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 lipooligosaccharides 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), 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 mycobacteria (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 lipooligosaccharides, are prominent and important phospholipids/lipoglycans. PI and PIMans are regarded as essential for membrane stability and thus for cell viability (3). Lipooligosaccharides are 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 XLI-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 mc2155 (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—Electrocompeetent cells of E. coli XLI-Blue and M. smegmatis mc2155 were prepared as described (22) and

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§ The abbreviations used are: PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PG, phosphatidylglycerol; CL, cardiolipin; PIMan, phosphatidylinositol mannoside; PIManα, phosphatidylglycerol 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’, kanamycin-resistant; Suc, sucrose; Suc’, sucrose-resistant; Str, streptomycin; Str’, streptomycin-resistant.

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electrotransfomed 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 PstI, SauI-, and SmaI-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 below). These plasmids were isolated in the following way: pUCpGspA carrying the M. smegmatis pgsA gene on a 2.0-kb PstI restriction fragment, pUCpGspA.S carrying the pgsA gene on a 2.6-kb SauI restriction fragment, and pUCpGspA.Sm carrying the pgsA gene on a 4.0-kb SmaI 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 previously (23), except that 100 μCi/ml [14C]acetate (specific activity of 54 mCi/mmol; NEN Life Science Products) to A492nm < 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 PstI restriction fragment (vector pUCpGspA.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 SmaI restriction fragment and cloned into the HindIII-cut and blunt-ended PstI vector PXYL4 vector (a pBluescript derivative carrying the suxE and lacZa color-coded marker) (26), yielding plasmid pPGSx. The Km cassette from pUC4K conferring kanamycin resistance and carried on a 1.2-kb HincII fragment was then cloned into the HindIII-cut and blunt-ended pPGSx plasmid, yielding plasmid pPGSxx. Finally, pPGSxK, the construct used for allelic exchange, was obtained by transferring a 4.2-kb BamHI fragment from pPGSxK containing pgsA::Km and yaeI into BamHI cut pBR27, 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 thermostable 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 conditional mutant. Cells harboring these complementing vectors were constructed by inserting the 1.8-kb SmaI restriction fragment from pUCpGspA.P into pBamHI-cut and blunt-ended pCG76. The second complementing vector, pCpGspA, was constructed by cloning the 2.6-kb blunt-ended SauI fragment from pUCpGspA.S into BamHI-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 [3H]inositol as a Substrate—M. smegmatis cultures collected at A600nm = 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 MgCl2). The cell suspension was subjected to 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. 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 (dipalmityl); Sigma), 0.4% CHAPS, 22 μCi of [3H]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 CHCl3/CH2OH (2.1, 3 ml/400 μl of reaction mixture), followed by centrifugation of the separated pellet. The pellet was then washed with 3 ml of CHCl3/CH2OH (2.1). The combined extracts were washed once with 0.9% NaCl (1.2 ml) and once with 1 ml of CHCl3/CH2OH/H2O (3:47:48) to yield the washed CHCl3/CH2OH (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 CHCl3/CH2OH (2.1). The combined CHCl3/CH2OH (2.1) extracts were washed once with 0.9% NaCl and once with CHCl3/CH2OH/H2O (3:47:48) to yield a fraction containing the phospholipids including PIManαβ. When phosphatidylinositol pentamannosides and linear LM were to be extracted, the insoluble pellet resulting from the CHCl3/CH2OH (2.1) extraction was further extracted overnight with CHCl3/CH2OH/H2O (10:10:3). Characterization of the various PIMans was carried out using various ion modes and by fast atom bombardment mass spectrometry analysis. One- and two-dimensional thin-layer chromatography patterns of the intact PIMans (16, 28), sugar analysis by gas chromatography, and fatty acid analysis by gas chromatography-mass spectrometry were performed 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 PIMαβs and LysyPIMαβs were analyzed.
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 F254 (Merck, Darmstadt, Germany). Solvents used for the analysis of phospholipids and PIMan-s were CHCl3/CH3OH/CH2O/HCOOH/H2O (35:15:8:2:0.3) and CHCl3/CH3OH/NH4OH/H2O (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-s. Two-dimensional TLCs were performed using solvent CHCl3/CH3OH/H2O (60:36:6) in the first dimension and solvent CHCl3/CH3OH/CH2O/HCOOH/H2O (40:25:3:6:3) in the second dimension. An α-naphthol spray (1% α-naphthol in ethanol), a Dittmer-Lester spray (30%), and a cupric sulfate spray (10% CuSO4 in 8% phosphoric acid solution) were used to detect carbohydrate-containing spots. The 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.

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 after radiolabeled 32P-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.

TABLE I

Phospholipid composition of M. smegmatis control and recombinant strains

| mcP/V16 | mcP/VpgsA | mcP/VpgsA2 | mcP/VpgsA3 |
|---------|-----------|------------|------------|
| %       | %         | %          | %          |
| Origin  | 2.8       | 2.3        | 1.9        | 7.2         |
| PIMan-s | 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        |

The 32P-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.

Fig. 1. Alignment of the sequences conserved in phosphatidylinositol synthases (PSS), phosphatidylglycerophosphate synthases (PGPS), and phosphatidylinositol synthases (PIS). The 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, GenBankTM/EBI Data Bank accession number D80822 (residues 31–80); and phosphatidylglycerophosphate synthase, accession number U87792 (residues 46–95); Eco, Escherichia coli (phosphatidylglycerophosphate synthase, accession number Z14127 (residues 116–165); and PI synthase, accession number M12299 (residues 35–84); Saccharomyces cerevisiae (PI synthetase, accession number D82982 (residues 36–85); Hsa, Homo sapiens (PI synthetase, accession number NM_006319 (residues 36–85); and M. tuberculosis (PS synthase, Rv0436c (residues 43–92); phosphatidylglycerophosphate synthase, Rv2746c (residues 62–111); and PI synthase, Rv2612c (residues 57–106) (31); Mle, Mycobacterium leprae (PS synthase, accession number AL035159 (residues 43–92); phosphatidylglycerophosphate synthase, accession number S72934 (residues 43–92); and PI synthetase, accession number AF265568 (residues 57–106).
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. 1b). Overproduction of PG thus appeared to be the cause of the slow growth of strain mc2/pVVpgsA3. The distribution of 32P1 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 mc2/pVVpgsA recombinant strain exhibited a PI synthase activity that was 2–2.5-fold greater than that of the other strains (Fig. 2a). A comparative time course of PI synthesis by the crude cell wall preparations of mc2/pVVpgsA and mc2/pVV16 further reflected the overproduction of PI synthase in the recombinant strain mc2/pVVpgsA (Fig. 2b), 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 was used in all subsequent homologous recombination experiments was cloned on a 2.0-kb PstI restriction fragment, a 2.6-kb SalI restriction fragment, and a 4.0-kb Smal fragment as described under “Experimental Procedures.” The fact that the M. tuberculosis pgsA gene gave only one signal when probed on hybridization membranes carrying PstI- or Smal-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 GenBankTM/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 Rv2611c located downstream of the pgsA gene was not determined and deposited in the GenBankTM/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 Rv2611c located downstream of the pgsA gene was not determined and deposited in the GenBankTM/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. 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**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 liquid medium at 30 °C prior to plating onto 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, lane 9). This clone was named mcCSU01. The remaining 24 clones resulted from illegitimate recombination events (data not shown). The genetic organization of the pgsA region mcCSU01 is shown in Fig. 3b. 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 mcCSU01 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 mc2/pVV16 control strain (data not shown). On plates, mcCSU01 colonies appeared after 6 days (instead of 3 days for the control strain) and had a dryer and rougher aspect than the mc2/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 mcCSU01 (Fig. 4). The analysis clearly showed that although mcCSU01 contains all of the basic phospholipids found in the mc2/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 (C16/C19) (28). In the positive ion mode, the compound afforded a dominant fragment ion at m/z 371, corresponding to lysomonoacylglycerol carrying a C19 fatty acid chain. These data thus suggest that the missing compound in strain mcCSU01 is lyso-PIMan2. Since triacylated forms of PIMan2 were found in mcCSU01 and characterized by TLC and sugar analysis (see “Experimental Procedures”), the lack of lyso-PIMan2 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 C19 fatty acid chain found at position sn-2) (3) and thus involved in the synthesis of lyso-PIMan2, a possi-
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Total lipids from mc^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^CSU01.

Fig. 4. Evidence for the lack of lyso-PIMan_2 in the single crossover strain mc^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^CSU01.

Fig. 5. Growth characteristics and lipid composition of the pgsA conditional mutant mc^CSU03 incubated at 30 and 42 °C. Shown are growth curves of strains mc^CSU02 (A), mc^CSU03 (B), and mc^2/pMV261 (C) incubated at 30 °C in LB-Km medium as static cultures (a). Saturated mc^CSU03 and mc^2/pMV261 (control strain) cultures grown at 30 °C were diluted in fresh LB-Km medium to A_600 = 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^CSU03. CFUs, colony forming units.
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−Suc−Str−XylE− clones obtained upon plating of mc2CSU02/pCG76 had undergone a second crossover event at the pgsA chromosomal locus (Fig. 3d, 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 mc2CSU02/pCGpis.1 and mc2CSU02/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 mc2CSU02 and mc2CSU02/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 mc2CSU02 (mc2CSU02/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 mc2CSU02/pCGpis.1 or mc2CSU02/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, mc2CSU02 complemented with pCGpis.2 is more likely to undergo gene replacement at the pgsA locus than mc2CSU02/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 mc2CSU03) 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 mc2CSU03 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 mc2CSU03 exhibited the same growth characteristics as the control strain mc2/pMV261 and as the single crossover strain mc2CSU02. 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. 5b, the number of viable mc2CSU03 cells started to decline after 8 h. In contrast, the control strain continued to grow exponentially. The growth of mc2CSU03 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 mc2CSU03 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 mc2CSU03 strain placed at 42 °C for different periods of time revealed that a dramatic loss of PI and PIMa was achieved in this strain (Fig. 5c). [14C]Acetate labeling of the lipids of mc2CSU03 placed at 42 °C allowed a quantification of this loss and showed that bacterial growth ceased after 8 h when the PI and PIMa 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 PIMa, 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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