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A *Mycobacterium smegmatis* Mutant with a Defective Inositol Monophosphate Phosphatase Gene Homolog Has Altered Cell Envelope Permeability

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A bacteriophage infection mutant (strain LIMP7) of *Mycobacterium smegmatis* was isolated following transposon mutagenesis. The mutant showed an unusual phenotype, in that all phages tested produced larger plaques on this strain compared to the parent strain. Other phenotypic characteristics of the mutant were slower growth, increased clumping in liquid culture, increased resistance to chloramphenicol and erythromycin, and increased sensitivity to isoniazid and several β-lactam antibiotics. Permeability studies showed decreases in the accumulation of lipophilic molecules (norfloxacin and chenodeoxycholate) and a small increase with hydrophilic molecules (cephaloridine); taken together, these characteristics indicate an altered cell envelope. The DNA adjacent to the transposon in LIMP7 was cloned and was shown to be highly similar to genes encoding bacterial and mammalian inositol monophosphate phosphatases. Inositol is important in mycobacteria as a component of the major thiol mycothiol and also in the cell wall, with phosphatidylinositol anchoring lipoarabinomannan (LAM) in the cell envelope. In LIMP7, levels of phosphatidylinositol dimannoside, the precursor of LAM, were less than half of those in the wild-type strain, confirming that the mutation had affected the synthesis of inositol-containing molecules. The *impA* gene is located within the histidine biosynthesis operon in both *M. smegmatis* and *Mycobacterium tuberculosis*, lying between the *hisA* and *hisF* genes.

The mycobacteria include species which are major human (*Mycobacterium tuberculosis* and *Mycobacterium leprae*) and animal (*Mycobacterium bovis* and *Mycobacterium paratuberculosis*) pathogens. Many of the characteristics of these bacteria seem to stem from the properties of their unique cell wall, a complex structure containing long-chain fatty acid molecules, the mycolic acids, that are covalently attached to the peptidoglycan by an arabinogalactan polymer. Species-specific glycolipids and peptidoglycolipids surround this polymer, forming the outer leaflet of a lipid bilayer (5). In addition, lipoarabinomannan (LAM) molecules are present in the envelope, although it is not certain in which part of the wall the lipid anchor lies. The cell envelope is a highly effective permeability barrier (36) which may be important for survival of pathogenic species inside the host environment.

We have been studying the structure and biosynthesis of the mycobacterial cell wall using the genetic approach of isolating mycobacteriophage infection mutants. When a phage attaches to specific cell surface receptors, then disruption of genes encoding enzymes involved in the biosynthesis of these receptors will cause resistance to infection by that phage. A large number of mycobacteriophages have been isolated, including many for *M. tuberculosis* strain typing (30), and there has been some success in identifying phage receptors. The receptor of phage D4 is thought to be an apolar glycopeptidolipid, probably involving an interaction with a methylated rhamnose (12), and there is evidence that D29 and DS6A adsorb to lipid components (8). More recently, it has been shown that D29 probably binds to a glycolipid, since *Mycobacterium smegmatis* mutants resistant to D29 infection contain altered pyruvylated, glycosylated acyltrehaloses (6).

Following the development of an efficient transposon mutagenesis system for the fast-growing nonpathogenic species *M. smegmatis* (16), we screened a bank of mutants for strains resistant to a variety of phages. During this process, we isolated a mutant on which all phages tested produced plaques larger than those on the wild-type strain. In this paper, we have investigated the phenotype of this mutant and identified the genetic lesion.

MATERIALS AND METHODS

Bacterial strains, bacteriophages, and plasmids. The bacteria, mycobacteriophages, and plasmids used in this study are listed in Table 1. *M. smegmatis* mc^2155 was grown in Lemco broth (5 g of Lab Lemco powder per liter, 5 g of Bacto Peptone per liter, 5 g of NaCl per liter, 0.05% Tween 80) or in 7H9 broth supplemented with 10% OADC (Difco, West Molesey, Sussex, United Kingdom) and 0.05% Tween 80. For phage infection, Tween 80 was omitted. For agar plates, Bacto Agar at 15 g/liter was added; for soft agar, Bacto Agar at 7.5 g/liter was added. *Escherichia coli* DH5α was grown in Luria-Bertani medium (Difco) with Bacto Agar (15 g/liter) added for plates. LIMP7 was maintained at more than 39°C at all times.

Phage infection assays. Totals of 100 to 300 PFU of phage were added to 200 μl of an overnight culture, mixed with 3 ml of soft top, and plated onto agar plates, and the plates were incubated for 2 days at 37°C.

Phage adsorption assays. *M. smegmatis* cultures were grown to an optical density at 600 nm (OD_{600}) of between 0.4 and 0.6 and then diluted to an OD_{600} of 0.1. A 900-μl volume of culture (10^7 cells) was added to 0.1 μl of phage suspension (10^6 PFU), and 2 μl of 1 M $CaCl_2$ was added. Suspensions were incubated at room temperature for 0 to 1 h and then centrifuged for 5 min to pellet bacteria. Supernatants were recovered and assayed for free phages in duplicate.

Phage growth assay. Overnight cultures of *M. smegmatis* were diluted 1/20 and grown to an OD_{600} of 0.1 (10^7 cells/ml). A total of 2×10^6 PFU of phage D56 was added to 1 ml of bacteria and allowed to adsorb for 20 min at room

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TABLE 1. Bacteria, bacteriophages, and plasmids used in this study

Strain, phage, or plasmid	Property	Reference or source[a]
Bacterial strains		
M. smegmatis mc^{2}155	Efficient transformation mutant	34
LIMP7 (*impA1*::Tn*611*)	Large-plaque mutant of mc^{2}155	This study
E. coli DH5α		Life Technologies, Paisley, United Kingdom
Mycobacteriophages		
AG1	Each mycobacteriophage used in this study forms larger plaques on LIMP7	John Grange
Battaglini		John Grange
C3		John Grange
D4		Jan Van Embden
D29		John Grange
D33		John Grange
D56		John Grange
DNA III 8		John Grange
GS4		Jan Van Embden
Legendre		John Grange
Minetti		John Grange
MX2		John Grange
Sedge		John Grange
Watson		John Grange
Plasmids		
pCG79	Temperature-sensitive Tn*611* delivery vector	16
pUC18		28
pROSE13	*Eco*RI-religated chromosomal DNA from LIMP7; Sm^r	This study
pIMP13	196-bp *Eco*RI-*Hin*dIII *impA* fragment from pROSE13 in pUC18	This study
pIMP14	Clone from a *Sau*3A partial digest library in pBluescriptII, with an insert of approximately 2 kb	This study

[a] John Grange, National Heart and Lung Institute, London, United Kingdom; Jan Van Embden, National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands.

temperature. The cells were pelleted and resuspended in 20 ml of 7H9 broth supplemented with OADC and 1 mM $CaCl_2$, and the suspensions were incubated at 37°C with shaking. A 1-ml volume was removed every 20 min, the cells were pelleted, and the supernatant was assayed for free phages.

DNA manipulations. Most DNA manipulation techniques are described by Sambrook et al. (32). Restriction enzymes, T4 DNA ligase, and *Taq* polymerase, together with enzyme buffers, were purchased from Promega, Ltd. (Southampton, United Kingdom), and used according to the manufacturer's instructions.

Recovery of the transposon from the chromosome. A 5-μg amount of genomic DNA from LIMP7 was digested with *Eco*RI and self-ligated overnight. Ligations were used to transform *E. coli* DH5α. Transformants were selected with 50 μg of streptomycin per ml, and plasmids were isolated with the Wizard minipreparation kit (Promega). Plasmids were analyzed by digestion with *Eco*RI and run on an 0.8% agarose gel. One plasmid was selected for further analysis and named pROSE13 (see Fig. 3). The 200-bp *Eco*RI-*Hin*dIII fragment was subcloned into pUC18 to create pIMP13 for DNA sequencing and labelling of probes.

DNA sequencing. Initially, pROSE13 was sequenced with primers ITCH4 (5′ GCA TGC TCA AGC TTC ACG 3′) and ITCH5 (5′ CCG TCA TCG TTC CGT CCG TCC 3′) generated to IS*6100* (16). pIMP13 was sequenced in both directions with M13 universal forward and reverse sequencing primers (Pharmacia Biotech, St. Albans, Herts, United Kingdom). pIMP14 was sequenced at both ends to ensure that it contained the entire *impA* gene, and 1,120 bp was sequenced in both directions, with a combination of plasmid deletions made by restriction digestion and religation and primer walking. Sequencing reactions were performed with an ABI PRISM cycle-sequencing kit (Perkin Elmer, Warrington, Cheshire, United Kingdom). The *M. smegmatis impA* sequence was translated and aligned with related sequences by using the Pileup program within the Genetics Computer Group (University of Wisconsin) software (11) at the Human Genome Mapping Project computing facility at Hinxton, United Kingdom (31). Database searches were carried out with the BEAUTY program (38) at the BCM Search Launcher Web site (http://kiwi.imgen.bcm.tmc.edu:8088/search-launcher/launcher.html) and with the BLAST program (2) on sequence data produced at the Sanger Centre, Hinxton, United Kingdom (http://www.sanger.ac.uk/pathogens/TB_blast_server.html), and are available in MycDB (4) (http://www.biochem.kth.se/MycDB/seqsearch_form.html).

Antibiotic sensitivity testing. A total of 5×10^5 cells were inoculated on 7H11 agar containing serial twofold dilutions of antibiotics. The results were read after 48 h of incubation at 37°C. For isoniazid (INH), cultures were grown to an OD_{600} of 0.6 to 0.8, diluted to an OD of 0.1 (10^7 cells/ml), and plated at appropriate dilutions onto Lemco agar containing INH.

Permeability assays. Norfloxacin and chenodeoxycholate accumulation was measured as described previously (22). The external concentration of norfloxacin was 25 μM, and the experiment was carried out at 23°C. The external concentration of chenodeoxycholate was 5 μM, and the experiment was carried out at 40°C. Cell wall permeability to β-lactam antibiotics was assayed by the method of Chambers et al. (10). [^{14}C]norfloxacin (specificity, 14.8 mCi/mmol) was a gift from Merck and Co. [^{14}C]chenodeoxycholate (specificity, 50 mCi/mmol) was obtained from NEN/DuPont.

TLC analysis. The extractable lipids were obtained by chloroform-methanol extraction of purified cell walls (10 mg [dry weight]) (15). These extracts were further deacylated in alkali (9). The glycerophosphate derivatives, recovered in the aqueous phase, were separated by thin-layer chromatography (TLC) on Kieselgel 60 silica gel plates (Merck). Two developing solvents were used, i.e., either isobutanol-pyridine-acetic acid-water (5:5:1:3) or 2-propanol-concentrated ammonia (2:1). Deacylated phosphatidylinositol dimmanoside (PIM_2), which was kindly provided by C. E. Ballou, was used as a standard.

Nucleotide sequence accession number. The sequence of *impA* has been deposited in the EMBL, GenBank, and DDJB Nucleotide Sequence Data Libraries under accession no. AF005905.

RESULTS

Transposon mutagenesis of *M. smegmatis*. *M. smegmatis* mc^{2}155 was mutagenized with Tn*611* by using a thermosensitive plasmid delivery system as previously described (16). During screening for phage-resistant mutants, a mutant, LIMP7, was identified that was remarkable in that all phages tested produced plaques larger than those on the parent strain (not shown). Therefore, we characterized the phenotype of this strain further.

Growth rate of *M. smegmatis* LIMP7. The mutant showed an increased tendency to clump in liquid culture and grew slightly more slowly than the wild-type strain (not shown). It also formed smaller colonies. One explanation for a large-plaque phenotype could be a lower growth rate, such that the lawn of plating cells took longer to grow to stationary phase, allowing

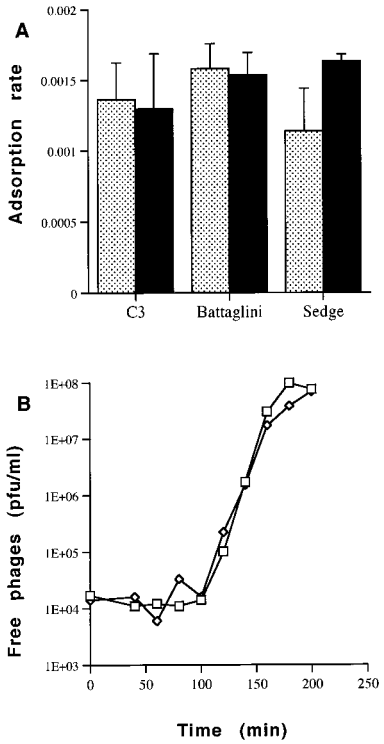

FIG. 1. (A) Adsorption of phages C3, Battaglini, and Sedge to wild-type *M. smegmatis* mc^{2}155 and LIMP7. Adsorption was carried out as described in Materials and Methods; results are expressed as numbers of phages adsorbed per CFU per minute. Stippled bars, mc^{2}155; filled bars, LIMP7. Lines above the bars indicate standard errors. (B) Time course of production of phage D56. *M. smegmatis* mc^{2}155 (squares) and the LIMP7 mutant (diamonds) were infected with D56, and free phages were assayed.

more time for phage growth. Alternatively, the cells might have continued growing for a longer period, due to altered sensing of the growth conditions. However, neither slow growth nor altered environmental sensing would explain the fact that differences in plaque size were visible after 1 day of incubation, while plaque development continued for at least 2 days.

Phage adsorption and growth. Another explanation for a general improvement in phage growth could be an improved adsorption of the phages. This was tested with three phages, Battaglini, C3, and Sedge. Phage adsorption was quantified by assaying for free phages remaining after 1 h of incubation with bacterial cultures. There were no differences observed for any of the phages tested between the wild type and the mutant (Fig. 1A). Phage Legendre was also assayed in a 30-min time course experiment, and, again, no differences were observed for the rate of adsorption (not shown). Thus, it seems unlikely that adsorption is responsible for the phenotype, although subtle differences cannot be ruled out. The multiplication and growth of phages were also assayed over time; again, there were no appreciable differences in either the latent period or the numbers of phage progeny obtained with several phages; an example is shown in Fig. 1B.

Antibiotic sensitivity. Alterations to the cell surface could also result in differences in the antibiotic sensitivity profile. The MICs of several antibiotics against LIMP7 were therefore measured, and the results are summarized in Table 2. Compared with parent strain mc^{2}155, LIMP7 showed slightly more resistance to chloramphenicol and erythromycin, both of which are hydrophobic molecules. In contrast, LIMP7 was more sensitive to several β-lactams, including ampicillin, cephaloridine, and cloxacillin, although the permeability of LIMP7 to cephaloridine was only slightly increased, from 1.2×10^{-6} cm/s for mc^{2}155 to 2.0×10^{-6} cm/s for LIMP7. When sensitivity to INH was assessed, there was no difference in MICs between the wild type and mutant as determined by colony number, but a qualitative difference was observed, in that colonies obtained from LIMP7 plated on 1 to 2 μg of INH per ml were significantly smaller (pinprick colonies) than those plated on 0 to 0.8 μg/ml, whereas the wild-type colonies were of a normal size at both concentrations. When higher concentrations of INH were used (5 to 10 μg/ml), a decrease in the colony size of the wild type could be seen (results not shown). Thus, LIMP7 is also more sensitive to INH.

TABLE 2. Antibiotic sensitivities of strain LIMP7

Antibiotic	MIC (μg/ml) for:	
	LIMP7	Wild type
Chloramphenicol	16	8–16
Erythromycin	16–32	8–16
Cloxacillin	128	256
Cephaloridine	<32	>128
Ampicillin	64	>128

Accumulation of norfloxacin and chenodeoxycholate. To determine the effect of the mutation on cell wall permeability, intact cells of LIMP7 were assayed for accumulation of two moderately lipophilic molecules, norfloxacin and chenodeoxycholate. The results are shown in Fig. 2. Accumulation of norfloxacin by LIMP7 was slightly lower than that in the parent

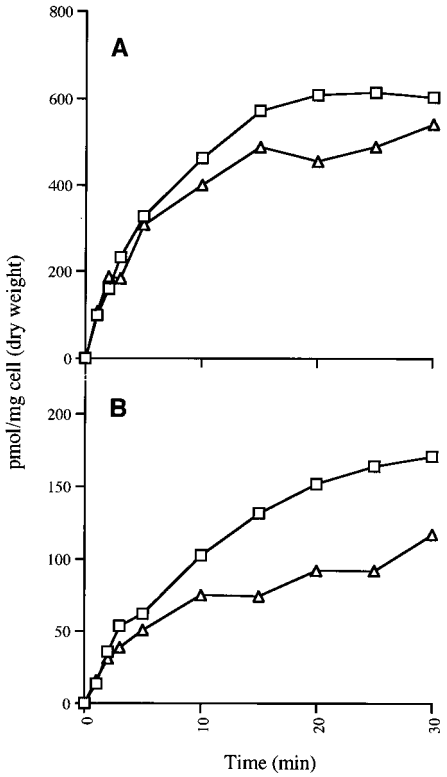

FIG. 2. Permeability assays. The accumulation of norfloxacin (A) and chenodeoxycholate (B) in *M. smegmatis* strains was measured. Squares, mc^{2}155; triangles, LIMP7.

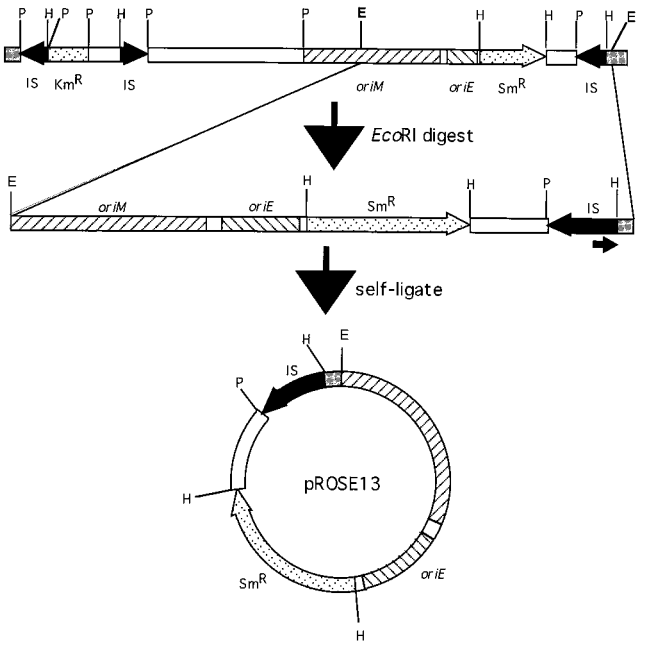

FIG. 3. Recovery of the transposon from LIMP7. The diagram at the top shows the arrangement of the transposon within the LIMP7 mutant chromosome. Transposition results in duplication of one of the copies of IS*6100*, resulting in three copies being present. In the LIMP mutant, the left-hand IS has been duplicated during transposition. Genomic DNA was digested with *Eco*RI, producing the fragment indicated (middle diagram), which carries both an *E. coli* replicon and an Sm^r gene. The digested DNA was religated and used to transform *E. coli*, resulting in the isolation of the plasmid pROSE13 (bottom diagram) containing 196 bp of *M. smegmatis* genomic DNA. The rightward arrow underneath the IS in the middle diagram shows the location of the primer used for sequence analysis. Restriction sites: E, *Eco*RI; H, *Hin*dIII; P, *Pst*I. IS, IS*6100*; Km^r, kanamycin resistance; *oriM*, mycobacterial origin of replication (temperature sensitive); *oriE*, *E. coli* origin of replication; Sm^r, streptomycin resistance.

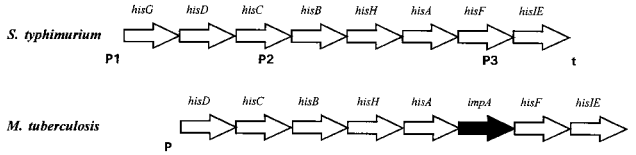

FIG. 4. Arrangement of *his* genes in *S. typhimurium* and *M. tuberculosis*. The *S. typhimurium* data are taken from Alifano et al. (1); the *M. tuberculosis* data are from the MycDB database (4). P, P1, P2, and P3, promoters; t, transcriptional terminator.

strain, mc^{2}155. The difference is more profound in the case of chenodeoxycholate, a more hydrophobic molecule than norfloxacin, with an approximately 50% decrease in the entry rate and level of accumulation compared with those of the parent strain.

Identification of the genetic lesion. In order to identify the gene which had been inactivated by the transposition event, we carried out experiments to subclone DNA adjacent to Tn*611*. When Tn*611* transposes, the entire plasmid becomes integrated, such that the resulting insert contains the transposon, the plasmid, and a third copy of IS*6100* (see Fig. 3). The orientation of the plasmid within the insert depends on which insertion element (IS) sequence has been duplicated. Southern hybridization with an IS*6100*-labelled probe confirmed that there was only one transposon present in the chromosome (not shown).

Chromosomal DNA from LIMP7 was digested with *Eco*RI, and after self-ligation streptomycin-resistant clones were obtained in *E. coli*. Restriction analysis of the plasmid (named pROSE13) isolated from a transformant showed that it contained only a single IS; thus, it was possible to sequence it by using an IS sequence-specific primer (Fig. 3). The chromosomal fragment was subsequently subcloned and sequenced in both orientations and was found to be 196 bp in length.

A database search showed that the encoded amino acid sequence was highly similar to those of bacterial and mammalian inositol monophosphate phosphatases (IMPases). Indeed, the transposon inserted within a segment encoding a Glu-Glu motif which is one of two conserved metal-binding domains (7). Therefore, we called the partially sequenced gene *impA*. One *M. leprae* and three *M. tuberculosis* homologs were found in the databases. Plasmids were isolated from an *M. smegmatis* plasmid library with the *impA* fragment as a probe, and the complete *impA* gene was sequenced. The *M. smegmatis impA* gene product was found to be 69% identical to one *M. tuberculosis* gene product (coding sequence 26658330_c1_243 from cosmid Y223 [GenBank accession no. AD000019]) and less similar (27 and 31% identity, respectively) to the other two (orf22c, pid:e321101 from cosmid MTCY5a6 [GenBank accession no. Z96072], and orf21c, pid:e290949 from cosmid MTCY3a2 [GenBank accession no. Z83867]). *M. smegmatis impA* and the *M. tuberculosis* gene from Y223 have the same arrangement, lying between *hisA* and *hisF* in the *his* operon (Fig. 4), providing further evidence that they are genuine homologs, and we therefore refer to both genes as *impA*. The *M. smegmatis* ImpA and all three *M. tuberculosis* gene products have the two sequence motifs found in other members of the IMPase family (25). There is a high level of similarity throughout the two ImpA proteins, while when *M. smegmatis* ImpA is compared to other gene products, the greatest similarity surrounds the two IMPase motifs.

Since *impA* lies in the middle of an operon, it was important to exclude polarity effects due to lack of expression of genes downstream of the transposon insertion. Therefore, we tested LIMP7 for auxotrophy; it was able to grow in minimal medium without histidine supplementation, whereas auxotrophs isolated previously following chemical mutagenesis (17) failed to grow. Therefore, we concluded that *his* genes downstream of *impA* were being expressed and that the phenotypes were not due to polarity effects.

Analysis of phosphatidylinositol dimannoside levels. Inositol is an important component of the mycobacterial cell wall, since the LAM is attached by a phosphatidylinositol (PtdIns) moiety. We therefore analyzed the mutant cells for alterations in the levels of PtdIns mannosides (PIMs). PIMs have been detected with various degrees of mannosylation, but the predominant form found is PIM_2, which is a precursor in LAM biosynthesis (Fig. 5). The PIMs were extracted, deacylated in alkali, and analyzed by TLC (Fig. 6). A major spot was observed migrating at the same position as the deacylated PIM_2 standard in both wild-type and mutant cells. The spot was stained with an α-naphthol and molybdate reagent (not shown), confirming the presence of sugar and phosphate residues. A different solvent system (2-propanol–ammonia [2:1]) gave the same results; thus, we concluded that the spot was deacylated PIM_2. Elution and determination of total phosphorus showed that the amount of PIM_2 in LIMP7 was 45% of that of the wild type.

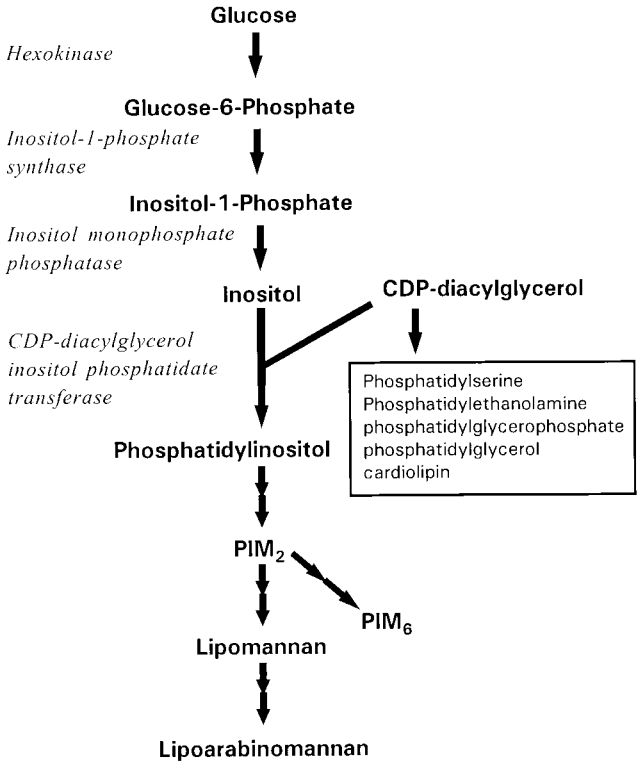

FIG. 5. Proposed pathway for inositol metabolism in mycobacteria.

DISCUSSION

The role of *impA*. We have shown that there is only one transposon in LIMP7 and that the transposon lies within a gene which is highly similar to those of IMPases. The fact that the *impA* gene lies in the *his* operon but that the mutant is not auxotrophic shows that polar effects are not responsible for the phenotypes observed. Therefore, we conclude that the phenotypes observed result from the loss of ImpA activity. The fact that LIMP7 is not an auxotroph means either that the *his* genes are expressed independently of the *impA* gene or that they may be part of a polycistronic message which is not disrupted by the presence of the transposon in the middle. The latter case seems unlikely due to the large size (approximately 19 kb) of the insertion. The expression could come from a promoter within the inserted DNA, or there may be secondary promoters in the *his* operon itself. The latter is a distinct possibility, since this has been shown to be the case for *Salmonella typhimurium* (1) (Fig. 4).

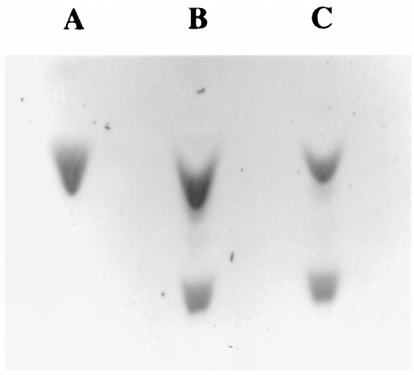

FIG. 6. TLC analysis of LIMP7. Deacylated phospholipids from equal amounts of cell walls of *M. smegmatis* mc^2155 and LIMP7 were spotted on TLC plates and developed twice in isobutanol-pyridine-acetic acid-water (5:5:1:3). The plate was charred with 5% H_2SO_4. (A) Deacylated PIM_2 standard; (B) mc^2155; (C) LIMP7.

It is possible to speculate on the role of such an enzyme. IMPases are enzymes involved in the conversion of inositol monophosphates to free inositol. In mammalian systems, this is important for regeneration of PtdIns, following the cleavage of PtdIns bisphosphate by phospholipase C into inositol triphosphate and diacylglycerol, both of which act as signalling molecules with multiple functions, including the mobilization of Ca^{2+} from intracellular storage (for a review, see the work of Bansal and Majerus [3]). However, the role of IMPases in bacteria is less clear. The *suhB* (also called *ssyA*) gene of *E. coli* is similar to those of IMPases, and the protein has IMPase activity (24). The gene was identified by isolating mutations (later shown to be insertional events) which suppressed certain temperature-sensitive alleles of *secY*, *rpoH* (*htpR*), and *dnaB* (24, 33, 39). It was suggested that the fact that the mutations suppress defects in basic metabolic processes such as protein secretion, DNA synthesis, and the heat shock response could be explained by effects on translational or posttranslational events, for example by increasing mRNA or protein stability, which allows a greater accumulation of a partially active protein. It is an intriguing idea that this effect is mediated by a signalling molecule analogous to the mammalian system. The existence of PtdIns in the *E. coli* membrane has not been conclusively proven; if present, it is a minor phospholipid (20).

In contrast to *E. coli*, mycobacteria contain abundant PtdIns, which plays a vital structural role in the cell wall, acting as the lipid anchor for LAM (5). In LAM biosynthesis, PtdIns is mannosylated (for a review, see the work of Lee et al. [21]) and then further glycosylated to form lipomannan (LM) and then LAM (Fig. 5). The reduction in the level of PIM_2 observed in LIMP7 is direct evidence that *impA* is involved in inositol production. In addition, the LAM of *M. smegmatis* is capped with phosphoinositol groups (19); although the donor of these groups is not known, they may come from PtdIns. These considerations suggest that PtdIns must be synthesized at a rapid rate by mycobacteria. An IMPase would be an essential enzyme in this biosynthetic pathway, since inositol is synthesized as *myo*-inositol-1-phosphate, which has to be converted to free *myo*-inositol by an IMPase (13) in order to accept the phosphatidic acid moiety from CDP-diglyceride for the generation of PtdIns (29) (Fig. 5). We detected weak cross-hybridizing bands in *M. smegmatis* and found three related sequences in a search of approximately two-thirds of the *M. tuberculosis* genome. Therefore, we hypothesize that there is at least one other IMPase in *M. smegmatis*, which partially compensates for the mutation, allowing the cells to survive. In addition to PtdIns, inositol is a component of the major mycobacterial thiol, mycothiol (MSH) (27, 35); MSH [2-(*N*-acetylcysteinyl) amido-2-deoxy-α-D-glucopyranosyl-(1→1)-*myo*-inositol] is found in most actinomycetes but appears to be restricted to these organisms (26). Although it has not been shown that MSH is made from inositol rather than inositol-1-phosphate, it is possible that a separate IMPase is used in its synthesis.

The location of *impA* within the *his* operons of *M. smegmatis* and *M. tuberculosis* is intriguing. The gene order is the same as that found in the *S. typhimurium his* operon, except for the lack of the first gene *hisG* (1, 17). We do not know exactly where the *M. tuberculosis* coding sequences start, but the sequence is consistent with the starts of *impA* and *hisF* being spliced precisely so that they overlap the upstream stop codons (not

shown). The rationale for linking these two separate activities in one operon is not immediately obvious. However, it has been shown in other systems that histidine biosynthetic enzymes are maintained at relatively high levels at all times, perhaps due to the high metabolic cost of synthesizing histidine and the consequent efficiency of the histidine transport system, so that when histidine biosynthesis is required there is not enough time to synthesize the enzymes (37). Furthermore, the separation of *hisG* from the main operon may mean that most transcriptional regulation occurs with this gene. If this is true, the location of *impA* may have no negative consequences, as might occur, for example, if it were repressed in the presence of high levels of histidine. This hypothesis is supported by the finding of open reading frames of unknown function in the *his* operons of several other organisms (1). The location of *impA* has been conserved between two very different mycobacterial species, and it will be interesting to see how widespread this arrangement is.

It is unclear at present how to explain the large-plaque phenotype of LIMP7 in terms of inositol metabolism. The ability of many different phages to produce larger plaques on strain LIMP7 could be explained by changes to different stages of the infective process. Initial, nonspecific adsorption could be more rapid, but no differences were detected. Changes to the specific receptors are unlikely to be the cause, because of the generality of the effect, although it could be imagined that alterations to the cell surface make several receptor molecules more accessible. Following injection of the phage DNA, replication and formation of progeny phages could be more efficient; alternatively, lysis of the bacteria and release of the phages could be more rapid. However, we could not see any gross difference in the latent period or the rate of phage release (Fig. 1B). Finally, the infection process could continue for a longer period, for example, due to slower cell growth, although we have argued against this above. All of these effects could stem from changes to the cell wall or general effects on the cell physiology such as those seen in *E. coli suhB* mutants.

However, the clumping phenotype, the permeability and antibiotic resistance studies, and the decrease in PIM_2 clearly point to an altered cell wall. A previous study suggested that the major site of penetration for the hydrophobic molecules norfloxacin and chenodeoxycholate is the lipid domain of the cell wall (23). These results, together with increased resistance to more hydrophobic antibiotics, suggest that the cell wall permeability of the mutant to lipophilic molecules is decreased. At the same time, there is increased sensitivity to more hydrophilic antibiotics, and we observed a small increase in accumulation of cephaloridine. The higher sensitivities to β-lactams and INH are not likely to be due to the altered growth rate of the mutant, since both are more active against rapidly growing cells (14, 18), whereas LIMP7 grows more slowly. It is surprising that a reduction in the PIM_2 level, which we predict will also reduce the level of the polar LAM molecules, improves permeability of polar molecules. Linking defined genetic defects with biochemical changes in the mycobacterial cell wall in this way is a powerful approach toward understanding both the biogenesis and the functioning of this complex structure.

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