *pimA* gene. The strongest signal detected corresponds to the 3.7 kb *BamHI* DNA fragment from *M. smegmatis* carried by the rescue plasmid, pCGpisB.

**b):** Southern blot analysis and expected hybridization profiles of a *pimA* allelic exchange mutant carrying the pVVpimA rescue plasmid (MYC1573) (lane 1) and mc²155 (lane 2). Chromosomal DNA was digested with *SphI* and *HindIII*. The probe used to perform the hybridization corresponds to the wild type 1580 bp *SphI* restriction fragment. The size of the *HindIII*-linearized pVVpimA vector is 6.94 kb.
Fig. 7: Essentiality of the pimA gene in *M. smegmatis*. Growth characteristics of the pimA conditional mutant MYC1572 at 30°C (a) and 42°C (b). Shown are growth curves of strain mc²155 (●) and MYC1572 (○) cultivated in LB-Tween80 and LB-Tween80-Km medium, respectively.
Figure 1:

a. 

b.
Figure 2:
Figure 3:

(a) Mass (m/z)

(b) Mass (m/z)
Figure 4:
Figure 5:

\[\text{Mass (m/z)}\]

\[\text{Intensity (\%)}\]

\[\text{Voyager Spec #1} \rightarrow \text{BC} \rightarrow \text{NF0.7} [\text{BP = 571.2, 46335}]\]

\[\text{Ac}_2\text{PI}\]

\[\text{Ac}_2\text{PIM}_1\]

\[863.6 \quad 887.6\]

\[1025.7 \quad 1049.7\]
Figure 6:

(a)

(b)
Figure 7:

a.

b.
Definition of the first mannosylation step in phosphatidylinositol mannoside synthesis: PimA is essential for growth of Mycobacteria

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