Mannose metabolism is required for mycobacterial growth

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Mycobacteria are the causative agents of tuberculosis and several other significant diseases in humans. All species of mycobacteria synthesize abundant cell-wall mannolipids (phosphatidylinositol mannosides, lipoarabinomannan), a cytoplasmic methylmannose polysaccharide and O-mannosylated glycoproteins. To investigate whether these molecules are essential for mycobacterial growth, we have generated a *Mycobacterium smegmatis* mannose auxotroph by targeted deletion of the gene encoding phosphomannose isomerase (PMI). The PMI deletion mutant displayed a mild hyperseptation phenotype, but grew normally in media containing an exogenous source of mannose. When this mutant was suspended in media without mannose, ongoing synthesis of both the mannolipids and methylmannose polysaccharides was halted and the hyperseptation phenotype became more pronounced. These changes preceded a dramatic loss of viability after 10 h in mannose-free media. Mannose starvation did not lead to detectable changes in cell-wall ultrastructure or permeability to hydrophobic drugs, or to changes in the rate of biosynthesis of other plasma-membrane or wall-associated phospholipids. These results show that mannose metabolism is required for growth of *M. smegmatis* and that one or more mannose-containing molecules may play a role in regulating septation and cell division in these bacteria.

INTRODUCTION

Mycobacteria are the causative agents of a number of important human diseases, including tuberculosis (*Mycobacterium tuberculosis*) and leprosy (*M. leprae*). Of the 1.86 billion people that are thought to be infected with *M. tuberculosis*, 16 million have active disease, resulting in more than 2 million deaths per year [1]. A very limited number of antibiotics are effective against *M. tuberculosis*, and the incidence of drug-resistant isolates is increasing. Consequently, there is an urgent need for new anti-mycobacterial drugs.

All mycobacteria are surrounded by a compositionally unique cell wall that is essential for the survival of pathogenic species in the phagolysosomes of human macrophages. The mycobacterial cell wall comprises a core complex of covalently linked peptidoglycan, arabinogalactan polysaccharide and long-chain mycolic acids (for reviews, see [2–4]). Additional glyco- and phospho-lipids may be incorporated into this structure or form a distinct outer lipid layer that intercalates into the layer of mycolic acids. Mannose-containing glycolipids, including the phosphatidylinositol mannosides (PIMs) and their hypermannosylated derivatives, lipomannan (LM) and lipoarabinomannan (LAM) are a major class of cell-wall glycolipids in all mycobacteria (Scheme 1B) [5–7]. The PIMs, LM and LAM are thought both to be incorporated into the plasma membrane and to be exposed on the cell surface [8,9]. There is a large body of evidence to suggest that the PIMs and LAM can act as ligands for host-cell receptors and contribute to the pathogenesis of *M. tuberculosis* [5,9,10]. However, the presence of these glycolipids in saprophytic species of mycobacteria suggest that they may have more general, but as yet undefined, roles in cell-wall biogenesis and growth. This notion is supported by the finding that enzymes involved in inositol metabolism, phosphatidylinositol synthesis and PIM synthesis appear to be essential for the viability of the rapidly growing species *M. smegmatis* [11–13].

Pathogenic and non-pathogenic species of mycobacteria synthesize a second major class of mannose-containing glycoconjugates termed the methylmannose polysaccharides (MMPs). The MMPs are cytoplasmic polysaccharides comprising linear chains of α1-4-linked mannose and 3-*O*-methylmannose residues (Scheme 1C) [14]. These polysaccharides adopt a helical conformation with a hydrophobic core that may accommodate a single fatty acid [15]. *In vitro* studies suggest that the MMPs can enhance the activity of the fatty acid synthase [16,17] and thus be indirectly involved in plasma-membrane and/or cell-wall synthesis. However, a *M. smegmatis* mutant with very low levels of MMP only exhibited subtle changes in fatty acid composition, and very little is known about the function of MMPs *in vivo* [18]. Finally, a limited number of glycoproteins in pathogenic and non-pathogenic species of mycobacteria are modified with short, O-linked mannose chains [19,20]. The function of protein mannosylation in mycobacteria is also unclear.

In the present study we generated a strain of *M. smegmatis* that lacks phosphomannose isomerase (PMI), a key enzyme in mannose metabolism. PMI is encoded by the *manA* gene and catalyses the interconversion of fructose 6-phosphate and mannose 6-phosphate, which is the only pathway for the *de*

Abbreviations used: cfu, colony-forming units; DIG, digoxygenin; HPTLC, high-performance TLC; Kan, kanamycin; LAM, lipoarabinomannan; LB, Luria–Bertani; LM, lipomannan; MALDI-TOF, matrix-assisted laser-desorption–ionization time-of-flight; MGLP, methylglucose lipopolysaccharide; (m/p)MMP, (mature/precursor) methylmannose polysaccharide (note: MMP does not, in the present paper, stand for matrix metalloproteinase); ORF, open reading frame; PIM, phosphatidylinositol mannosides; (di)acylPIM*X*, PIM species with one or two ester-linked fatty acids on the first mannose residue (linked to C-2 of inositol) or the inositol headgroup of the diacyl phosphatidylinositol lipid moiety and a total of *X* mannose residues; PMI, phosphomannose isomerase; Str, streptomycin.

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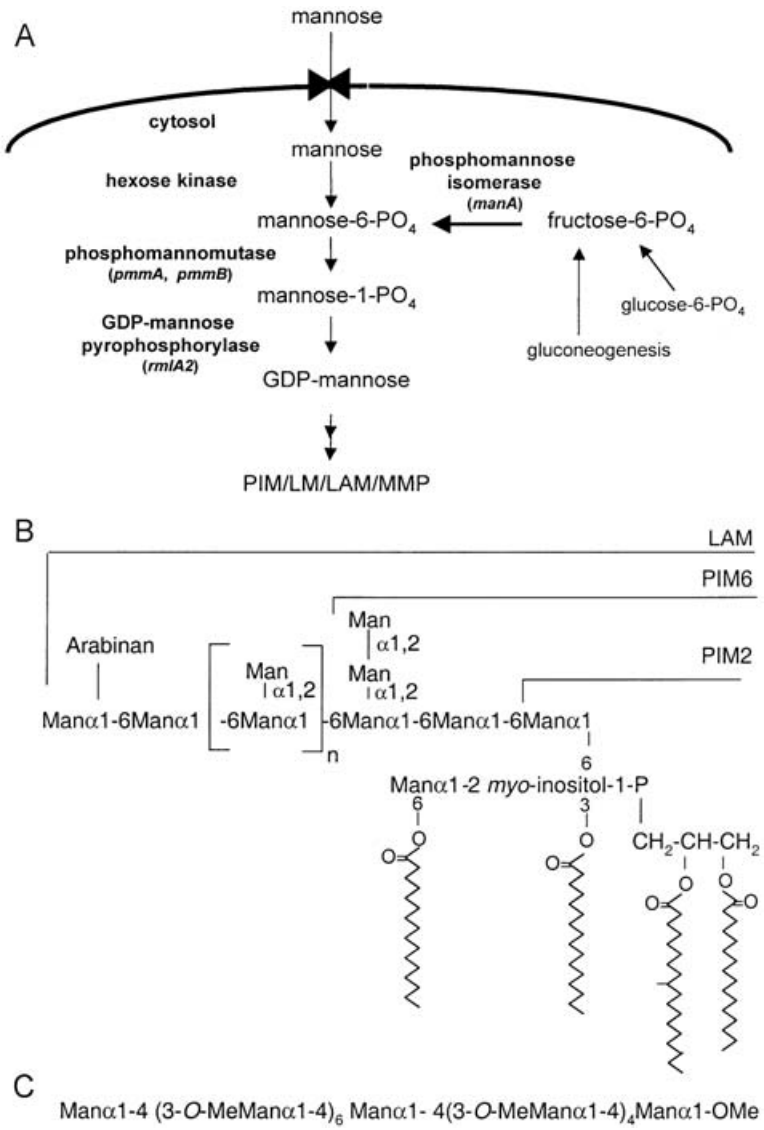
The sequence of the *manA* open reading frame from *M. smegmatis* mc^{2}155 genomic DNA has been submitted to the DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession number AF527174.



Scheme 1 Pathway of mannose biosynthesis and major mannoglycoconjugates in mycobacteria

(**A**) Proposed pathway of mannose biosynthesis in mycobacteria. Homologues for each step from *M. tuberculosis* H37Rv are included [39]. Abbreviations: -1-PO_4 and -6-PO_4, 1- and 6-phosphate. (**B**) Structure of PIMs/LAM. (**C**) Structure of MMP. Similar structures are present in both pathogenic and non-pathogenic (*M. smegmatis*) mycobacteria species.

novo synthesis of mannose (Scheme 1A). We hypothesized that the PMI deletion mutant should not be able to synthesize mannose-containing molecules in the absence of an exogenous source of mannose. Analysis of this mutant showed that mannose metabolism is essential for growth of *M. smegmatis* and suggested that mannose-containing molecules may have a role in regulating septation and cell division without perturbing other pathways of lipid biosynthesis.

EXPERIMENTAL

Bacterial strains and growth conditions

Escherichia coli XL-1 Blue MRF′ (Stratagene), *M. smegmatis* mc^2155 [21] and derivative strains were grown in Luria–Bertani (LB) medium at 37 °C. The MAN1 mutant was routinely cultivated in LB media containing 5 mM mannose. For biochemical studies, mycobacterial strains were grown in M9 minimal medium [22] with 0.05 % v/v Tween 80. Streptomycin (Str) and kanamycin (Kan) (20 μg/ml), ampicillin (100 μg/ml) and 10 % (w/v) sucrose were added as required. Cultures were also supplemented with 5 mM mannose where indicated.

Cloning and genetic manipulation

Cloning procedures were performed according to standard protocols [22]. Electrotransformation was performed using a Bio-Rad Gene Pulser, and *E. coli* cells were prepared for transformation according to the manufacturer's recommendations. *M. smegmatis* cells were prepared for electrotransformation as previously described [21]. Genomic DNA was extracted as previously described [23]. Probes for Southern blotting were labelled with digoxygenin (DIG)-labelled dNTPs using a Roche DIG labelling kit, and membranes were developed according to the

manufacturer's instructions. Restriction endonucleases and DNA-modification enzymes were obtained from Pharmacia.

Disruption of the *manA* gene in *M. smegmatis*

PCR was employed to amplify 1 kbp regions flanking the *manA* open reading frame (ORF) from *M. smegmatis* mc^{2}155 genomic DNA. The primer pairs A (5′-CCGGAATTCA-TGGCGGGGGCCCAGGTGCGGG-3′) and B (5′-GAATTGG-TTCGAGGTTAAACGACCCGGGCGGCGC-3′) were used for the upstream region, and C (5′-AAACGACCCGGGCGGCGCT-GCACCGCCACCGCAC-3′) and D (5′-CTAGTCTAGACGGT-GCTGCGCGCGGCGGGGTCGC-3′) were used for the down-stream region. The amplicons were annealed, relying on 6 bp of complementarity introduced with primers B and C, and used as a template for spliced overlap extension PCR using primers A and D. The resultant 2 kbp amplicon was cloned into pBluescript SK(+) using *Eco*RI and *Xba*I restriction-endonuclease recognition sites included in primers A and D, creating pJP3873. A Str-resistance gene [24] was cloned into pJP3873 at a unique *Sma*1 site, creating pJP3874. The resulting recombination cassette was subcloned from pJP3874 into p*KmobsacB* [24] using *Eco*RI and *Xba*I sites to create the homologous recombination construct pJP3875. This plasmid was electroporated into *M. smegmatis* mc^{2}155, and mutants that had undergone consecutive rounds of homologous recombination were finally selected on the basis of Str-resistance, growth in the presence of sucrose and Kan-sensitivity. A complementation vector was constructed after PCR amplifying the *manA* ORF from *M. smegmatis* mc^{2}155 genomic DNA using the primer pair E (5′-GCGGATCCAGTGCATCTGCTACGGGGA-3′) and F (5′-TTGGATCCACAAGAGCCTGGCCGCCAC-3′). These primers included *Bam*HI restriction-endonuclease recognition sites. The amplicon was cloned into the mycobacterial shuttle vector pMV261 [25] using the unique *Bam*HI site, creating the *manA* complementation vector pJP3876. The pJP3876 and pMV261 (control) plasmids were introduced into *M. smegmatis* mc^{2}155 and MAN1 by electrotransformation.

Monitoring growth/viability

Mycobacterial growth was followed by measuring colony-forming units (cfu), monitoring attenuance (D_{600}) of cultures or by measuring the total protein concentration [26]. Culture viability was assessed by adding 25 μM [5,6-^{3}H]uracil (0.8 μCi, 33.1 Ci/mmol; PerkinElmer Life Sciences) to a 1 ml aliquot of the culture. After a 10 min incubation, cells were washed twice with PBS, lysed with 1 M NaOH at 100 °C and radioactivity measured by liquid-scintillation counting after neutralization with 5 M HCl.

PMI assays

Cytosolic extracts of mycobacteria were obtained by sonication (MSE Soniprep 150 instrument; 10 × 30 s at 25 μ, 0 °C) in 50 mM Hepes/NaOH (pH 7.4), containing 2 mM EGTA, 2 μM leupeptin, 0.1 mM PMSF and 0.1 mM N^{α}-*p*-tosyl-L-lysylchloromethane ('N^{α}-*p*-tosyl-L-lysine chloromethyl ketone', 'TLCK'). Intact cells were removed by centrifugation (13 000 ***g***, 10 min) and PMI activity measured as described previously [27].

Metabolic labelling

M. smegmatis mc^{2}155 or MAN1 cells were grown to mid-exponential phase in M9 medium containing 5 mM mannose, washed in M9 media (minus mannose), then pulse-labelled with [2-^{3}H]mannose (50 μCi/5 ml; 17 Ci/mmol; ICN) in M9 medium for 30 min. Mycobacteria were harvested by centrifugation and equal aliquots suspended in M9 media (10 ml) with or without 5 mM mannose. Aliquots (1 ml) were removed at indicated time points for analysis. *M. smegmatis* mc^{2}155 and MAN1 were also continuously labelled for 6–20 h with *myo*-[2-^{3}H]inositol (25 μCi/5 ml; 17 Ci/mmol; Amersham Pharmacia), [1-^{14}C]acetate (50 μCi/5 ml; Amersham Pharmacia) or L-[*methyl*-^{3}H]-methionine (25 μCi/5 ml; PerkinElmer Life Sciences) after being preincubated in the presence or absence of mannose for 9 h. Uptake of L-[*methyl*-^{3}H]methionine required the addition of a mixture of MEM amino acids (Gibco) to the M9 medium (1 ml in 200 ml of medium) prior to labelling.

Preparation of PIM, LAM and MMP fractions

PBS-washed cell pellets were extracted twice with chloroform/methanol (2:1, v/v) then chloroform/methanol/water (1:2:0.8, by vol.). Water was added to the combined supernatants to give final chloroform/methanol/water proportions of 1:2:1.4 (by vol.) and the upper phase, containing both PIM and MMP, was dried under a stream of N_2 gas, resuspended in 0.1 M ammonium acetate containing 5 % (v/v) propan-1-ol and loaded on to a small column (500 μl) of octyl-Sepharose (Amersham Pharmacia) equilibrated in the same buffer. MMP was eluted in 0.1 M ammonium acetate containing 5 % propan-1-ol (2 ml), whereas PIM was eluted with 30, 40 and 50 % propan-1-ol (1 ml each). The bound fractions were pooled, dried, and combined with the chloroform/methanol/water (1:2:1.4, by vol.) organic lower phase for high-performance TLC (HPTLC) analysis (PIM fraction). The unbound fractions were treated with bovine alkaline phosphatase (Sigma) in 0.1 M NH_4HCO_3 (100 units, 37 °C, 2 h) then freeze-dried to remove buffer salts (MMP, low-molecular-mass metabolite fraction). LM/LAM was extracted from the delipidated pellet by four refluxes in 50 % (v/v) ethanol at 100 °C. The combined 50 %-ethanol extracts were dried under N_2 and the LM/LAM purified by octyl-Sepharose chromatography as described above (LM/LAM fraction). The monosaccharide composition of these fractions was determined by GLC–MS after hydrolysis in 2 M trifluoroacetic acid (100 °C, 2 h) and conversion of the released monosaccharides into their corresponding alditol acetates [28].

Analysis of PIM, LAM and MMP fractions

The degree of acylation of the major steady-state pools of *M. smegmatis* PIMs was determined using a matrix-assisted laser-desorption–ionization time-of-flight (MALDI-TOF) mass spectrometer. PIM-enriched fractions were suspended in 30 % (v/v) propan-1-ol, mixed with an equal volume of freshly prepared saturated α-cyano-4-hydroxycinnamic acid in 60 % propan-1-ol and analysed on a Voyager System 4180 MALDI-TOF mass spectrometer (PerkinElmer) in the linear negative-ion mode. The accelerating voltage was 20 000 V, the grid voltage applied was between 94 and 96 % and the guide wire voltage was 0.05 %.

Metabolically labelled PIMs were deacylated in methanolic 0.2 M NaOH for 1 h at 37 °C, and the reaction mixture neutralized with 1 M acetic acid. The samples were dried under a stream of N_2, partitioned between butan-1-ol (400 μl) and water (200 μl) and the deacylated phosphoglycans recovered in the lower aqueous phase. The aqueous fraction was dephosphorylated in ice-cold 48 % (w/v) HF (50 μl) for 48 h and neutralized with a saturated solution of LiOH. After removal of the LiF precipitate

by centrifugation, the supernatant was desalted on a mixed-bed column (400 μl) of AG50-X12 (H^+) over AG3 × 8 (OH^-) (Bio-Rad).

Metabolically labelled lipids were resolved on aluminium-backed silica-gel-60 HPTLC sheets (Merck) developed in chloroform/methanol/1 M ammonium acetate/13 M NH_3/water (180:140:9:9:23, by vol.). The ^{3}H-labelled MMPs were resolved on HPTLC sheets developed twice in the same dimension in butan-1-ol/ethanol/water (4:3:3, by vol.) [29]. The neutral glycan fractions were resolved by HPTLC by development in propan-1-ol/acetone/water (9:6:5, by vol.) [29]. Radiolabelled components were detected by fluorography after coating the HPTLC sheets with EA Wax (EA Biotech, Clarkston, Glasgow, Scotland, U.K.) and exposure to BioMax MR film (Kodak) at − 80 °C.

Electron microscopy

M. smegmatis mc^{2}155 or MAN1 cells were washed in PBS, fixed with glutaraldehyde (2.5 %) (ProSciTech, Thuringowa Central, Qld, Australia) and Ruthenium Red (0.05%) in PBS (2 h, 4 °C), then sequentially washed in dilutions of PBS (100, 66, 50, 33 and 0 %) in water. Bacteria were post-fixed with 1 % (w/v) OsO_4 and Ruthenium Red (0.05 %) in water (2 h, 4 °C), washed in water, then pelleted into agarose (1 % at 60 °C) and dehydrated in a graded acetone series over 20 h. After embedding in Spurrs resin [30], ultrathin sections were cut using a Leica Ultracut R microtome. Sections were stained with uranyl acetate (20 min) and Reynold's lead citrate stain (4 min) and observed using a Philips CM120 BioTWIN transmission electron microscope.

RESULTS

Disruption of *manA* in *M. smegmatis*

Possible homologues of the genes encoding PMI (*manA*), phosphomannomutase (*pmmA*, *pmmB*) and GDP-mannose pyrophosphorylase (*rmlA2*) occur in the genomes of *M. tuberculosis*, *M. leprae* and *M. smegmatis* (Scheme 1A), suggesting that the pathway of mannose metabolism in mycobacteria is similar to that found in all eukaryotes. The ORF of the *M. smegmatis manA* gene contained 1227 bp and encoded a predicted 408-amino-acid protein. Comparative analysis of the amino acid sequence of the putative PMI proteins from *M. smegmatis* with other homologues showed it to possess 74–76% sequence identity with the *M. tuberculosis* and *M. leprae* PMI enzymes and 24 % identity with human PMI.

As PMI is expected to be essential for the *de novo* synthesis of mannose, we hypothesized that targeted deletion of *manA* should convert the wild-type strain, mc^{2}155, into a mannose auxotroph. The *manA* deletion mutant was made using a recombination construct containing the Str-resistance gene (*Str*) flanked by 1 kbp of non-coding sequence from either side of the *manA* gene (Figure 1A). Genes for kanamycin resistance and levansucrase were included on the vector portion of the recombination construct. After two rounds of selection for single and double homologous recombination events, several viable colonies were recovered on mannose-containing plates. Southern-blot analysis of *Pst*1 digests confirmed that these colonies lacked a genomic copy of *manA* (Figure 1B). One of these, designated MAN1, was chosen for further analysis. MAN1 lacked detectable PMI enzymic activity (< 400 times that in mc^{2}155) (Figure 1C). However, complementation of both MAN1 and wild-type mc^{2}155 with a plasmid containing *M. smegmatis manA* under the control of a GroEL promoter, resulted in high levels of PMI activity

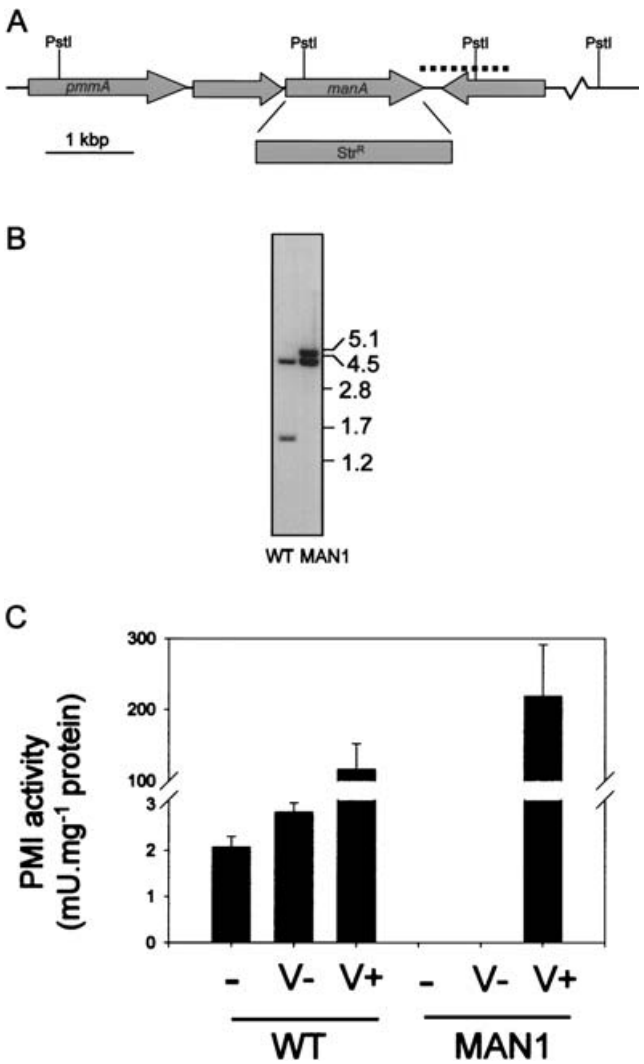


Figure 1 Identification and targeted deletion of the *M. smegmatis* PMI gene

(**A**) *Pst*I ('PstI') restriction map of the *M. smegmatis manA* locus. The broken line (▪▪▪▪) indicates complementary sequence used for a DIG-labelled probe for Southern blotting. The functions of the predicted genes immediately flanking *manA* are unknown. Str^R, Str-resistance gene. (**B**) Southern-blot analysis of *Pst*I-restriction-enzyme-digested *M. smegmatis* mc^{2}155 (wild-type, 'WT') and *M. smegmatis* Δ*manA* ('MAN1') genomic DNA. The sizes of DNA standards are indicated in kbp. (**C**) PMI activity in mc^{2}155 (wild-type, 'WT') or in MAN1 containing no plasmid (–), pMV261 vector control (V–) or pJP3876 complemented strains with intact *manA* under the control of a GroEL promoter (V +). mU is milliunits.

in both strains (Figure 1C). These studies showed that the *M. smegmatis manA* gene encoded an active PMI enzyme and that this gene has been successfully disrupted in MAN1.

Mannose is essential for the growth of MAN1 cells

The growth rate of the MAN1 strain was compared with wild-type mc^{2}155 in M9 medium with or without mannose. When stationary-phase MAN1 cells were inoculated into fresh medium containing 5 mM mannose, they grew at the same rate as wild-type mc^{2}155 (Figure 2A). However, in the absence of exogenous mannose, growth ceased after one or two cell divisions, as determined by measurements of D_{600} (Figure 2A), protein yield and cfu (results not shown). Growth of MAN1 in M9 medium required at least 5 μM exogenous mannose and was not

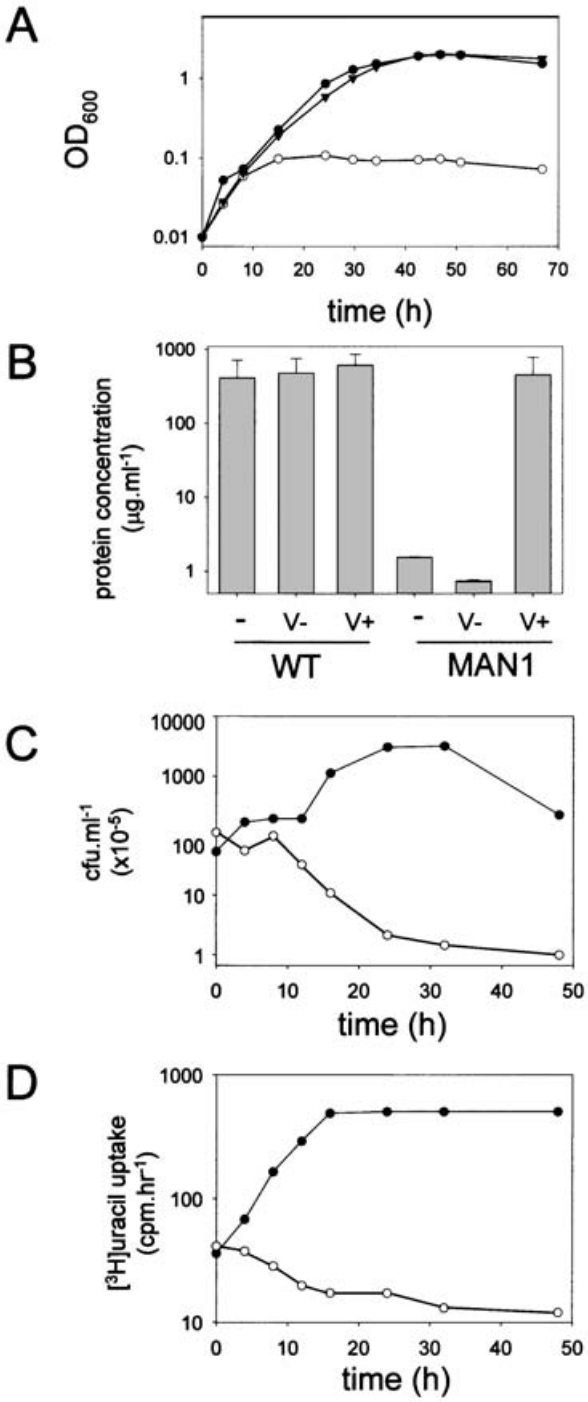


Figure 2 ***M. smegmatis*** **MAN1 is dependent on mannose for growth**

(**A**) Growth of MAN1 cells in M9 medium with (▼) or without (○) 5 mM mannose. Growth of mc^2155 grown in M9 medium minus mannose (●). 'OD_{600}' is D_{600}. (**B**) Protein yield from wild-type ('WT') and MAN1 cells containing no plasmid (–), vector control (V +) or complemented strain with intact *manA* (V +) after growth in M9 medium lacking mannose for 150 h. (**C** and **D**) Exponential-phase MAN1 cells were transferred into medium with (●) or without (○) mannose, and cell growth and viability measured at indicated time points by determining cfu (**C**) or rate of [5,6-^{3}H]uracil uptake (**D**).

inhibited by concentrations up to 50 mM (results not shown). Complementation of MAN1 with an episomal copy of *manA* restored prototrophy (Figure 2B) and wild-type growth rates (results not shown).

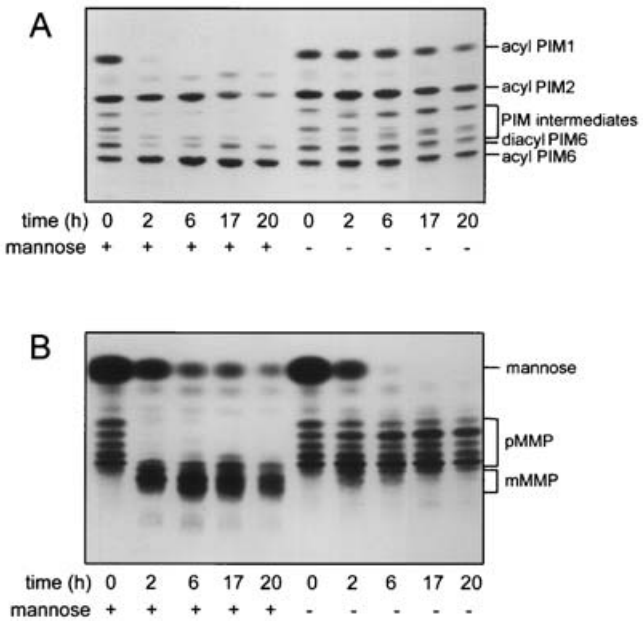


Figure 3 Synthesis of PIM and MMPs in mannose-starved MAN1

MAN1 cells were grown in the presence of 5 mM mannose, pulse-labelled with [2-^{3}H]mannose (30 min), then suspended in M9 medium with or without 5 mM mannose. Cell aliquots were removed at indicated time intervals and labelled PIMs (**A**) and MMPs (**B**) extracted and analysed by HPTLC. The PIM intermediates shown in (**A**) have been characterized as acyl-PIM3, acyl-PIM4 and acyl-PIM5. The ladder of bands that run above mature MMP (mMMP) in (**B**) are proposed to be pMMP.

We next examined whether MAN1cells remained viable during mannose starvation. MAN1 cells were grown to mid-exponential growth phase in M9 medium containing 5 mM mannose, than resuspended at the same cell density in fresh media with or without mannose. The MAN1 cells inoculated into mannose-containing media continued to grow, while bacteria in the medium lacking mannose did not divide and started to lose viability rapidly after 10 h (Figure 2C). The loss of viability was preceded by a decrease in metabolic activity, as indicated by uptake of [5, 6-^{3}H]uracil (Figure 2D). These results suggested that mannose metabolism was essential for both the growth and viability of rapidly dividing *M. smegmatis*.

Mannose glycoconjugate biosynthesis and catabolism in MAN1

We next examined the biosynthesis and catabolism of mannose-containing glycoconjugates in MAN1 grown in the presence and absence of mannose. MAN1 cells in mid-exponential growth phase were pulse-labelled with [2-^{3}H]mannose in M9 medium, then resuspended in fresh medium with or without exogenous mannose. As shown in Figure 3(A), [^{3}H]mannose was initially incorporated into a number of PIM precursor species which 'chased' into mature PIMs within 2 h when pulse-labelled cells were suspended in medium with 5 mM mannose. These mature PIM species were shown to be acyl and diacyl species of PIM2 and PIM6 by MALDI-TOF MS of steady-state pools (Figure 4A) and HPTLC analysis of both the native [^{3}H]mannose-labelled PIMs (Figure 4B) and [^{3}H]mannose-labelled glycan headgroups (Figure 4C). Similar analyses have shown that the PIM precursor species labelled in the initial pulse were acyl-PIM1, acyl-PIM3, acyl-PIM4 and acyl-PIM5 (results not shown). When [^{3}H]mannose-pulse-labelled MAN1 cells were suspended

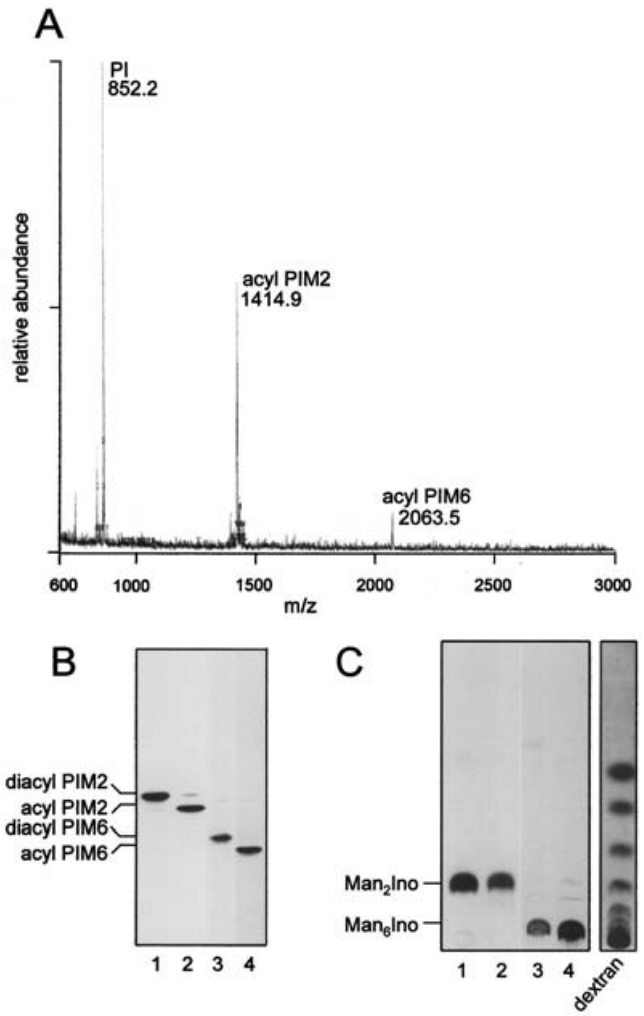


Figure 4 Characterization of *M. smegmatis* PIMs

(**A**) Partially purified PIMs were analysed by MALDI-TOF MS in the negative-ion mode, with data collected between *m/z* 600 and 3000. The major ions at *m/z* 852, 1415 and 2063 represent phosphatidylinositol, acyl-PIM2 and acyl-PIM6. (**B**) HPTLC purification of major PIM species labelled with [^{3}H]mannose in *M. smegmatis* mc^{2}155. The relative migration of authentic PIM species identified by MS is indicated along the left-hand side. (**C**) HPTLC profile of [^{3}H]mannose-labelled neutral glycans generated after deacylation and dephosphorylation of PIM species. 'Ino' means inositol.

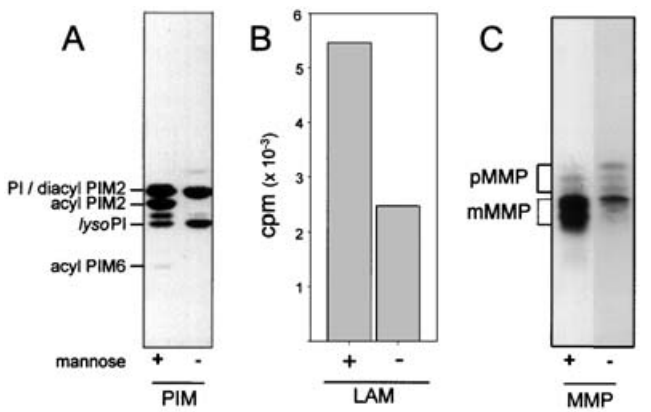


Figure 5 [^{3}H]Inositol and [*methyl*-^{3}H]methionine labelling of PIMs and MMPs in MAN1

MAN1 cells were cultured in the presence (+) or absence (–) of mannose for 10 h, then continuously labelled with [^{3}H]inositol for 10 h. (**A**) HPTLC analysis of total [^{3}H]inositol-labelled lipids. Labelled phosphatidylinositol ('PI') is only incorporated into PIM species when exogenous mannose is present in the medium. '*lyso*PI' is lysophosphatidylinositol. (**B**) Recovery of [^{3}H]inositol in the LM/LAM fraction. (**C**) MAN1 cells were cultured in the presence (+) or absence (–) of mannose for 10 h, then continuously labelled with [*methyl*-^{3}H]methionine for 10 h. MMPs were extracted, then analysed by HPTLC. Note that [^{3}H]methyl groups are incorporated into both mMMP and pMMP species.

in media without mannose, the ^{3}H label was not chased into mature end products (Figure 3A). Significantly, label in the earliest PIM species detected in these analyses, acyl-PIM1, was very inefficiently chased over 20 h (Figure 3A). These results showed that the conversion of precursor glycolipids into mature PIMs is effectively halted in the absence of exogenous mannose.

The flux of [^{3}H]mannose into mature MMPs was also halted in the absence of exogenous mannose. After a short pulse, the non-lipidic MMPs were resolved as a ladder of bands by HPTLC (Figure 3B). The bands with the slowest HPTLC mobility were shown by GLC–MS compositional analysis to contain mannose and 3-*O*-methylmannose and co-migrated with mature MMP (mMMP) (results not shown). The faster-migrating species are likely to be MMP precursors (pMMP) as ^{3}H label in these species rapidly chased into mMMP in the presence of exogenous mannose (Figure 3B). These species were also labelled with [*methyl*-^{3}H]methionine (Figure 5C), which is converted into *S*-adenosylmethionine and used to methylate mannose residues in the elongating MMP chains [31]. Significantly, [^{3}H]mannose label in the pMMP did not chase into mMMP when pulse-labelled cells were suspended in medium without mannose (Figure 3B), demonstrating that synthesis of the MMPs is also rapidly halted in the absence of exogenous mannose.

MAN1 were also continuously labelled with [^{3}H]inositol and [*methyl*-^{3}H]methionine to measure *de novo* synthesis of PIM/LAM and MMP respectively. Incorporation of [^{3}H]inositol into the PIMs, but not phosphatidylinositol and *lyso*-phosphatidylinositol species, was completely inhibited when MAN1 cells were preincubated for 9 h in media lacking exogenous mannose (Figure 5A). Incorporation of [*methyl*-^{3}H]methionine into MMP was also greatly reduced in the absence of exogenous mannose (Figure 5C). However, incorporation of [^{3}H]inositol into LAM was only reduced by $\approx$60 % in the absence of exogenous mannose (Figure 5B). The residual incorporation of [^{3}H]inositol in the absence of exogenous mannose may reflect the addition of inositol to the non-reducing termini of pre-existing pools of mature LAM [32]. Collectively, these results suggested that biosynthesis of all mannose-containing molecules is rapidly halted in the absence of an exogenous source of mannose. The pulse–chase labelling experiments also showed that none of these glycoconjugates are catabolized during mannose starvation.

MAN1 cells exhibit a hyperseptation phenotype

We next examined whether the growth phenotype of mannose-starved MAN1 was associated with changes in either cell-wall permeability or structure. Mannose-starved MAN1 cells did not exhibit increased sensitivity to the hydrophobic drugs, rifampicin or novobiocin, compared with mannose-replete MAN1 or mc^{2}155, indicating that cell-wall permeability had not changed dramatically (results not shown) [33]. Furthermore, there was no evidence for general perturbation of cell-wall structure in thin sections of mannose-starved MAN1 (Figures 6A and 6B). However, MAN1 cells were significantly shorter than wild-type

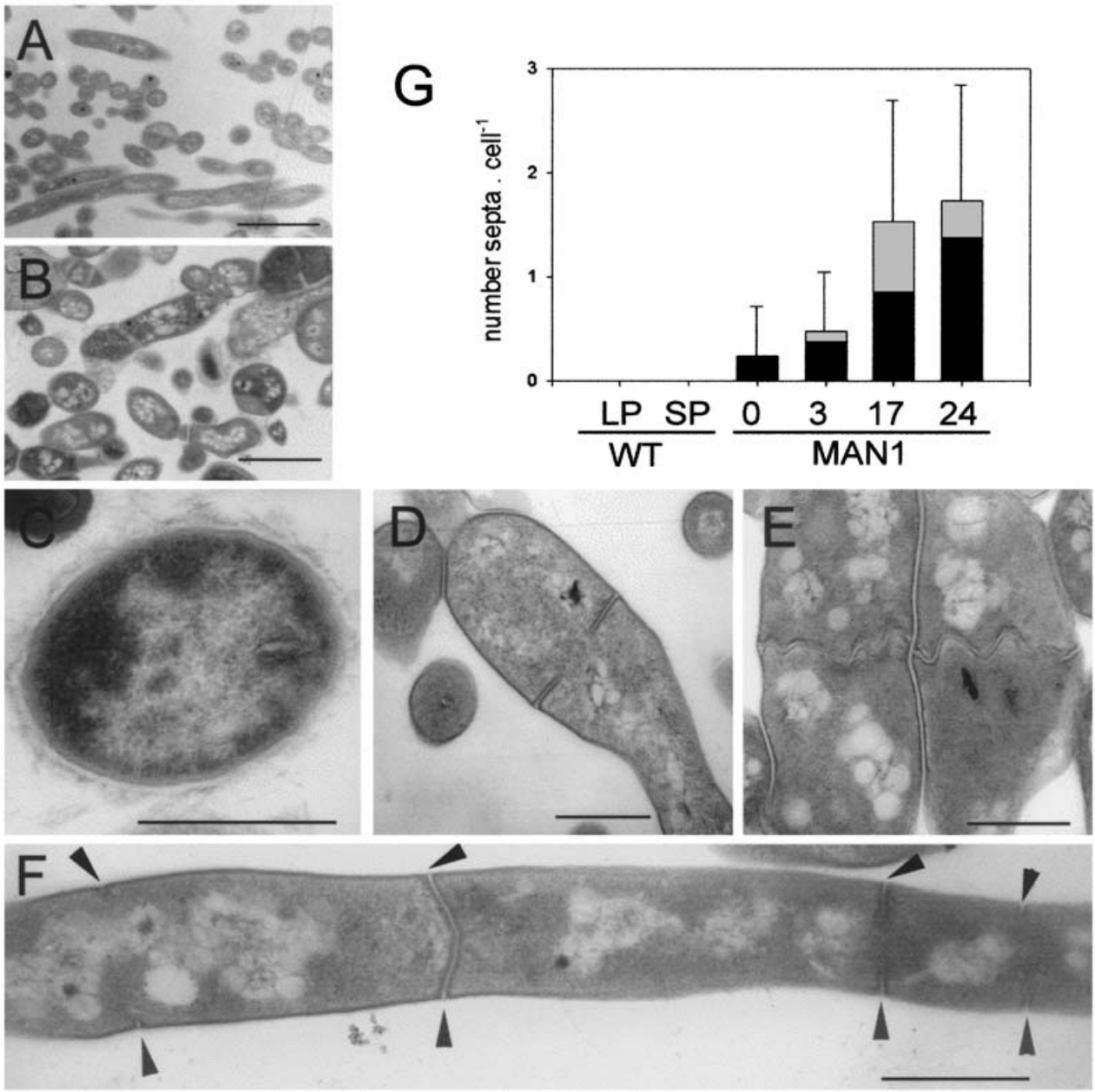


Figure 6 Ultrastructure of wild-type and mannose-starved MAN1

Mycobacteria were cultured in mannose-free M9 medium for 24 h, before being fixed for electron microscopy. (**A**) mc^{2}155. (**B**) MAN1. (**C–F**) Higher magnification of mannose-starved MAN1 showing shedding of extracellular material (**C**) and the appearance of multiple septa (arrows) that are often incomplete or distorted (**D–F**). (**G**) The number of septa in exponential-phase ['log phase' ('LP')] or stationary-phase ('SP') mc^{2}155 or MAN1 cells after incubation in mannose-free media for 0, 3, 17 or 24 h. Septa were counted in longitudinal cell profiles (>2 μm in length) in electron micrographs. The black and grey portions of the bar represent the number of complete and incomplete septa present respectively (means $\pm$ S.D. for 50 cells). Scale bars in (**A**) and (**B**) represent 2 μm; those in (**C**)–(**F**) represent 500 nm.

M. smegmatis cells [4.73 $\pm$ 1.40 μm (mean $\pm$ S.D) versus 7.43 $\pm$ 1.76 μm] and exhibited a mild hyperseptation phenotype in the presence of mannose which was exacerbated in the absence of exogenous mannose (Figures 6C–6G). In the presence of mannose, approximately a quarter of MAN1 cells (24/100 cells) contained a septum, compared with < 1 % of wild-type mc^{2}155 (Figure 6G) cells. An increase in the number of complete or incomplete septa was evident after exponential-phase MAN1 cells were suspended in medium lacking mannose for 3 h, and nearly all cells contained one or more septa after 17 h (174 septa/100 cells) (Figure 6G). The septa in these cells were often incomplete and/or were distorted (Figures 6E and 6F). Mannose-starved MAN1 also released large amounts of extracellular material that accumulated in the interstitial spaces of cell clumps (Figure 6C). This material was not detected in MAN1 grown in the presence of mannose or in wild-type cells (results not shown). As the hyperseptation phenotype was evident when cells were grown in the presence of mannose, and was rapidly exacerbated in the absence of exogenous mannose, it is likely to be an early consequence of perturbations in mannose metabolism.

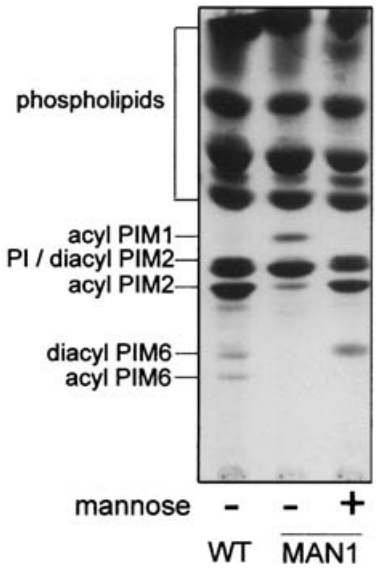


Figure 7 Phospholipid biosynthesis is not perturbed in mannose-starved MAN1

MAN1 cells were grown in medium with or without mannose for 10 h, then continuously labelled with [^{14}C]acetate for 20 h. Total lipids recovered in the lower chloroform phase of the biphasic chloroform/methanol/water (1:2:1.4, by vol.) mixture were analysed by HPTLC. This fraction is depleted of polar PIM6 species, which partition into the upper aqueous phase, but contains apolar PIM2 species.

Inhibition of mannoglycoconjugate biosynthesis does not affect fatty acid synthesis

A similar hyperseptation phenotype can be induced by treating mycobacteria with inhibitors of fatty acid biosynthesis [34]. Given that cytoplasmic MMPs are thought to regulate the activity of fatty acid synthases [16,17], we investigated whether fatty acid biosynthesis was affected in mannose-starved MAN1. As shown in Figure 5, the biosynthesis of phosphatidylinositol was not affected in mannose-starved MAN1. To extend this observation, MAN1 cells were incubated in the presence or absence of mannose for 10 h, then continuously labelled with [^{14}C]acetate. As shown in Figure 7, the *de novo* synthesis of the major phospholipid classes was quantitatively and qualitatively similar in the presence or absence of mannose. In contrast, the synthesis of the major PIM species, acyl-PIM2, was greatly decreased in mannose-starved MAN1 (Figure 7, second lane). This result suggests that perturbation of mannose metabolism does not result in gross changes in fatty acid biosynthesis.

DISCUSSION

Mannose is a key component in several cell-wall and intracellular molecules in mycobacteria, including an abundant family of mannolipids (PIM/LM/LAMs), a cytoplasmic MMP, and a limited number of O-mannosylated glycoproteins. These molecules are synthesized by both pathogenic and non-pathogenic species of mycobacteria, raising the possibility that they fulfil important, but as yet undefined, 'housekeeping' functions in these organisms. In the present study we have generated a *M. smegmatis* mannose auxotroph by targeted deletion of the *manA* gene encoding PMI. When grown in the presence of an exogenous supply of mannose, the MAN1 cells grew at the same rate as wild-type *M. smegmatis*, but were significantly shorter than wild-type cells and displayed a mild hyperseptation phenotype. When suspended in medium lacking mannose, this hyperseptation phenotype became more pronounced and cell division ceased. These changes preceded the exponential loss of viability that occurred after 10 h in mannose-free medium. Disruption of mannose metabolism thus appears to have an early effect on processes involved in septation and cell division and subsequently leads to loss of viability.

The phenotype of the MAN1 mutant is likely to be due to perturbations in the rate of synthesis of mannose-containing molecules. Specifically, the processing of prelabelled PIMs and MMP intermediates was immediately halted when exponential-phase MAN1 cells were suspended in mannose-free medium, suggesting that intracellular pools of mannose precursors (mannose 6-phosphate, GDP-mannose) are rapidly depleted under these conditions. In contrast, stationary-phase cells underwent one or two cell doublings when suspended in mannose-free media (as shown by increase in both cfu and cellular protein) before cell division ceased (Figure 2A). Stationary-phase cells may contain higher steady-state levels of either the mannose precursors and/or mannose-containing glycoconjugates to allow a limited number of cell divisions in the absence of ongoing mannose metabolism. Interestingly, these analyses showed that there was very little turnover of PIM or MMP precursors or end products during the 20 h mannose starvation period. A similar lack of turnover of metabolically labelled intermediates was observed if MAN1 or wild-type cells were suspended in media lacking a carbon source (J. H. Patterson and M. J. McConville, unpublished work). These results are consistent with the notion that these very abundant cellular components are metabolic end products and do not have a significant reserve function.

Our finding that mannose metabolism is essential for growth of *M. smegmatis* is supported by the studies of Jackson and colleagues [12,13], who recently showed that *pgsA* and *pimA* (encoding phosphatidylinositol synthase and the first mannosyltransferase in PIM biosynthesis respectively), are also required for growth and viability of *M. smegmatis*. The loss of viability of MAN1 cells in the absence of exogenous mannose is thus likely to be due, at least partly, to the cessation of PIM biosynthesis. While the precise role of the PIMs and LAMs in mycobacterial cell-wall biogenesis and growth remain unclear, it is possible that they are essential bulk components of the cell wall and/or that they modulate specific processes in plasma-membrane and cell-wall compartments. Our current analyses suggest that the growth defect in mannose-starved MAN1 is not due to gross changes in the steady-state pools of PIM or LAM, or the rate of synthesis of other cell-wall and plasma-membrane phospholipids. The permeability of the mannose-starved MAN1 was also unchanged, as indicated by the sensitivity of the mutant to the hydrophobic drugs rifampicin and novobiocin. In contrast, *M. smegmatis* mutants with defects in arabinogalactan biosynthesis displayed significantly increased sensitivity to these drugs [33]. While these results do not preclude the possibility that the PIMs and LAM are important bulk plasma-membrane or cell-wall components, they raise the possibility that one or more of these glycolipids may have a more direct role in regulating processes involved in cell division or cell viability.

The phenotype of the mannose auxotroph could also reflect changes in the steady-state levels or rate of biosynthesis of other mannose-containing molecules, such as the intracellular pools of MMP [16,17] or O-mannosylated glycoproteins [19,35]. In this respect we have recently found that selective disruption of PIM biosynthesis in an *M. smegmatis* inositol auxotroph, in the presence of ongoing MMP biosynthesis and protein glycosylation, does not result in the hyperseptation phenotype seen in the MAN1 mutant (R. Haites, Y. Morita, J. H. Patterson, M. J. McConville and H. Billman-Jacobe, unpublished work). The MMPs [and the

structurally related methylglucose lipopolysaccharide (MGLP)] have been shown to sequester fatty acyl-CoAs in *in vitro* assays and could directly or indirectly affect the properties of the plasma membrane and cell wall by regulating fatty acid synthesis [16,17]. However, depletion of cellular levels of both MMP and MGLP in *M. smegmatis* (to 7 and 50 % of wild-type levels respectively) had no effect on growth, and only resulted in subtle changes in fatty acid composition [18]. Similarly, gross changes in the synthesis of cell-wall or plasma-membrane phospholipids were not observed when MMP synthesis was inhibited in MAN1. Furthermore, the growth phenotype of mannose-starved MAN1 was not reversed by supplementation of the culture media with fatty acids to by-pass any defects in fatty acid biosynthesis (results not shown). It is possible that pre-existing pools of MMPs are sufficient to sustain normal rates of fatty acid synthesis in mannose-starved MAN1 cells and/or that the MGLPs compensate for any decrease in MMP levels.

These studies show that mannose metabolism is essential for the growth and viability of *M. smegmatis*, suggesting that enzymes involved in the synthesis of mannose donors, or downstream mannosyltransferases, may be potential drug targets in pathogenic species of mycobacteria. In this respect it is notable that four mycobacterial mannosyltransferases that catalyse early steps in PIM biosynthesis share little homology with the mannosyltransferases of animal cells [13,36–38]. The mannose auxotroph characterized in the present study will provide an useful cell line for further investigating the specific functions of mannose-containing glycoconjugates in cell-wall assembly and cell division.

Preliminary sequence data were obtained from The Institute for Genomic Research website at http://www.tigr.org. Sequencing of *M. smegmatis* mc^{2}155 was accomplished with support from the National Institute of Allergy and Infectious Diseases (NIAID), Bethesda, MD, U.S.A. We thank Dr Y. Morita (Department of Biochemistry and Molecular Biology, University of Melbourne, Parkville, Victoria, Australia) for critical reading of the manuscript. This work was supported by the Australian National Health and Medical Research Council, the United Nations Development Programme (UNDP)/World Bank/World Health Organization (WHO) Special Programme for Research and Training in Tropical Diseases, and a Wellcome Trust major equipment grant for the GLC–MS and MALDI-TOF MS. M.J.M. is a Howard Hughes International Scholar.

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