Role of the pks15/1 Gene in the Biosynthesis of Phenolglycolipids in the Mycobacterium tuberculosis Complex

EVIDENCE THAT ALL STRAINS SYNTHESIZE GLYCOSYLATED p-HYDROXYBENZOIC METHYL ESTERS AND THAT STRAINS DEVOID OF PHENOLGLYCOLIPIDS HARБOR A FRAMESHIFT MUTATION IN THE pks15/1 GENE*

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Diesters of phthiocerol and phenolphthiocerol are important virulence factors of Mycobacterium tuberculosis and Mycobacterium leprae, the two main mycobacterial pathogens in humans. They are both long-chain β-diols, and their biosynthetic pathway is beginning to be elucidated. Although the two classes of molecules share a common lipid core, phthiocerol diesters have been found in all the strains of the M. tuberculosis complex examined although phenolphthiocerol diesters are produced by only a few groups of strains. To address the question of the origin of this diversity 8 reference strains and 10 clinical isolates of M. tuberculosis were analyzed. We report the presence of glycosylated p-hydroxybenzoic acid methyl esters, structurally related to the type-specific phenolphthiocerol glycolipids, in the culture media of all reference strains of M. tuberculosis, suggesting that the strains devoid of phenolphthiocerol derivatives are unable to elongate the putative p-hydroxybenzoic acid precursor. We also show that all the strains of M. tuberculosis examined and deficient in the production of phenolphthiocerol derivatives are natural mutants with a frameshift mutation in pks15/1 whereas a single open reading frame for pks15/1 is found in Mycobacterium bovis BCG, M. leprae, and strains of M. tuberculosis that produce phenolphthiocerol derivatives. Complementation of the H37Rv strain of M. tuberculosis, which is devoid of phenolphthiocerol derivatives, with the fused pks15/1 gene from M. bovis BCG restored phenolphthiocerol glycolipid production. Conversely, disruption of the pks15/1 gene in M. bovis BCG led to the abolition of the synthesis of type-specific phenolphthiocerol glycolipid. These data indicate that Pks15/1 is involved in the elongation of p-hydroxybenzoic acid to give p-hydroxyphenylalkanoates, which in turn are converted, presumably by the PpsA-E synthase, to phenolphthiocerol derivatives.

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Two distinctive features resides in the variety of lipid compounds with unusual structures, many of them being unique to mycobacteria (1). Consistent with this singularity, genome sequence data for several mycobacterial species reveal that a large portion of the genes may be involved in lipid metabolism (~250 genes in Mycobacterium tuberculosis) (3). Therefore there is a large avenue for future research toward the definition of the biological functions of specific lipids, some of them important for pathogenicity. Understanding the biosynthetic pathways leading to the production of virulence factors or essential components of the cell envelope may help to identify new targets for the development of antituberculous drugs and represents an important post-genomic challenge for the near future.

Among the few genes that have been clearly associated with a defined metabolic pathway in M. tuberculosis and shown to play a role in the pathogenicity of the tubercle bacillus are the cluster of genes involved in the biosynthesis of phthiocerol and phenolphthiocerol dimycocerosates (4–6). These compounds and their relatives are constituents of the mycobacterial cell envelope produced by only some of the slow growers in particular the major pathogens such as Mycobacterium leprae, Mycobacterium ulcerans, Mycobacterium marinum, and members of the M. tuberculosis complex (7). Phthiocerol and its relatives consist of a long-chain β-diol, which occurs naturally as diesters of polymethyl-branched fatty acids (Fig. 1). According to the mycobacterial strain, the asymmetric centers bearing the methyl branches in these fatty acids may be of the D- or the L-series and are known as mycocerosates and phthiocieranates, respectively (7). The mycobacterial species that produce phthiocerol dimycocerosates (DIMs) or phthiocerol diphthioceranates (DIPs) may also syn-
thesize structurally related substances, called phenolphthiocerol and its relatives, in which the lipid core is ω-terminated by an aromatic nucleus, probably derived from p-hydroxybenzoic acid (see Ref. 1). In these substances the hydroxyl group of the phenol moiety is usually glycosylated by a type- or species-specific mono-, tri-, or tetra-saccharide unit (8, 9) leading to phenolphthiocerol glycolipids, also called phenolic glycolipids (PGLs) (Fig. 1). Numerous studies have established the usefulness of PGLs, notably PGL-1 from *M. leprae*, in the serodiagnosis of leprosy and tuberculosis (10–13). In addition some of these glycolipids exhibit biological activities *in vitro* that may be relevant to the pathogenesis of mycobacterial infections. For instance, PGL-1 from *M. leprae* has been reported to inhibit the proliferation of T lymphocytes after stimulation with concanavalin A (14); this observation has been extended to PGLs produced by other mycobacterial species (15). Furthermore, PGL-1 seems to be associated with resistance to intracellular killing by macrophages (16) and promotes phagocytosis of *M. leprae* by macrophages and Schwann cells through binding, respectively, to complement component C3 or laminin-2 (17, 18). Recently, the molecular basis of the interaction between *M. leprae* and Schwann cells was established: the saccharide moiety of PGL-1 binds the α2LG1, α2LG4, and α2LG5 modules of the peripheral nerve laminin α2 chain (18).

The structural similarities between DIMs, DIPs, phenolphthiocerol derivatives, and PGLs have led to the proposal of a biosynthesis pathway for these diols involving a common branch in which two different precursors, either C22-C24 fatty acid or p-hydroxyphenylalkanoic acid, are elongated by three malonyl-CoA and two methylmalonyl-CoA units to yield a common lipid core (Fig. 1) (19). This postulate was supported by the identification in the *Mycobacterium bovis* BCG and *M. tuberculosis* genomes of a group of 5 polyketide synthase genes (named *ppsA-E*) that contain the domains required for these sequential elongation steps. As predicted, disruption of *ppsB* and *ppsC* in *M. bovis* BCG led to a mutant strain unable to synthesize PGLs or DIMs (19). Mycocerosic acids, the class of polymethyl-branched fatty acids that esterify the phthiocerol and phenolphthiocerol of members of the *M. tuberculosis* complex, *M. leprae*, *Mycobacterium kansasi*, and *Mycobacterium gastri* (7), are synthesized by another polyketide synthase, Mas (20, 21). The *mas* and *ppsA-E* genes are clustered on the *M. tuberculosis* chromosome in a region that has been later
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shown to contain 6 other genes encoding acyl-CoA synthase and transporters important for DIM biosynthesis and translocation (4–6, 22). In species that produce DIMs or DIPs, all the strains do so; however many of these strains do not contain phenolphthiocerol derivatives (1, 23). For instance, phenolphthiocerol derivatives are produced by only a few strains of M. tuberculosis, notably those belonging to the subspecies M. tuberculosis Canetti (24, 25). We investigated the intraspecies differences in the production of PGLs in M. tuberculosis. We demonstrate the occurrence of glycosylated p-hydroxybenzoic acid methyl esters structurally related to the type-specific PGLs in the culture media of all the strains of M. tuberculosis, suggesting that the strains devoid of phenolphthiocerol derivatives are unable to elongate the putative p-hydroxybenzoic acid precursor (p-HBA). We also provide evidence that the polypektine synthase gene pks15I is involved in the elongation of p-hydroxybenzoic acid derivatives (p-HBAD) to form p-hydroxyphenylalkanoates, which in turn are converted, presumably by the PpaA-E synthase, to phenolphthiocerol derivatives. This demonstration is based on the observation that: (i) p-hydroxybenzoate derivatives are produced in every tested strain of M. tuberculosis, (ii) that the disruption of the pks15I gene of M. bovis BCG abolished the production of the pbois-specific PGL but not that of DIMs, and (iii) that the production of PGL-tb by M. tuberculosis H37Rv can be restored by introducing the pks15I gene of M. bovis BCG. Consistent with this result is the finding that strains of M. tuberculosis devoid of PGL are natural mutants with a frameshift mutation in pks15I.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Culture Conditions—M. tuberculosis H37Rv (ATCC 27294), H37Ra (ATCC 25177), Erdman (ATCC 35801), B1Rv (ATCC 35818), B1Ra (ATCC 35819), Canetti (Collection Institut Pasteur 140010059), Mt103 (a clinical isolate from Institut Pasteur, Paris), and M. bovis BCG 1173P2 (the Pasteur strain) were grown at 37 °C on Sauton’s medium as surface biofilm for biochemical analyses. M. tuberculosis H37Rv and M. bovis BCG were also grown on Middlebrook 7H9 medium (Difco) supplemented with ADC (0.2% dextrose, 0.5% bovine serum albumin fraction V, 0.0063% beef catalase) and antibiotics (am) where indicated, or on solid Middlebrook 7H11 medium (Difco) supplemented with OADC (0.005% oleic acid, 0.2% dextrose, 0.5% bovine serum albumin fraction V, 0.085% NaCl, 0.0003% beef catalase). Kanamycin (km), hygromycin (Hyg), and sucrose were added when required. The Amplification program consisted of one cycle of 10 min at 95 °C, 1 min at 60 °C, and 1 min at 72 °C, and a final 10 min at 72 °C. The PCR product was analyzed by electrophoresis in 0.8% agarose gel. The ~2.7-kb fragment was gel-purified using the QiAQuick gel extraction purification kit and inserted into the pGEM-T easy vector according to the manufacturer’s recommendations (Promega, Lyon, FR) to give pCIG12. A kanamycin resistance cassette formed by the fkm cassette from pHE4fkm (29) flanked by two res site from transposon yb was inserted between the two XhoI sites of pks15I generating a 1068-bp deletion. The resulting plasmid was named pCG134. The 4.5-kb PmeI fragment from pCG134 containing the disrupted pks15I construct was inserted at the Smal site of pQJ200 (30) leading to pWM8. M. bovis BCG was electrotansformed as previously described, and transformants were selected on OADC (0.005% oleic acid, 0.2% dextrose, 0.5% bovine serum albumin fraction V, 0.085% NaCl, 0.0003% beef catalase), Kana- mycin (km), hygromycin (Hyg), and sucrose were added when required. The Amplification program consisted of one cycle of 10 min at 95 °C followed by 30 cycles of 1 min at 95 °C, 1 min at 55 °C and 1 min at 72 °C, and a final 10 min at 72 °C. One clone gave the pattern corresponding to the insertion of pWM80 via a single homologous recombination event. This strain was renamed PMM1 and grown in 5 ml of 7H9, ADC, km, Tween at 37 °C until saturation. Dilutions of this culture were plated on 7H11, OADC, km, sucrose and incubated at 37 °C for 3 weeks. Four clones were then analyzed further. Saturated 5-ml liquid cultures of these four clones were prepared, and total DNA was extracted. The four DNA preparations were then analyzed using primers pks1C, pks1D, pks1E, res1, and res2. The PCR products were analyzed by electrophoresis in 0.8% agarose gel. The ~3.4-kb and ~2.9-kb fragments were gel-purified using the QiAQuick gel extraction purification kit. The 5′-PCR fragment was digested with BglII and SpeI and inserted between the BamHI and SpeI of pMIP12H to give pWM15. The 3′-PCR fragment was digested with NsiI and SpeI and ligated to pWM15 cut with NsiI and SpeI. The resulting plasmid, pWM18, contains the entire pks15I gene from M. bovis BCG under the control of the pBlAF promoter and 7-bp downstream from a Shine-Dalgarno sequence. In the second strategy, we used the M. bovis BCG BAC library constructed by Brosch et al. (33). An 8-kbp fragment was obtained from BAC X203 following digestion with enzymes NruI and StuI. This fragment containing the entire pks15I gene from M. bovis BCG was gel-purified using QiAQuick gel extraction kit and inserted into the same primers (BglII and SpeI) as above. This fragment was ligated with SpeI and AscI and the 8.3-kbp fragment containing the entire pks15I gene was gel-purified and cloned into pMIP12H digested with SpeI and NdeI. The resulting plasmid, named pPET1, contains the gene pks15I in the same orientation as the pBlAF promoter. The resulting plasmid, named pPET1, contains the gene pks15I in the same orientation as the pBlAF promoter. The resulting plasmid, named pPET1, contains the gene pks15I in the same orientation as the pBlAF promoter. The resulting plasmid, named pPET1, contains the gene pks15I in the same orientation as the pBlAF promoter.
sterilized by filtration through 0.2-μm pore size membrane and concentrated to one-tenth of the initial volumes. The cells were left in CHCl3/CH3OH 2:1, v/v, for 2 days at room temperature to kill bacteria, and lipids were extracted twice with CHCl3/CH3OH 1:1, v/v, washed twice with water, and dried. Lipids from culture media were obtained by adding admixture (TLC). Briefly, the various extracts were dissolved in CHCl3 to give a final lipid concentration of 20 mg/ml. Equivalent volumes of each extract were deposited on silica gel G 60 plates (20 × 20 cm, Merck), which were run in petroleum ether-diethylether (90:10, v/v) and CHCl3/CH3OH (95:5, v/v) for the detection of DIMs and PGLs, respectively. Glycolipids and DIMs were visualized by spraying the plates with 0.2% anthrone (w/v) in concentrated H2SO4 and H2SO4, respectively, following by heating.

Glycolipids were purified as previously described (24). Crude lipid extracts from cells or culture medium were chromatographed on a Florisil (60–100 mesh) column and eluted with a series of concentrations of CH3OH (0, 1, 2, 3, 4, 5, 10, 50, and 100%) in CHCl3. Each fraction was analyzed by TLC on silica gel G 60 plates (0.3 mm, 20 × 20 cm, Merck) using CHCl3/CH3OH, 95:5, v/v as the solvent system. Glycolipids were visualized by spraying the plates with 0.2% anthrone (w/v) in concentrated H2SO4, followed by heating. When necessary, glycolipids were additionally purified by preparative chromatography on silica gel G 60 plates (0.3 mm, 20 × 20 cm, Merck) using CHCl3/CH3OH, 95:5, v/v as the developing solvent.

Nuclear Magnetic Resonance (NMR) Spectroscopy—Chemical shift (δ) values were determined using a Bruker standard pulse-field gradient program cosygpmf (35), with 0.41 s acquisition time, 4096 data points in the F2 dimension and 512 increments in the F1 dimension. The data matrix was zero-filled in the F1 dimension to give a matrix of 4096 × 4096 points, and a shifted sine-bell apodization function was applied prior to Fourier transformation. Similarly, nuclear Overhauser spectroscopy (NOESY) (36) was employed using a Bruker standard pulse-field gradient program inv4gp and inv4gplrnd (34). After a 2 h-period, the mixture was partitioned into two phases by adding 2 volumes of CH3OH and 1 volume of CHCl3 to 0.8 volume of their per-methylated derivatives using the conventional Hakomori procedure (33). Briefly, the reaction was incubated for 2 h and stopped by adding 1 ml of H2O and sodium thiosulfate. Fatty acid trideuteriomethyl esters and per-O-deuteriomethylated substances of the phenolphthiocerol family were extracted from CHCl3, washed with water, dried under nitrogen, and dissolved in diethyl ether for analysis by GC and GC/MS.

GC and GC/MS Analysis—GC analyses were performed using a Girdel series 30 instrument equipped with an OV1 capillary column (0.3 mm x 25 m) using helium gas (0.7 bar) with a flame ionization detector at 310 °C. The temperature program was from 60 to 310 °C, at 5 °C/min. GC/MS analyses were performed on a Hewlett-Packard 5890 X mass spectrometer (electron energy, 70 eV) working in both electron impact (EI) and chemical ionization (CI) modes using NH3 as the reagent gas (CINH3), coupled with a Hewlett-Packard 5890 series II gas chromatograph fitted with a similar OV1 column (0.3 mm x 12 m). Infrared (IR) and Ultraviolet (UV) Spectroscopy—IR and UV spectroscopy were performed using a PerkinElmer model 177 machine and an UVikon 925 Double Beam UV/VIS spectrophotometer (Kontron instruments), respectively.

RESULTS

Evidence for the Production of p-Hydroxybenzoate Derivatives by M. tuberculosis—All strains of M. tuberculosis examined to date synthesize DIMs (41, 7), but only few of them produce the structurally related PGLa (24, 25, 42, 43). However many sera from tuberculous patients in diverse geographic regions contain antibodies specifically recognizing the major PGL of the Canetti subspecies (PGL-tb) (11–13). The seroreaction with glycolipids is dependent on the carbohydrate moiety of the lipid antigens (10), and therefore the presence of DIMs and glycolipids by the various strains was analyzed by thin-layer chromatography (TLC). Briefly, the various extracts were dissolved in diethyl ether for analysis by GC and GC/MS.

The Na/K exchange reaction was performed by mixing 5 μl of sample (dissolved in chloroform) with 2 μl of a 10% KI solution in CH3OH/H2O, 7:3. The mixture was then directly analyzed by MALDI-MS.

The various constituents of PGL were structurally characterized as their per-O-methylated derivatives using the conventional Hakomori procedure (33). Briefly, the reaction was incubated for 2 h and stopped by adding 1 ml of H2O and sodium thiosulfate. Fatty acid trideuteriomethyl esters and per-O-deuteriomethylated substances of the phenolphthiocerol family were extracted from CHCl3, washed with water, dried under nitrogen, and dissolved in diethyl ether for analysis by GC and GC/MS.
fractionated in culture media (C). Lipid extracts dissolved in CHCl₃ were run in CHCl₃/CH₃OH (95:5, v/v). Glycolipids were visualized and cells (Cₜₚₘ) and purification of the two major A-Spectra were obtained in CHCl₃ (7.24 ppm).

Concentrations of CH₃OH (0, 1, 2, 3, 4, 5, 10, 50, and 100%) in CHCl₃ were analyzed by TLC using CHCl₃/CH₃OH, 95:5 (v/v) as the solvent system.

Extracts. TLC area where spots corresponding to expected PGL are indicated. B. Lipid fractions eluted from a Florisil column with a series of concentrations of CH₃OH (0, 1, 2, 3, 4, 5, 10, 50, and 100%) in CHCl₃ were analyzed by TLC using CHCl₃/CH₃OH, 95:5 (v/v) as the solvent system.

PGL-tb, p-hydroxybenzoate II (data not shown) confirmed the nature of the sugar residues in the two PGL-tb and established the 1→3 α-linkages between the three sugar units constituting PGL-tb. The molecular mass of the native PGL-tb was determined by MALDI-TOF mass spectrometry (43). p-HBAD-II exhibited an ion peak at m/z 669 Da. To determine the composition of this peak, the sample was mixed with potassium iodide, resulting in a 16 mass-unit shift (peak at m/z 685). This indicated that the observed mass corresponded to pseudomolecular mass and established a molecular mass of 646 Da for the native p-HBAD-II.

Comparative analyses of the two-dimensional COSY-DQF ¹H NMR spectra of peracetylated PGL-tb (44), p-HBAD-I, and p-HBAD-II (data not shown) confirmed the nature of the sugar residues in the two p-HBADs and established the 1→3 α-linkages between the three sugar units constituting p-HBAD-II. The molecular mass of the native p-HBA-II was determined by MALDI-TOF mass spectrometry (38). p-HBA-II exhibited an ion peak at m/z 669 Da. To determine the composition of this peak, the sample was mixed with potassium iodide, resulting in a 16 mass-unit shift (peak at m/z 685). This indicated that the observed mass corresponded to pseudomolecular mass and established a molecular mass of 646 Da for the native p-HBA-II. Following transesterification, using sodium ethanolate, a 14 mass-unit shift of the pseudomolecular mass of p-HBA-II was observed (683 Da instead of 669 Da), demonstrating the presence of a methyl ester in the native p-HBA-II molecule. This ester function in the proximity of the phenolic group explains the observed deshielding of the phenolic proton resonances (Fig. 3A), relative to those of PGL-tb (Fig. 3B). Following trimethylsilylation, a pseudomolecular mass of 885 Da was found for the per-TMS p-HBAD-II, indicating the presence of 3 TMS linked to 3 free hydroxyl groups of the sugar moiety. The structure of p-HBAD-II can thus be established.
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Fig. 4. Scheme of the analytical techniques used to establish the structure of p-HBAD-I and p-HBAD-II.

(Fig. 4) as tri-O-methyl-fucosyl-(α1→3)-rhamnosyl-(α1→3)-2-O-methyl-rhamnosyl- α-p-hydroxybenzoic acid methyl ester, the glycosylated phenolic moiety of PGL-tb (24). Similarly, experiments with p-HBAD-I (data not shown) demonstrated that its structure, 2-O-methyl-rhamnosyl- α-p-hydroxybenzoic acid methyl ester, is identical to the glycosylated phenolic moiety of the major PGL from M. bovis also present in PGL-producing M. tuberculosis strains as a minor compound (7).

Pks1/15 May Be Involved in the Biosynthesis of Phenolphthiocerol—Since all the strains of M. tuberculosis examined produced both DIMs and p-HBADs, the main reason that now explains the lack of production of PGLs by most strains of M. tuberculosis resides in the fact that these strains cannot elongate p-HBA to produce PGLs, due to mutations in gene(s) coding for the elongation enzyme(s). The proposed biosynthetic pathway leading to phenolphthiocerol (45) involves the elongation of p-HBA by an enzyme that adds eight or nine malonyl-CoA units to form p-hydroxyphenylalkanoic acid, the putative substrate of PpsA-E (Fig. 1). These reactions may be catalyzed by either fatty acid synthase (FAS) or a specific polyketide synthase (Pks). If a Pks is involved in this catalysis, the enzyme would need to contain a β-ketoacyl synthase domain (KS), an acyltransferase domain (AT), a β-ketoacyl reductase domain (KR), a deshydratase domain (DH), an enoylreductase domain (ER), and an acylcarrier protein domain (ACP). Bioinformatic scanning of the M. tuberculosis pks genes revealed several candidates: pks15/1, pks2, pks3/4, pks5, pks7, pks8/17, pks12, ppsC, and mas. Three of these have been demonstrated to be involved in the biosynthesis of known compounds: mycoserosic acids for mas (21, 20), pthioceranic and hydroxyphthioceranic acids for pks2 (46), and the late steps in the biosynthesis of phthiocerol and phenolphthiocerol for ppsC (19). We focused on pks15/1 because (i) its two adjacent open reading frames (ORFs) map on M. tuberculosis chromosome close to the gene cluster involved in the biosynthesis of the structurally related DIMs, including several genes shown to be involved in the biosynthesis of PGL in M. bovis BCG (3, 4); (ii) pks15/1 and the adjacent region is highly conserved in M. leprae (85% identity at the amino acid level over the full-length putative Pks15 and Pks1 proteins), a species that produces PGL-1 (Table I); and (iii) in M. leprae there is a single fused pks15/1 ORF whereas in M. tuberculosis H37Rv the two are separate. Accordingly, we speculated that when fused, Pks15/1 may be involved in the elongation of p-HBA with malonyl-CoA to yield p-hydroxyphenylalkanoic acid. To test this hypothesis, we compared the DNA sequence corresponding to the end of pks15 and beginning of pks1 for various reference strains of M. tuberculosis and M. bovis. A 520-bp fragment was amplified by PCR from the pks15/1 junction region of four strains, sequenced, and compared with available sequences (Fig. 5). Sequence alignments showed, in M. tuberculosis H37Rv, Mt103, CDC1551, and Erdman, when compared with the M. bovis sequence, the deletion of a single base, a guanine, causing a frameshift and thus the two ORFs for pks15/1. In contrast, M. bovis strain AF2122/97 (spoligotype 9), M. bovis BCG, M. tuberculosis (strain 210), and M. tuberculosis Canetti exhibited no frameshift. Compared with the M. bovis sequence, however, an insert of 6 bp is present in the pks15/1 ORFs of the last two strains of M. tuberculosis. Importantly, this insertion does not cause a frameshift in pks15/1 but adds two codons. M. tuberculosis H37Rv, Mt103, and Erdman do not produce PGLs whereas M. bovis BCG and M. tuberculosis Canetti do (Fig. 2). PGL production thus correlates with the presence of a single ORF for pks15/1. Consequently, the observed frameshift mutation in strains devoid of PGLs may have led to the inactivation of pks15/1, which in turn may have caused the inability of the strains to produce phenolphthiocerol derivatives. To ascertain this hypothesis, the DNA sequence corresponding to the end of pks15 and beginning of pks1 of ten clinical isolates of M. tuberculosis were sequenced, and the production of PGLs by the strains was determined. As expected, the nine strains that do not produce PGL had a deletion of a single base, leading to a frameshift, and an occurrence of the two ORFs for pks15/1. In contrast, the only strain that does synthesize PGL-tb exhibited a Canetti-like sequence (data not shown). It was thus concluded that strains of M. tuberculosis devoid of PGL are natural mutants with frameshift mutation in pks15/1.

Complementation of M. tuberculosis H37Rv—Assuming that...
a frameshift mutation has occurred in strains of the M. tuberculosis complex that do not produce PGL and has led to the inactivation of pks15/1, it should be possible to complement these strains for the production of PGL. The representative H37Rv strain of M. tuberculosis was used to test this hypothesis. The full-length pks15/1 gene from M. bovis BCG was placed under the control of the strong mycobacterial promoter pBlaf in the shuttle plasmid, pMIP12H. The resulting plasmids, pWM16 or pPET1, were used for electroporation of H37Rv. The transformants were grown in liquid culture, and the plasmids (14 kb for pWM16 and 16 kb for pPET1) were used for electrotransformation of their lipid content was determined by TLC analysis (Fig. 6A). Unlike their parent strain, several transformants (2 of 4 for pWM16 and 2 of 2 for pPET1) produced a major glycoconjugate with mobility similar to that of PGL-tb from the Canetti strain of M. tuberculosis. The conflicting results with different transformants from the same transformation experiment were found to result from plasmid rearrangements, probably due to the size of the plasmids (14 kb for pWM16 and 16 kb for pPET1) (data not shown). The glycolipid was purified from the transformants. Acid methanolysis of the transformant glycolipids and PGL-tb, followed by trimethylsilylation and GC analysis showed that both compounds were composed of tri-O-methylfucose, rhamnose, and 2-O-methyl-rhamnose. MALDI-TOF mass spectrometry analysis confirmed the loss of a tetra-O-methyl-trisaccharide moiety from both compounds and still showed the same 28 mass unit difference between the two phenolphthiocerol dimycocerosate moieties of the molecules (data not shown). Analysis of the per-O-methylation products of the two phenolphthiocerol dimycocerosates by GC identified the major fatty acyl components of both molecules as C29 (n, n' = 18; p, p' = 3; Fig. 1). The phenolphthiocerol from PGL-tb consisted most exclusively of C35 (m2 = 15; Fig. 1) whereas that from the transformants was composed of an equimolar amount of C35 (m2 = 15; Fig. 1) and C37 (m2 = 17; Fig. 1). Thus, the difference in the phenolphthiocerol chain length explains the observed 28 mass unit difference between the molecular masses of the two PGLs. We concluded that the complementation of a M. tuberculosis strain defective in the production of PGL by the pks15/1 gene from M. bovis BCG fully restores the production of the glycolipid.

Disruption of pks15/1 in M. bovis BCG by Allelic Exchange and Biochemical Characterization of the M. bovis BCG pks15/1::km Mutant—To further establish the involvement of pks15/1 in the biosynthesis of phenolphthiocerol-derived lipids, we constructed a pks15/1-inactivated mutant of M. bovis BCG. A kanamycin resistance cassette flanked by arms, 1 kb and 800 bp identical to parts of pks15/1, was cloned into the vector pJQ200 to give plasmid pWM08. This construct was trans-
ferred by electroporation into *M. bovis* BCG. A total of 76 kmR transformants were obtained in three different experiments and screened by PCR using primers pks1C/H11001
pks1D, pks1C/H11001/H9024
km1, or pks1E/H11001/H9024
km2. One clone, named PMM1, gave an amplification pattern consistent with insertion of the plasmid by a single recombination event at the pks15/locus, i.e.
no signal with pks1C/H11001
pks1D, pks1E/H11001
res1 and a 1.4-kb PCR product with primers pks1C/H11001/H9024
km1 or pks1C/H11001
res2 (Fig. 8). The second homologous recombination event was selected by plating serial dilutions of a PMM1 culture on solid medium containing kanamycin and sucrose. PCR analysis of four isolates from these counterselective plates using pks1C+pks1D, pks1C+res2, or pks1E+res1 primers revealed that two of them corresponded to an allelic replacement of the wild-type allele of pks15/1 by the disrupted allele (Fig. 8). This was confirmed by Southern hybridization analysis (data not shown). One of these strains, named PMM3, was analyzed by TLC for the production of PGL. As expected, PMM3 did not produce the specific PGL of *M. bovis* (Fig. 6) but still produced amounts of DIMs comparable to those of the parent strain. These data demonstrate that in *M. bovis* pks15/1 is essential for the biosynthesis of PGLs but is not required for DIM synthesis.

**DISCUSSION**

The main objective of the present study was to decipher the biosynthesis of PGL in mycobacteria, thereby to determine why most strains of *M. tuberculosis* are unable to synthesize this glycolipid although they produce the structurally related DIM. We constructed a pks15/1-inactivated mutant of *M. bovis* BCG, and it was deficient in the production of PGL. Complementation with pks15/1 from *M. bovis* BCG endowed *M. tuberculosis* strain H37Rv with the ability to produce PGL. Thus, Pks15/1 is involved in the biosynthesis of PGL in members of the *M. tuberculosis* complex. This finding is also supported by the demonstration that the strains of *M. tuberculosis* that do not produce PGL contain a frameshift mutation in pks15/1. We propose that Pks15/1 catalyzes the elongation of p-hydroxybenzoic acid with malonyl-CoA units to form p-hydroxyphenylalkanoic acid derivatives, which in turn are used by PpsA-E to yield phenolphthiocerol and its relatives (Fig. 1). This proposal is consistent with several observations. First, Pks15/1 contains all the domains (KS, AT, KR, DH, ER, ACP) required for the catalysis of this reaction. Second, the *M. bovis* BCG pks15/1::km mutant synthesizes amounts of DIM similar to those produced by the wild-type strain, showing that the enzymatic machinery required for the production of both mycocerosic acids and the lipid core shared by phthiocerol and phenolphthiocerol is still functional in the mutant strain. Third, glycosylated p-hydroxybenzoic acid methyl esters are found in all the strains, including those devoid of PGL, suggesting that p-hydroxybenzoic acid is available. This latter finding raises the question of whether the glycosylation of phenolphthiocerol in PGLs occurs on the p-hydroxybenzoic acid, on the p-hydroxyphenylalkanoic acid or on the phenolphthiocerol dimycoserosates. Thurman et al. (47) showed that aglycosyl compounds, phenolphthiocerol and phenolphthiadiolone dimycoserosates, re-
covered from the lipid extracts of *M. microti* could be glycosylated by acellular extracts of the bacterium, suggesting that the glycosylation is the final step in the biosynthetic pathway of PGL. In contrast, we isolated glycosylated \(p\)-hydroxybenzoic acid methyl esters from the culture fluids of *M. tuberculosis*, indicating that the glycosylation may be an early step of the biosynthesis of PGLs. This issue remains to be clarified. In this respect, it is noteworthy that \(pks15/1\) maps on the *M. tuberculosis* chromosome very close to genes encoding enzymes involved in other steps of the PGL biosynthetic pathway in particular \(mas\) and \(fadD28\). The genetic organization of this genomic region is well conserved between *M. tuberculosis* and *M. leprae* and the gene sequences are also very similar (Table I). Some of these genes encode two putative methyltransferases and three glycosyltransferases (two in *M. leprae*), both classes of enzymes being required for the transfer and modification of the carbohydrate moieties of PGLs. Therefore these genes may also be involved in the biosynthesis of PGLs. The inactivation of these genes and biochemical characterization of the resulting mutants should elucidate this issue.

An important issue raised by the isolation of glycosylated \(p\)-hydroxybenzoic acid methyl esters from the culture fluids of *M. tuberculosis* is whether these compounds are genuine intermediates in the biosynthesis of phenol glycolipids. Three lines of evidence suggest that this is not the case. First, biosynthetic intermediates are expected to be found either in the cytosol or the plasma membrane where the enzymatic machinery is postulated to be located. Second, in all known pathways that
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involve fatty acid synthases or polyketide synthases, the acid function of the substrate is esterified either by an acyl carrier proteins or coenzyme A before being transferred onto the enzyme. Therefore the methyl found in p-HBAD is likely to prevent the use of these molecules as substrate for Pks15/1. Third, the production of PGL-tb by M. tuberculosis H37Rv complemented by pks15/1 from M. bovis BCG does not abolish the secretion of p-HBAD into the culture filtrate of the strain. As a consequence we propose that p-HBAD are final products derived from a precursor of the phenolglycolipids biosynthetic pathway.

The finding that strains of M. tuberculosis devoid of PGL-tb are natural mutants of pks15/1 deserves consideration. M. bovis and M. tuberculosis strains can be grouped into three clusters according to the sequence of the pks15/1 junction. The first group is composed of M. bovis BCG and M. bovis strain AF2122/97 (spoligotype 9) and exhibits a single ORF for pks15/1 allowing (at least in the case of M. bovis BCG) the expression of a functional synthase. The second group consists of strains H37Rv, CDC1551, Erdman, and M103 of M. tuberculosis and contains a deletion of a single nucleotide leading to a frameshift mutation knocking out the pks15/1 gene. The last group, including M. tuberculosis Canetti and strain 210, carries an insertion of 6 nucleotides not found in M. bovis BCG, resulting in the expression of a functional Pks15/1 synthase with two extra amino acids. Each group includes both strains that have been maintained in laboratories for many decades and recent clinical isolates, showing that inactivation of pks15/1 cannot be solely a consequence of repeated passages on synthetic media. Sreevatsan et al. (48) proposed an evolutionary scenario for the strains of the M. tuberculosis complex and have classified the strains into four groups; strains of group I, like those belonging to the group of M. bovis, are descended from a common ancestor whereas strains of groups 2 and 3 have sequentially diverged from group I. Brosch et al. (49) confirmed the lineage for M. tuberculosis but suggest that, before the appearance of any other member of the M. tuberculosis complex, M. tuberculosis strain Canetti diverged from the common ancestor of the M. tuberculosis strains of today. Beijing strains, which include strain 210, belong to the first group whereas strains CDC1551 and Erdman of M. tuberculosis are classified in group 2 and strain H37Rv in group 3. If the evolutionary scenario proposed by Brosch et al. (49) is correct then, strains with frameshift mutations would have arisen by deletion of 7 nucleotides of a group I type strain whereas M. bovis strains would have arisen from a 6-bp deletion in a common ancestor.

Numerous roles have been attributed to PGLs, notably PGL-1 from M. leprae. It has been implicated in several processes at different steps of the infection cycle: tropism for Schwann cells or phagocytes; the resistance of the leprosy bacillus to intracellular killing; and immunomodulatory activities (18, 50, 14). However, analysis of the contribution of PGL-1 to the pathogenicity of M. leprae has been hampered by the failure of the bacterium to grow on laboratory media and its extremely slow growth in animal models. In this context, recombinant strains of the M. tuberculosis complex expressing the PGL-1 of M. leprae and analysis of these strains in various models may be the best way to study the role of this glycolipid in the pathogenesis of leprosy. In other PGL-producing mycobacterial pathogens, the role of these molecules in mycobacterial virulence is poorly documented. Many clinical isolates of M. tuberculosis do not produce PGL, and this does not suggest a role of PGL-tb in the pathogenesis of tuberculosis. However, pathogenicity and virulence are complex phenotypic traits. For example the lack of a defined virulence factor may be compensated by the overproduction of other factors. Epidemiological data and laboratory work show that the different clinical isolates from M. tuberculosis do not exhibit all the same virulence or dissemination patterns (51, 52). Accordingly, the generation of mutants devoid of PGL-tb, and investigations of their virulence and the histological features of infected animals would clarify the contribution of PGL-tb to the pathogenicity of M. tuberculosis. The observation that p-HBAD are present in the culture fluids of all the strains of M. tuberculosis examined raises the question of their role in virulence of the tubercle bacillus. These compounds are properly located to interact with host cells and contain the oligosaccharide moiety of PGL, which seems to be essential for the various activities found for the PGL of M. leprae (18, 50, 14). Once again the best way to address this question will be the generation and characterization of M. tuberculosis mutants devoid of p-HBAD. To this regard, the identification of genes likely to be involved in the biosynthesis of these compounds (Table I) opens the way for such studies. Besides, the occurrence of p-HBAD in M. tuberculosis and the elucidation of the structure of its glycosylated moiety that constitutes the epitopes recognized by antibodies (10) now explains the reactivity of most tuberculous sera to PGL-tb (11, 12, 13) despite the small percentage of clinical isolates that produce PGL-tb.

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