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Phosphatidylinositol Is an Essential Phospholipid of Mycobacteria*

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Phosphatidylinositol (PI) and metabolically derived products such as the phosphatidylinositol mannosides and linear and mature branched lipomannan and lipoarabinomannan are prominent phospholipids/lipoglycans of *Mycobacterium* sp. believed to play important roles in the structure and physiology of the bacterium as well as during host infection. To determine if PI is an essential phospholipid of mycobacteria, we identified the *pgsA* gene of *Mycobacterium tuberculosis* encoding the phosphatidylinositol synthase enzyme and constructed a *pgsA* conditional mutant of *Mycobacterium smegmatis*. The ability of this mutant to synthesize phosphatidylinositol synthase and subsequently PI was dependent on the presence of a functional copy of the *pgsA* gene carried on a thermosensitive plasmid. The mutant grew like the control strain under permissive conditions (30 °C), but ceased growing when placed at 42 °C, a temperature at which the rescue plasmid is lost. Loss of cell viability at 42 °C was observed when PI and phosphatidylinositol dimannoside contents dropped to ~30 and 50% of the wild-type levels, respectively. This work provides the first evidence of the essentiality of PI to the survival of mycobacteria. PI synthase is thus an essential enzyme of *Mycobacterium* that shows promise as a drug target for anti-tuberculosis therapy.

The increased incidence of tuberculosis during the last decades and the emergence of multidrug-resistant strains have made it clear that there is a need for new chemotherapeutic agents (1). The mycobacterial cell envelope is the site of action of many of the first-line antimycobacterial agents (2). A better understanding of the biochemistry and genetics of the pathways leading to the synthesis of envelope components required for cell viability or survival in the host will provide a basis for the rational design of new drugs.

Among the potentially attractive drug targets are the enzymes involved in the synthesis of the main mycobacterial phospholipids: phosphatidylethanolamine (PE),[1] phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylglycerol (PG), and cardiolipin (CL) (3). It is expected that a deficiency in some of these phosphoglycerides would affect the structural and functional organization of the mycobacterial plasma membrane, presumably resulting in the death of the bacterium. Contrary to PE, PG, PS, and CL, which are frequently encountered in all living organisms, PI is an essential phospholipid of eukaryotic cells (4–7), but has seldom been found in prokaryotic cells. Actually, the distribution of PI in prokaryotes seems to be confined to some actinomycetes (*Mycobacterium*, *Corynebacterium*, *Nocardia*, *Micromonospora*, *Streptomyces*, and *Propionibacterium*) (3, 8–12), to myxobacteria (13), and to *Treponema* (14). In *Mycobacterium* sp., PI and metabolically derived molecules (15) of which PI constitutes a lipid anchor to the cell envelope, such as phosphatidylinositol mannosides (PIMans) (16), linear lipomannan (LM), (15), and mature branched LM and lipoarabinomannan (17), are prominent and important phospholipids/lipoglycans. PI and PIMans are regarded as essential for membrane stability and thus for cell viability (3). Lipoarabinomannan is an important modulator of the immune response in the course of tuberculosis and leprosy (18) as well as a key ligand in the interactions between *Mycobacterium tuberculosis* and macrophages that ultimately may facilitate the survival of the tubercle bacillus within phagocytic cells (19). Finally, although PI is regarded as an essential component of the mycobacterial cell wall, positive proof of an essential role of this molecule was lacking. Previously, *de novo* synthesis of PI in mycobacterial cell wall extracts had been described involving the exchange of the CMP moiety of CDP-diacylglycerol (DAG) for inositol (20). In the present study, we undertook the identification of the gene encoding the *M. tuberculosis* PI synthase and addressed the question of the essentiality of this gene through the construction of a conditional mutant of *Mycobacterium smegmatis*.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions—Escherichia coli XL1-Blue, the strain used in this study for cloning experiments, was routinely propagated in LB-Lennox medium (10 g/liter peptone from casein, 5 g/liter yeast extract, and 5 g/liter sodium chloride; Life Technologies, Inc.) at 37 °C. *M. smegmatis* strain mc^{2}155 (21) was routinely grown at 30, 37, or 42 °C in LB-Lennox liquid medium supplemented with 0.05% Tween 80. LB-Lennox medium was used as the solid medium for all bacteria. Antibiotics were added at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 20 μg/ml; hygromycin, 50 μg/ml; and streptomycin, 20 μg/ml. When required, 10% sucrose was added to the solid medium.

Cloning Procedures, Construction of DNA Libraries, Colony Hybridization, and Southern Analysis—Electrocompetent cells of *E. coli* XL1-Blue and *M. smegmatis* mc^{2}155 were prepared as described (22) and

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF265558.

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[1] The abbreviations used are: PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PG, phosphatidylglycerol; CL, cardiolipin; PIMan, phosphatidylinositol mannoside; PIMan$_2$, phosphatidylinositol dimannoside; LM, lipomannan; DAG, diacylglycerol; PCR, polymerase chain reaction; kb, kilobase; ORF, open reading frame; MOPS, 4-morpholinepropanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; Km, kanamycin; Km^r, kanamycin-resistant; Suc, sucrose; Suc^r, sucrose-resistant; Str, streptomycin; Str^r, streptomycin-resistant.

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electrotransformed using a Gene Pulser unit (Bio-Rad). Purification of DNA restriction fragments and PCR fragments was performed using the QIAquick nucleotide removal kit, the QIAquick gel extraction kit, and the QIAquick PCR purification kit (QIAGEN Inc., Chatsworth, CA). Plasmids were isolated from *E. coli* XL1-Blue using the QIAprep miniprep kit (QIAGEN Inc.).

Partial *M. smegmatis* chromosomal DNA libraries were constructed using *Pst*I-, *Sal*I-, and *Sma*I-digested and dephosphorylated "ready-to-clone" pUC18 vectors (Appligene, Illkirch, France). These libraries were transformed into *E. coli* XL1, and colonies harboring a plasmid carrying the *M. smegmatis pgsA* gene were identified by colony hybridization using the *M. tuberculosis pgsA* gene as a probe (PCR-amplified using primers A1.a/A1.b; see below). Three plasmids were isolated in this way: pUCpgsA.P carrying the *M. smegmatis pgsA* on a 2.0-kb *Pst*I restriction fragment, pUCpgsA.S carrying the *pgsA* gene on a 2.6-kb *Sal*I restriction fragment, and pUCpgsA.Sm carrying the *pgsA* gene on a 4.0-kb *Sma*I restriction fragment. They were used for DNA sequencing of the *M. smegmatis pgsA* gene and surrounding ORFs.

Southern blot analysis and colony hybridization were performed as described (23). Sequences of double-stranded plasmids were obtained by Macromolecular Resources (Colorado State University) using an ABI Prism 377 automated DNA sequencer. DNA sequence comparisons were done by BLAST analysis (National Center for Biotechnology Information) (24). Sequences were assembled and processed by using the DNA Strider program (Commissariat à l'Energie Atomique, Gif-sur-Yvette, France).

Overexpression of the M. tuberculosis pgsA, pgsA2, and pgsA3 Genes in M. smegmatis—Standard PCR strategies with *Vent* DNA polymerase (New England Biolabs, Inc.) were used to amplify the *M. tuberculosis* H37Rv *pgsA*, *pgsA2*, and *pgsA3* genes. PCR amplifications consisted of one cycle of denaturation (95 °C, 10 min), followed by 30 cycles of amplification that included denaturation (95 °C, 1 min), annealing (61 °C, 1 min), and primer extension (72 °C, 1 min). The primers used were as follows: A1a (5′-ccccccccccatatgagcaagctgcccttcctgtcc-3′) and A1b (5′-cccaagcttccggtcgccctttccaggaatc-3′) for the *pgsA* gene, A2a (5′-ccccccccccatatggagccggtgctcacgcag-3′) and A2b (5′-cccaagcttgccacgttcaccagcgttctg-3′) for the *pgsA2* gene, and A3a (5′-ccccccccccatatgagcaggtcaacccgttattc-3′) and A3b (5′-cccaagcttgctggcggtctggcggatcccc-3′) for the *pgsA3* gene. They were designed to provide PCR-amplified fragments containing an *Nde*I and a *Hin*dIII restriction site, enabling direct cloning into the pVV16 expression vector, a derivative of pMV261 (25) harboring a kanamycin resistance marker, a hygromycin resistance marker, the *phsp60* promoter, and a six-histidine tag for the expression of C-terminal His_6-tagged fusion proteins. The resulting expression vectors, named pVVpgsA, pVVpgsA2, and pVVpgsA3, were transformed into *M. smegmatis* mc^2155, and transformants were selected on LB-Km-hygromycin plates.

The expression of the PgsA, PgsA2, and PgsA3 proteins in the recombinant strains of *M. smegmatis* was checked by immunoblotting using mouse monoclonal anti-His antibodies (Penta-His antibody, QIAGEN Inc.). *M. smegmatis* crude extracts were prepared by harvesting cultures of the recombinant strains ($A_{600\ nm}$ = 0.6–0.7), washing them twice with cold phosphate-buffered saline, subjecting them to probe sonication for 3 min in the form of 3 × 60-s pulses with 60-s cooling intervals between pulses, and removing the unbroken cells and bacterial debris by centrifugation of the sonicate at 10,000 × *g* for 15 min. Protein concentration was estimated using the BCA protein assay kit (Pierce). SDS-polyacrylamide gel electrophoresis and immunoblot experiments were carried out as described previously (23), except that 100 μg of proteins were loaded onto the gels. The protocol used for immunodetection was as recommended by QIAGEN Inc. Transfer membranes were incubated with the anti-His antibody diluted 1:1000 and with an alkaline phosphatase-conjugated goat anti-mouse IgG secondary antibody diluted 1:2000 (Sigma).

Whole Cell Radiolabeling Experiment—For radiolabeling of whole *M. smegmatis* cells with $^{32}P_i$, *M. smegmatis* was grown in LB-Lennox medium supplemented with 0.05% Tween 80, 200 μg/ml *myo*-inositol, 20 μg/ml glycerol, and kanamycin at 37 °C under agitation. 1 μCi/ml $^{32}P_i$ (carrier-free; specific activity of 1–60 Ci/mmol; NEN Life Science Products) was added to the medium when cultures reached $A_{600\ nm}$ = 0.05 (or $A_{600\ nm}$ = 0.01 for strain mc^2/pVVpgsA3). The incubation was terminated after 24 h when cells reached mid-log phase (early log phase for mc^2/pVVpgsA3). Cultures were then pelleted and washed once with phosphate-buffered saline prior to lipid extraction.

For radiolabeling of the *M. smegmatis* conditional mutant with [^{14}C]acetate, the conditional mutant strain was first grown at 30 °C in LB-Lennox medium supplemented with 0.05% Tween 80 and kanamycin. This culture was then diluted in fresh medium containing 0.5 μCi/ml [^{14}C]acetate (specific activity of 54 mCi/mmol; NEN Life Science Products) to $A_{600\ nm} < 0.1$ and placed at 42 °C for different periods of time prior to lipid extraction.

Plasmids Used for the Construction of the M. smegmatis pgsA Conditional Mutant—A pUC18 vector harboring the *M. smegmatis pgsA* gene on a 2.0-kb *Pst*I restriction fragment (vector pUCpgsA.P) was isolated by colony hybridization using the *M. tuberculosis pgsA* gene (PCR-amplified with primers A1.a/A1.b) as a probe. The *M. smegmatis pgsA* gene and flanking regions were excised from this plasmid on a 1.8-kb *Sma*I restriction fragment and cloned into the *Hin*dIII-cut and blunt-ended pXYL4 vector (a pBluescript derivative carrying the *xylE* colored marker) (26), yielding plasmid pPGSX. The *Km* cassette from pUC4K conferring kanamycin resistance and carried on a 1.2-kb *Hin*cII fragment was then cloned into the *Hin*dIII-cut and blunt-ended pPGSX plasmid, yielding plasmid pPGSXK. Finally, p27PGSXK, the construct used for allelic exchange, was obtained by transferring a 4.2-kb *Bam*HI fragment from pPGSXK containing *pgsA::Km* and *xylE* into *Bam*HI-cut pPR27, a temperature-sensitive mycobacterial vector carrying the counter-selectable marker *sacB* (26).

pCG76, a *Mycobacterium*/*E. coli* shuttle plasmid derived from pCG63, harboring a mycobacterial thermosensitive origin of replication and a streptomycin resistance cassette (27), was used to carry functional copies of the *M. smegmatis pgsA* gene in the *M. smegmatis pgsA* mutant. One of these complementing vectors, pCGpis.1, was constructed by inserting the 1.8-kb *Sma*I restriction fragment from pUCpgsA.P into *Bam*HI-cut and blunt-ended pCG76. The second complementing vector, pCGpis.2, was constructed by cloning the 2.6-kb blunt-ended *Sal*I fragment from pUCpgsA.S into *Bam*HI-cut and blunt-ended pCG76. These temperature-sensitive vectors are able to replicate at 30 °C, but not at 42 °C.

Cell-free Assay for Phosphatidylinositol Synthesis Using [^{3}H]Inositol as a Substrate—M. smegmatis cultures collected at $A_{600\ nm}$ = 0.6–0.7 were washed, and cells were resuspended at 4 °C in buffer A (50 mM MOPS (pH 7.9), 5 μM β-mercaptoethanol, and 10 mM $MgCl_2$). The cell suspension was subjected to probe sonication (1-cm probe, Soniprep 150, MSE, Sussex, United Kingdom) for 10 min in the form of 10 × 60-s pulses with 90-s cooling intervals between pulses. After the whole sonicate was centrifuged at 27,000 × *g* for 15 min at 4 °C, the pellet was resuspended in buffer A. The crude cell wall fraction was obtained upon centrifugation of this resuspended pellet at 3000 rpm for 10 min to remove cell debris and unbroken cells. Proteins were kept frozen in small aliquots at −70 °C. Crude cell wall fractions were assayed for PI synthase activity in a final volume of 400 μl (20). Each reaction mixture contained crude cell wall preparation (~1 mg of protein), 0.1 mM ATP, 5 mM glucose, 300 μM CDP-diacylglycerol (dipalmitoyl; Sigma), 0.4% CHAPS, 22 μCi of [^{3}H]inositol (2.5 μM final concentration; specific activity of 22 Ci/mmol; NEN Life Science Products), and buffer A up to 400 μl. The mixture was incubated at 37 °C for the indicated times. At the end of incubations, the reactions were terminated by the addition of $CHCl_3$/CH_3OH (2:1, 3 ml/400 μl of reaction mixture), followed by centrifugation to separate the pellet. The pellet was extracted once more with 3 ml of $CHCl_3$/CH_3OH (2:1). The combined extracts were washed once with 0.9% NaCl (1.2 ml) and once with 1 ml of $CHCl_3$/CH_3OH/H_2O (3:47:48) to yield the washed $CHCl_3$/CH_3OH (2:1) lipids. Since no labeled product other than PI was found in the washed lipid extract, quantification of the reaction was performed directly by scintillation counting of the washed lipid extract.

Analytical Methods—Lipids from labeled and unlabeled cells were extracted by two consecutive overnight extractions in 4 ml of $CHCl_3$/CH_3OH (2:1). The combined $CHCl_3$/CH_3OH (2:1) extracts were washed once with 0.9% NaCl and once with $CHCl_3$/CH_3OH/H_2O (3:47:48) to yield a fraction containing the phospholipids including $PIMan_2$s. When phosphatidylinositol pentamannosides and linear LM were to be extracted, the insoluble pellet resulting from the $CHCl_3$/CH_3OH (2:1) extraction was further extracted overnight with $CHCl_3$/CH_3OH/H_2O (10:10:3). Characterization of the various PIMans followed earlier work (28) and was based on fast atom bombardment mass spectrometry analysis, one- and two-dimensional thin-layer chromatographic patterns of the intact PIMans (16, 28), sugar analysis by gas chromatography, and fatty acid analysis by gas chromatography-mass spectrometry (29). $PIMan_2$s were analyzed by fast atom bombardment mass spectrometry analysis either directly in the negative ion mode or as acetyl derivatives (100 μl of pyridine/acetic anhydride (1:1, v/v) for 24 h) in the positive ion mode. Fast atom bombardment mass spectrometry analysis was performed on a Fisons VG AutoSpec mass spectrometer with a cesium ion gun operating at 25 kV. Samples (30 μg) were applied to a *m*-nitrobenzyl alcohol matrix. Triacylated $PIMan_2$ and lyso-$PIMan_2$

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were purified by preparative TLC in the solvent systems described below.

TLC was conducted in one- and two-dimensions on aluminum-backed plates of Silica Gel 60 F_{254} (Merck, Darmstadt, Germany). Solvents used for the analysis of phospholipids and $PIMan_2$s were $CHCl_3$/CH_3OH/CH_3COOH/HCOOH/H_2O (35:15:6:2:0.3) and $CHCl_3$/CH_3OH/NH_4OH/H_2O (65:25:0.5:3.6), respectively. Analysis of phosphatidylinositol pentamannosides and linear LM was performed in the same solvent as the one used for $PIMan_2$s. Two-dimensional TLCs were performed using solvent $CHCl_3$/CH_3OH/H_2O (60:30:6) in the first dimension and solvent $CHCl_3$/CH_3OH/CH_3COOH/H_2O (40:25:3:6) in the second dimension. An α-naphthol spray (1% α-naphthol in ethanol), a Dittmer-Lester spray (30), and a cupric sulfate spray (10% $CuSO_4$ in an 8% phosphoric acid solution) were used to detect carbohydrate-containing lipids, phosphorus-containing lipids, and all organic compounds, respectively. Autoradiograms were obtained by exposing chromatograms to Kodak X-Omat AR films at −70 °C usually for 1–4 days. Plates were also scanned for radioactivity using a Bio-Scan System 200 imaging scanner with Autochanger 3000, or relevant spots were scraped off for scintillation counting. L-α-Phosphatidylethanolamine (Sigma), L-α-phosphatidyl-L-serine (Sigma), L-α-phosphatidylinositol (Sigma), L-α-phosphatidyl-DL-glycerol (Sigma), and cardiolipin (ICN Pharmaceuticals Inc.) commercial standards were used to identify the mycobacterial phospholipids on thin-layer chromatograms.

RESULTS AND DISCUSSION

Identification of the Phosphatidylinositol Synthase Gene of M. tuberculosis

Expression of the M. tuberculosis pgsA, pgsA2, and pgsA3 Genes in M. smegmatis—The search for the mycobacterial PI synthase gene was based on the fact that all the enzymes (with the exception of phosphatidylserine synthases of Gram-negative bacteria) capable of catalyzing the transfer of a free alcohol (inositol, serine, or glycerol) onto CDP-diacylglycerol share a common motif in their primary sequence, named the CDP-alcohol phosphatidyltransferase signature (Prosite accession number PS00379). Screening of the *M. tuberculosis* H37Rv genome (31) for this motif revealed that four ORFs, *pgsA*, *pgsA2*, *pgsA3*, and *pssA*, potentially encoded enzymes carrying this signature. The presence of the *psd* gene encoding a phosphatidylserine decarboxylase responsible for the synthesis of PE from PS adjacent to the *pssA* gene in addition to sequence similarities the *M. tuberculosis* PssA enzyme shares with known PS synthases (32) (Fig. 1*a*) strongly suggest that the *pssA* gene encodes a PS synthase. Therefore, this gene was not considered to be a PI synthase candidate gene. The three other genes, *pgsA*, *pgsA2*, and *pgsA3*, were PCR-amplified and placed under the control of the *phsp60* promoter (25) in the mycobacterial expression vector pVV16, yielding plasmids pVVpgsA, pVVpgsA2, pVVpgsA3. These three plasmids were electrotransformed into *M. smegmatis* mc^2155. The production of recombinant proteins in each recombinant strain was checked using anti-His antibodies (data not shown). Interestingly, in LB-Km liquid medium at 37 °C, the mc^2/pVVpgsA and mc^2/pVVpgsA2 strains exhibited the same growth rate as the mc^2/pVV16 control strain, whereas mc^2/pVVpgsA3 exhibited a much slower growth due to an extended lag period that lasted ≅24 h instead of 10–12 h for all the other strains (data not shown). On LB-Km solid medium at 37 °C, mc^2/pVVpgsA3 colonies appeared after 8 days instead of 3 days for the other strains.

Phospholipid Composition of the Recombinant Strains—The phospholipid composition of the three recombinant strains and that of the control strain were analyzed by measuring the distribution of labeled $^{32}P_i$ among the major lipid classes. $^{32}P_i$ labeling was performed as described under "Experimental Procedures." After 24 h of labeling, when bacterial cultures reached mid-log phase (early log phase for mc^2/pVVpgsA3), bacteria were pelleted, and their lipids were extracted and analyzed. This experiment was performed on two different

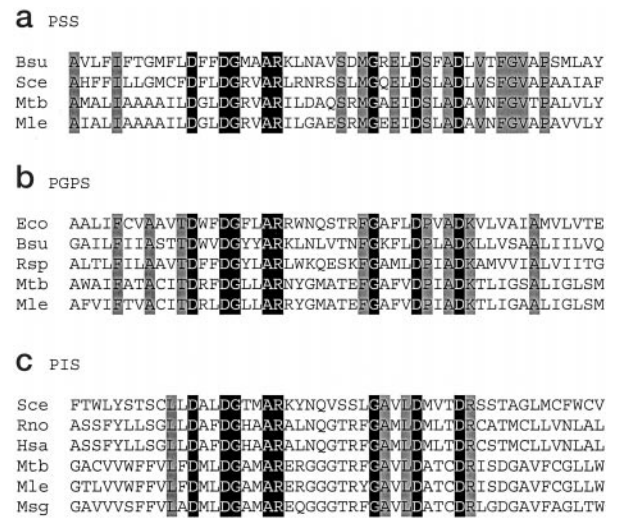

FIG. 1. **Alignment of the sequences conserved in phosphatidylserine synthases (*PSS*), phosphatidylglycerophosphate synthases (PGPS), and phosphatidylinositol synthases (*PIS*).** Amino acid residues of the CDP-alcohol phosphatidyltransferase signature (Prosite accession number PS00379) are shown in *black boxes*. The residues conserved within the enzyme groups are shown in *gray boxes*. *Bsu*, *Bacillus subtilis* (PS synthase, GenBank™/EBI Data Bank accession number D38022 (residues 31–80); and phosphatidylglycerophosphate synthase, accession number U87792 (residues 46–95)); *Eco*, *Escherichia coli* (phosphatidylglycerophosphate synthase, accession number M12299 (residues 35–84)); *Sce*, *Saccharomyces cerevisiae* (PS synthase, accession number Z14127 (residues 116–165); and PI synthase, accession number J02697 (residues 45–94)); *Rsp*, *Rhodobacter sphaeroides* (phosphatidylglycerophosphate synthase, accession number U29587 (residues 42–91)); *Rno*, *Rattus norvegicus* (PI synthetase, accession number D82928 (residues 36–85)); *Hsa*, *Homo sapiens* (PI synthetase, accession number NM_006319 (residues 36–85)); *Mtb*, *M. tuberculosis* (PS synthase, Rv0436c (residues 43–92); phosphatidylglycerophosphate synthase, Rv2746c (residues 62–111); and PI synthase, Rv2612c (residues 57–106) (31)); *Mle*, *M. leprae* (PS synthase, accession number AL035159 (residues 43–92); phosphatidylglycerophosphate synthase, accession number S72934 (residues 43–92); and PI synthetase, accession number Z96801 (residues 57–106)); *Msg*, *M. smegmatis* (PI synthetase, accession number AF265558) (residues 57–106)).

TABLE I
Phospholipid composition of M. smegmatis control and recombinant strains

The $^{32}P_i$-labeled lipids were extracted and separated by TLC as described under "Experimental Procedures," and radioactivity distribution among phospholipids was determined using a Bio-Scan System 200 imaging scanner.

	mc^2pVV16	mc^2pVVpgsA	mc^2pVVpgsA2	mc^2pVVpgsA3
	%	%	%	%
Origin	2.8	2.3	1.9	7.2
$PIMan_2$	4.5	3.9	4.7	6.5
PI	23.4	34.5	21.3	18.0
PE	31.6	29.1	31.7	24.1
PG	0.4	0.6	0.3	23.5
CL	37.3	29.6	40.1	20.7

mc^2/pVVpgsA transformants, two different mc^2/pVVpgsA2 transformants, and one mc^2/pVVpgsA3 transformant. Table I shows the radiolabeled phospholipid composition of each strain in one typical experiment. The mc^2/pVVpgsA2 strain exhibited the same pattern of $^{32}P_i$ distribution as the mc^2/pVV16 control strain. The mc^2/pVVpgsA recombinant strain exhibited a slightly higher quantity of PI (PI represented 34% of the total phospholipids as compared with 21–23% for the mc^2/pVVpgsA2 and control strains), suggesting that *pgsA* may encode a PI synthase. The mc^2/pVVpgsA3 strain had a completely different pattern of $^{32}P_i$ distribution, with the appearance of a new highly labeled compound that was barely detected in the other

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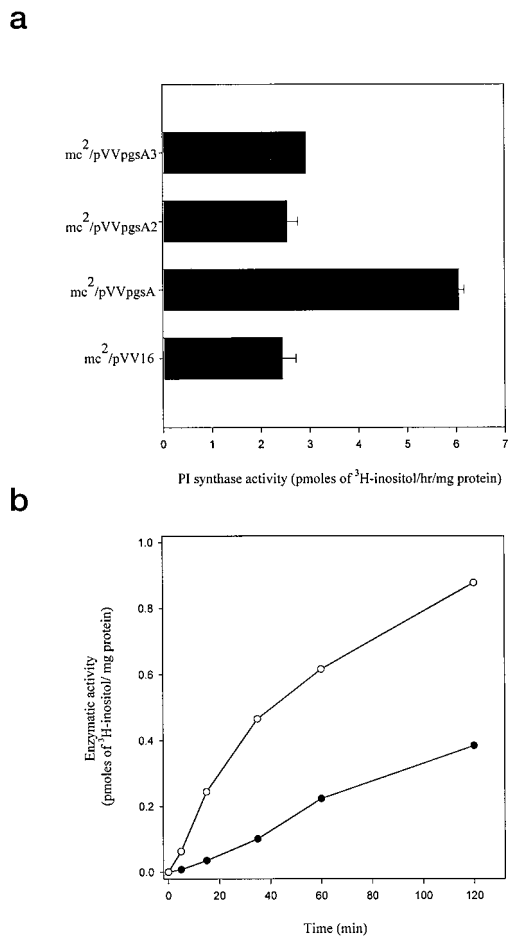

FIG. 2. **PI synthase activity in *M. smegmatis* control and recombinant strains.** *a*, PI synthase activity in cell wall preparations from *M. smegmatis* control and recombinant strains. Incubations for 1 h at 37 °C were performed as described under "Experimental Procedures." Each point represents the mean of two determinations. Amounts of substrate consumed did not exceed 1% in each case. *b*, time course of PI synthesis by *M. smegmatis* mc^2/pVV16 (●) and mc^2/pVVpgsA (○) cell wall preparations.

strains. This compound comigrated with the phosphatidylglycerol commercial standard, suggesting that *pgsA3* encodes a phosphatidylglycerophosphate synthase. This result is consistent with the sequence similarities that the PgsA3 enzyme of *M. tuberculosis* shares with other known PG synthases (Fig. 1*b*). Overproduction of PG thus appeared to be the cause of the slow growth of strain mc^2/pVVpgsA3. The distribution of $^{32}P_i$ among other PI-containing compounds such as PIMans and linear LM was identical for all strains (data not shown).

PI Synthase Cell-free Assays—PI synthase cell-free assays were performed on crude cell wall preparations of each strain. Results are presented in Fig. 2 (*a* and *b*). After a 1-h incubation at 37 °C, the cell wall preparation of the mc^2/pVVpgsA recombinant strain exhibited a PI synthase activity that was 2–2.5-fold greater than that of the other strains (Fig. 2*a*). A comparative time course of PI synthesis by the crude cell wall preparations of mc^2/pVVpgsA and mc^2/pVV16 further reflected the overproduction of PI synthase in the recombinant strain mc^2/pVVpgsA (Fig. 2*b*), strongly suggesting that the mycobacterial PI synthase is encoded by the *pgsA* gene.

Although no obvious function could be attributed to the PgsA2 enzyme from these experiments, it is likely that this enzyme is involved in the synthesis of cardiolipin. It has been suggested that mycobacteria possess a eukaryotic type of cardiolipin synthase activity involving the transfer of a phosphatidyl group from CDP-diacylglycerol to PG to form CL and CMP (33). In yeast, the Crd1p enzyme (34) that catalyzes this reaction carries the same CDP-alcohol phosphatidyltransferase signature as the one found in the *M. tuberculosis* PgsA, PgsA2, and PgsA3 proteins. Moreover, although it has been suggested that mycobacteria are also able to make CL from the usual prokaryotic reaction, PG + PG → CL + glycerol (33), no protein carrying the characteristic amino acid motifs of bacterial cardiolipin synthases (35) was found in the genome of *M. tuberculosis* H37Rv. This suggests that the synthesis of CL from PG and CDP-diacylglycerol may be prevalent in mycobacteria and catalyzed by an enzyme of the same family as the yeast Crd1p protein, presumably PgsA2.

Construction of a Lethal Mutation in the Synthesis of PI of M. smegmatis

To determine if PI is an essential phospholipid of *Mycobacterium* sp., we constructed a *pgsA* conditional mutant of *M. smegmatis*. The experimental approach used to construct a null mutation in the *pgsA* gene made no assumption of whether the mutation would be lethal or not. It uses a two-step homologous recombination procedure to achieve allelic exchange at the *pgsA* locus of *M. smegmatis* (22) and a mycobacterial temperature-sensitive plasmid (27) to perform complementation experiments. The genetic studies were performed in *M. smegmatis* rather than in *M. tuberculosis* because temperature-sensitive vectors that are efficiently lost under nonpermissive conditions are only available for *M. smegmatis* (27).

Isolation of the M. smegmatis pgsA Gene—The *M. smegmatis pgsA* gene to be used in all subsequent homologous recombination experiments was cloned on a 2.0-kb *Pst*I restriction fragment, a 2.6-kb *Sal*I restriction fragment, and a 4.0-kb *Sma*I fragment as described under "Experimental Procedures." The fact that the *M. tuberculosis pgsA* gene gave only one signal when probed on hybridization membranes carrying *Pst*I- or *Sma*I-cut *M. smegmatis* chromosomal DNA suggested that the *pgsA* gene was present only in one copy in the genome of *M. smegmatis* (data not shown). The sequences of the *M. smegmatis pgsA* gene and surrounding ORFs were determined and deposited in the GenBank™/EBI Data Bank under accession number AF265558. The *pgsA* genomic region in *M. smegmatis* showed an organization similar to that found in *M. tuberculosis* and *Mycobacterium leprae*. Interestingly, in all these species, *pgsA* is the second ORF of a cluster of three to four ORFs that seem to be organized as an operon. The function of the product potentially encoded by ORF Rv2613c located upstream of the *pgsA* gene was annotated as unknown (31). The product of ORF Rv2611c located downstream of the *pgsA* gene showed only weak similarities to acyltransferases of *Chlamydophila pneumoniae* (Protein Identification Resource accession number B72119; 42% similarity on a 185-amino acid overlap) and *Campylobacter jejuni* (GenBank™/EBI Data Bank accession number AAF31766) (47% similarity on a 159-amino acid overlap). The existence of Rv2610c, potentially encoding a mannosyltransferase within the cluster of genes, strongly suggests that the Rv2610c enzyme could be involved in the very first transfer of mannose residues onto PI, leading to the synthesis of phosphatidylinositol monomannoside. The mannosyltransferase responsible for the synthesis of PIMan$_2$s has recently been identified (36). For clarity purposes, the homologs of Rv2613c, Rv2611c, and Rv2610c in *M. smegmatis* were given the same names as their *M. tuberculosis* counterparts throughout this report.

Achievement of a Single Homologous Recombination Event at the pgsA Locus of M. smegmatis and Characterization of the

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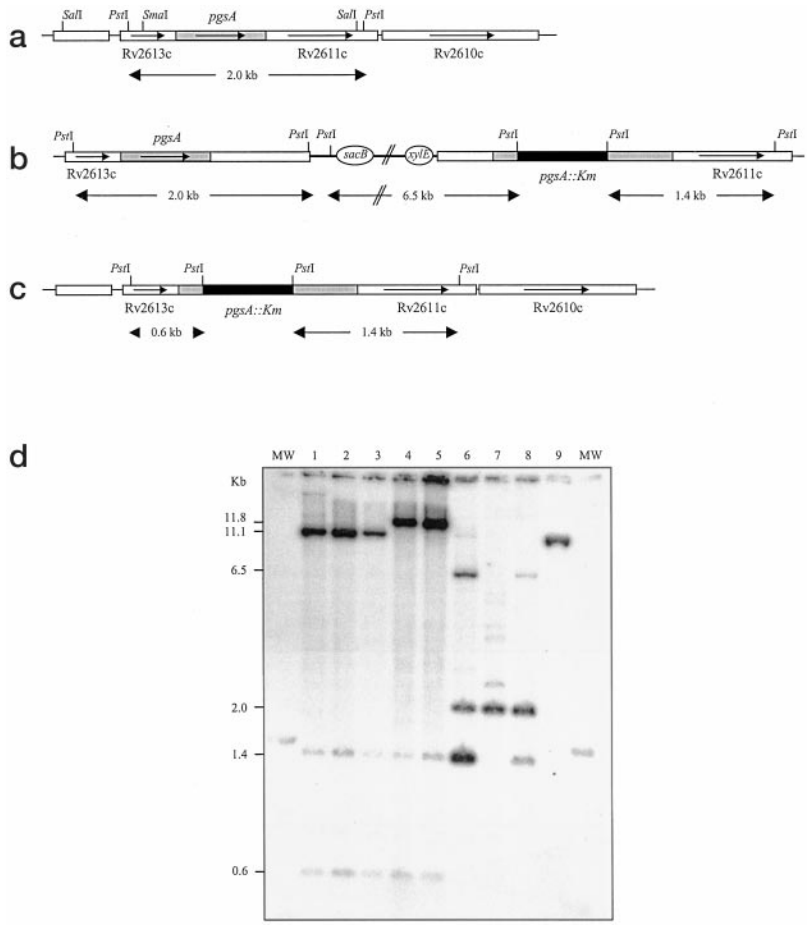

FIG. 3. **Genetic organization of the *pgsA* chromosomal region in the wild-type strain mc^{2}155, the single crossover strain mc^{2}CSU01, and the *M. smegmatis pgsA* conditional mutants.** *a–c*, genetic organization, partial restriction map, and expected hybridization profiles of the *pgsA* regions in the wild-type strain mc^{2}155, the single crossover strain mc^{2}CSU01, and the conditional mutant strain mc^{2}CSU03, respectively; *d*, Southern blot analysis of wild-type mc^{2}155 (*lane 7*), mc^{2}CSU01 (*lane 6*), *pgsA* allelic exchange mutants (conditional mutants complemented with plasmid pCGpis.2 (*lanes 1–3*) and with plasmid pCGpis.1 (*lanes 4* and *5*)), a Km^r-Suc^r-Str^r-XylE$^-$ clone obtained upon plating of mc^{2}CSU02/pCG76 onto LB-Km-Suc plates (*lane 8*), and plasmid pCGpis.2 (*lane 9*). *Lane MW* contains molecular size markers in kilobases. Chromosomal DNA was digested with *Pst*I. The probe used to perform the Southern blot hybridization was the *M. smegmatis* 2.0-kb *Pst*I restriction fragment from pUCpgsA.P carrying the *pgsA* gene.

Rv2611c Gene—The system we used to achieve gene replacement at the *pgsA* locus of *M. smegmatis* is based upon the use of pPR27, a temperature-sensitive plasmid of mycobacteria harboring the *sacB* counter-selectable marker (26). A disrupted copy of the *M. smegmatis pgsA* gene (*pgsA::Km*) containing an inactivated *pgsA* gene flanked by truncated Rv2613c and Rv2611c ORFs was constructed as described under "Experimental Procedures." The *pgsA::Km* construct and the *xylE* colored marker (37) were inserted into pPR27. The resulting plasmid (p27PGSXK) was electroporated into *M. smegmatis*, and kanamycin-resistant (Km^r) transformants were selected on LB-Km plates at 30 °C. One transformant was then propagated in LB-Km liquid medium at 30 °C prior to plating onto LB-Km plates at 42 °C. Since the temperature-sensitive plasmid is able to replicate at 30 °C but not at 42 °C, the Km^r colonies that appear on plates at 42 °C necessarily have integrated all or part of the p27PGSXK plasmid into their chromosome by homologous recombination or by illegitimate recombination. Out of 25 such Km^r clones that were analyzed by Southern hybridization, only one had integrated the vector at the *pgsA* locus through a single homologous recombination event (Fig. 3, *b* and *d*, *lane 6*). This clone was named mc^{2}CSU01. The remaining 24 clones resulted from illegitimate recombination events (data not shown). The genetic organization of the *pgsA* region in mc^{2}CSU01 is shown in Fig. 3*b*. As shown, because a truncated operon was used to construct the disrupted copy *pgsA::Km*, the insertion of p27PGSXK at the *pgsA* locus, although not affecting the expression of the Rv2613c ORF, presumably affected the expression of the Rv2611c ORF by depriving it of its natural promoter. This should not be the case if Rv2611c is transcribed independently from Rv2613c and *pgsA*. Similarly, the expression of the putative mannosyltransferase gene Rv2610c may also have been affected if expressed from the same promoter as the three preceding ORFs.

When the growth characteristics of mc^{2}CSU01 were studied in LB liquid medium at 37 °C, this strain showed a great tendency to clump and exhibited a slower growth rate than the mc^2/pVV16 control strain (data not shown). On plates, mc^{2}CSU01 colonies appeared after 6 days (instead of 3 days for the control strain) and had a dryer and rougher aspect than the mc^2/pVV16 colonies. Assuming that the function of the Rv2611c product is linked to PI synthesis, we extracted and analyzed by TLC the lipids of strain mc^{2}CSU01 (Fig. 4). The analysis clearly showed that although mc^{2}CSU01 contains all of the basic phospholipids found in the mc^2/pVV16 control strain (CL, PG, PE, and PI), it lacks one compound migrating within the PI and PIMan region. The purification of this compound from the control strain by preparative TLC and its analysis by fast atom bombardment mass spectrometry analysis in the negative ion mode gave a mass (*m*/*z* 1175) consistent with that of a diacylated dimannophosphoinositide (C_{16}/C_{19}) (28). In the positive ion mode, the compound afforded a dominant fragment ion at *m*/*z* 371, corresponding to lysomonoacylglycerol carrying a C_{19} fatty acid chain. These data thus suggest that the missing compound in strain mc^{2}CSU01 is lyso-PIMan$_2$. Since triacylated forms of PIMan$_2$s were found in mc^{2}CSU01 and characterized by TLC and sugar analysis (see "Experimental Procedures"), the lack of lyso-PIMan$_2$ in this strain seems more likely due to the alteration of the expression of Rv2611c rather than to that of the putative mannosyltransferase. Therefore, these data suggest that Rv2611c is an acyltransferase responsible for the cleavage of one fatty acid chain of PI (most likely the C_{16} fatty acid chain found at position *sn*-2) (3) and thus involved in the synthesis of lyso-PIMan$_2$, a possi-

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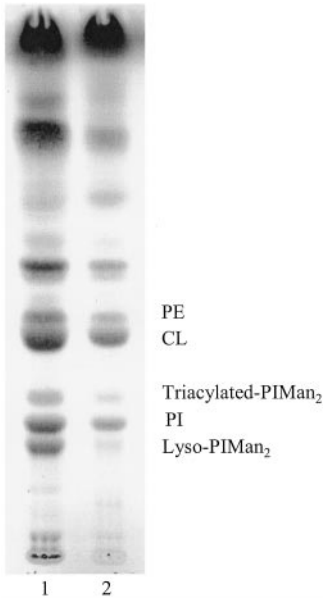

FIG. 4. **Evidence for the lack of lyso-PIMan$_2$ in the single crossover strain mc^{2}CSU01.** Total lipids were separated by TLC in the solvent system $CHCl_3/CH_3OH/NH_4OH/H_2O$ (65:25:0.5:4) and detected with a cupric sulfate spray. *Lane 1*, total lipids from mc^2/pVV16; *lane 2*, total lipids from mc^{2}CSU01.

ble precursor of LM and lipoarabinomannan (38, 39). The observations concerning the growth characteristics of mc^{2}CSU01 in both liquid and solid media are consistent with the description Parish and collaborators (40) made earlier of the growth characteristics of an inositol-monophosphate synthase mutant (*impA*) of *M. smegmatis* affected in the synthesis of PIMans.

The growth phenotype associated with mc^{2}CSU01 was found to be very unstable since after 5–10 generations in liquid medium, the strain systematically recovered a growth rate identical to that of the control strain at the same time as it recovered its ability to synthesize lyso-PIMan$_2$ (data not shown). The most likely explanation for such a reversion is that the region located directly upstream of Rv2611c in mc^{2}CSU01 (Fig. 3*b*) progressively accumulated mutations leading to the formation of a cryptic promoter capable of driving the expression of Rv2611c. One such modified mc^{2}CSU01 strain (named strain mc^{2}CSU02) that had recovered a normal growth (Fig. 5*a*) and the ability to synthesize lyso-PIMan$_2$ was selected and used to perform all subsequent genetic steps leading to the construction of the *M. smegmatis pgsA* mutant.

Construction of the pgsA Conditional Mutant of M. smegmatis—In a first attempt to isolate a *pgsA* mutant of *M. smegmatis*, strain mc^{2}CSU02 was grown to saturation in LB medium and then plated onto LB-Km-Suc plates at 37 °C. This selection step should lead to the selection of clones having undergone a second intrachromosomal crossover event leading to the excision of the vector and to the replacement of the functional copy of the *pgsA* gene by the disrupted one, *i.e.* allelic exchange mutants. Sucrose resistance appeared at a frequency of 10^{-4}, and all 3800 Km^r-Suc^r clones subsequently tested for their XylE phenotype exhibited a yellow color when sprayed with catechol, suggesting that none of them was an allelic exchange mutant. Instead, these clones had probably undergone some mutations in the *sacB* gene that rendered them resistant to sucrose. Since the failure to disrupt the *pgsA* gene in this first experiment is not an absolute measure of the essentiality of that gene, we next proceeded to the construction of a *pgsA* conditional mutant of *M. smegmatis*.

For this, mc^{2}CSU02 was electroporated with three different

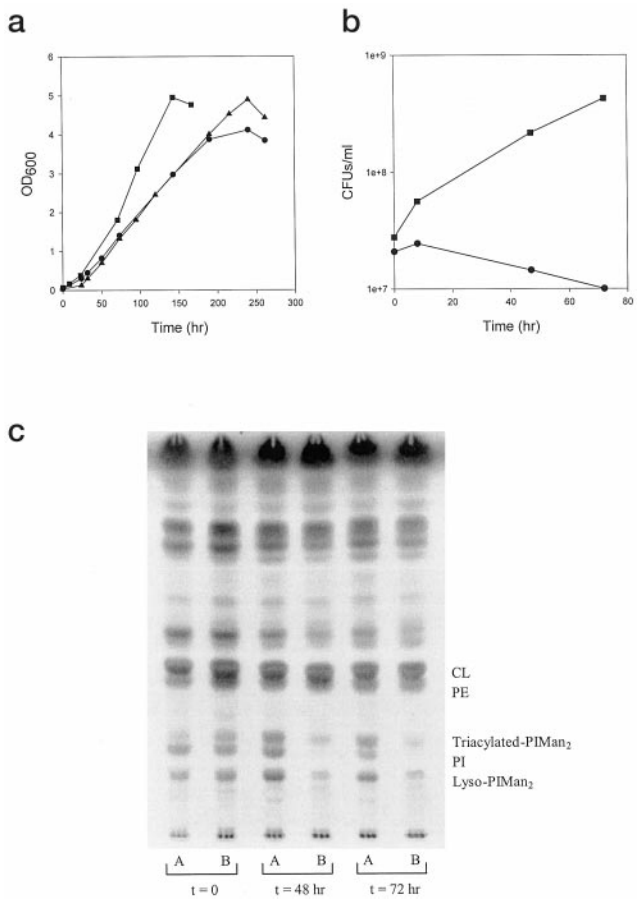

FIG. 5. **Growth characteristics and lipid composition of the *pgsA* conditional mutant mc^{2}CSU03 incubated at 30 and 42 °C.** Shown are growth curves of strains mc^{2}CSU02 (▲), mc^{2}CSU03 (●), and mc^2/pMV261 (■) incubated at 30 °C in LB-Km medium as static cultures (*a*). Saturated mc^{2}CSU03 and mc^2/pMV261 (control strain) cultures grown at 30 °C were diluted in fresh LB-Km medium to $A_{600\ nm} < 0.1$ and incubated at 42 °C. At different time points, the number of viable cells in the cultures was evaluated by plating serial dilutions onto LB-Km plates at 30 °C (*b*), and the lipid compositions of the strains were analyzed by TLC (solvent system: $CHCl_3/CH_3OH/NH_4OH/H_2O$ (65:25:0.5:3.6)) (*c*). *A lanes*, strain mc^2/pMV261; *B lanes*, mc^{2}CSU03. *CFUs*, colony forming units.

derivatives of the temperature-sensitive pCG76 plasmid: (i) the empty pCG76 vector, (ii) a pCG76 vector carrying the *M. smegmatis pgsA* gene flanked by the last 370 base pairs of Rv2613c and the first 858 base pairs of Rv2611c (pCGpis.1), and (iii) a pCG76 vector carrying the complete *M. smegmatis* Rv2613c and *pgsA* genes including the promoter region of the operon (pCGpis.2). Transformants were selected on LB-Km-Str plates at 30 °C, a temperature at which the pCG76 plasmid is able to replicate. One mc^{2}CSU02/pCGpis.1, one mc^{2}CSU02/pCGpis.2, and one mc^{2}CSU02/pCG76 transformant were picked and cultured in LB-Km-Str medium at 30 °C before being plated onto LB-Km-Suc-Str plates at 30 °C to select for allelic exchange mutants. Sucrose resistance occurred at a frequency of 10^{-4} for all transformants. The percentages of Km^r-Suc^r-Str^r-XylE$^-$ allelic exchange candidates among Km^r-Suc^r-Str^r colonies obtained with each transformant were 3% for mc^{2}CSU02/pCG76 (1924 colonies tested), 6% for mc^{2}CSU02/pCGpis.1 (3477 colonies tested), and 32% for mc^{2}CSU02/pCGpis.2 (913 colonies tested). A further analysis by Southern hybridization of the Km^r-Suc^r-Str^r-XylE$^-$ colonies obtained revealed that all of the allelic exchange candidates obtained upon plating of mc^{2}CSU02/pCGpis.1 and mc^{2}CSU02/pCGpis.2 (two and eight colonies analyzed, respectively) (Fig. 3, *c* and *d*, *lanes 1–5*) had

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indeed undergone gene replacement at the *pgsA* chromosomal locus. These *pgsA* conditional mutants thus harbor a nonfunctional *pgsA::Km* gene on their chromosome and a functional *pgsA* gene carried by a temperature-sensitive vector in their cytoplasm. In contrast, none of the analyzed Km^r-Suc^r-Str^r-$XylE^-$ clones obtained upon plating of mc^2CSU02/pCG76 had undergone a second crossover event at the *pgsA* chromosomal locus (Fig. 3*d*, *lane 8*). Instead, they probably carried mutations affecting both the *sacB* and the *xylE* genes that rendered them resistant to sucrose and negative to catechol testing.

In conclusion, allelic exchange at the *pgsA* chromosomal locus was achievable only when using the complemented mc^2CSU02/pCGpis.1 and mc^2CSU02/pCGpis.2 strains, *i.e.* only when a rescue copy of the *pgsA* gene was provided to the bacterium. Moreover, the inability to achieve allelic exchange at the *pgsA* locus of mc^2CSU02 and mc^2CSU02/pCG76 seems to be attributable to the *pgsA* gene alone and not to polar effects of the mutation affecting the expression of adjacent genes since supplying an extra copy of the sole *pgsA* gene to mc^2CSU02 (mc^2CSU02/pCGpis.1 carries no other complete gene than *pgsA*) is sufficient to achieve allelic exchange at the *pgsA* locus. Therefore, these results strongly suggest that the *pgsA* gene is essential to mycobacteria. The difference in the percentage of allelic exchange candidates obtained when using mc^2CSU02/pCGpis.1 or mc^2CSU02/pCGpis.2 (6 and 32%, respectively) probably reflects the fact that the Rv2613c and *pgsA* genes are expressed from the same promoter. The expression of the *pgsA* gene is probably best from the pCGpis.2 plasmid, which carries the two complete Rv2613c and *pgsA* genes with their promoter region, than from pCGpis.1, on which *pgsA* is deprived of its natural promoter and probably expressed from a cryptic promoter. Therefore, mc^2CSU02 complemented with pCGpis.2 is more likely to undergo gene replacement at the *pgsA* locus than mc^2CSU02/pCGpis.1.

Characterization of the pgsA Conditional Mutant of M. smegmatis—To conclusively provide evidence that the *pgsA* gene is essential to *M. smegmatis*, we investigated the ability of a pCGpis.1-complemented *pgsA* mutant of *M. smegmatis* (named strain mc^2CSU03) to survive at 42 °C, a temperature at which the pCGpis.1 vector is unable to replicate. pCGpis.1 rather than pCGpis.2 was chosen to complement the mutant to ensure that the survival of the conditional mutant throughout the experiment was dependent on the expression of the *pgsA* gene alone and not on that of Rv2613c. The growth characteristics of the *pgsA* temperature-sensitive mutant mc^2CSU03 at 30 and 42 °C are presented in Fig. 5 (*a* and *b*, respectively). As expected, at 30 °C, the temperature-sensitive pCGpis.1 vector replicated, and mc^2CSU03 exhibited the same growth characteristics as the control strain mc^2/pMV261 and as the single crossover strain mc^2CSU02. After a shift of temperature from 30 to 42 °C, the temperature-sensitive plasmid was progressively cured from the population of bacteria, and as shown in Fig. 5*b*, the number of viable mc^2CSU03 cells started to decline after 8 h. In contrast, the control strain continued to grow exponentially. The growth of mc^2CSU03 for one to two generations after the temperature shift is consistent with the way the temperature-sensitive plasmid is cured. Indeed, if one considers that this pAL5000 derivative (41) is present in three to five copies in the cytoplasm of mc^2CSU03 grown under permissive conditions (30 °C), this strain should be able to undergo one or two more divisions when placed at 42 °C before the number of copies of functional *pgsA* gene becomes less than one per cell and the bacteria start to die.

Finally, these data prove that the *pgsA* gene is essential to the survival of *M. smegmatis*. Moreover, they show that the activity of the PgsA enzyme in the conditional mutant placed at 42 °C cannot be compensated by any other enzyme of *M. smegmatis*.

An analysis by TLC of the lipid composition of the mc^2CSU03 strain placed at 42 °C for different periods of time revealed that a dramatic loss of PI and $PIMan_2$s occurred in this strain (Fig. 5*c*). [^{14}C]Acetate labeling of the lipids of mc^2CSU03 placed at 42 °C allowed a quantification of this loss and showed that bacterial growth ceased after 8 h when the PI and $PIMan_2$s content of the mutant strain dropped to 30 and 50% of the wild-type levels, respectively.

Conclusions

In this study, we provide evidence that the PI synthase encoded by the *pgsA* gene is an essential enzyme of mycobacteria. The growth arrest of the *pgsA* temperature-sensitive mutant of *M. smegmatis* at 42 °C paralleled the loss of PI and PIMans, showing for the first time that part or all of these molecules and derived products (LM and lipoarabinomannan) serve essential functions in the mycobacterial cell wall. Construction of null mutations in the Rv2611c and mannosyltransferase (Rv2610c) genes will be required to precisely define whether it is PI itself or some of its metabolically derived products that are essential to the bacterium. Finally, because of its localization in the cell envelope, its lack of sequence homology to mammalian PI synthases, its different kinetic characteristics, the existence of selective inhibitors (20), and its essential role in mycobacteria, PI synthase appears to be a good potential drug target for antimycobacterial therapy.

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