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Attenuation of *Mycobacterium tuberculosis* by Disruption of a *mas*-Like Gene or a Chalcone Synthase-Like Gene, Which Causes Deficiency in Dimycocerosyl Phthiocerol Synthesis

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Tuberculosis is one of the leading preventable causes of death. Emergence of drug-resistant tuberculosis makes the discovery of new targets for antimycobacterial drugs critical. The unique mycobacterial cell wall lipids are known to play an important role in pathogenesis, and therefore the genes responsible for their biosynthesis offer potential new targets. To assess the possible role of some of the genes potentially involved in cell wall lipid synthesis, we disrupted a *mas*-like gene, *msl7*, and a chalcone synthase-like gene, *pks10*, with phage-mediated delivery of the disruption construct, in which the target gene was disrupted by replacement of an internal segment with the hygromycin resistance gene (*hyg*). Gene disruption by allelic exchange in the case of each disruptant was confirmed by PCR and Southern blot analyses. Neither *msl7* nor *pks10* mutants could produce dimycocerosyl phthiocerol, although both could produce mycocerosic acids. Thus, it is concluded that these gene products are involved in the biosynthesis of phthiocerol. Both mutants were found to be attenuated in a murine model, supporting the hypothesis that dimycocerosyl phthiocerol is a virulence factor and thus the many steps involved in its biosynthesis offer potential novel targets for antimycobacterial therapy.

Two billion people are latently infected with *Mycobacterium tuberculosis*, and 5 to 10% of them will develop active tuberculosis at some time during their life (World Health Organization, http://www.who.int/gtb/publications/globrep01/, 2001). Currently 8 million new cases of active tuberculosis are diagnosed annually, with 2 million deaths per year. With the advent of multidrug-resistant tuberculosis, this disease has become a major public health problem. The Centers for Disease Control and Prevention have designated drug-resistant *M. tuberculosis* a class C organism, with the potential to create major public health problems in population centers. Effective defense against this pathogen requires identification of new targets for development of antimycobacterial drugs. *M. tuberculosis* is a highly successful pathogen because it can evade host defenses and mycobacterial drugs. The unusually lipid-rich (50 to 60%) cell walls of this pathogen constitute an impermeable barrier that helps the pathogen enter monocytes/macrophages and grow in them (4, 9, 16, 18). Thus, it is not surprising that this pathogen contains a large number of genes involved in lipid synthesis.

Identification of the genes involved in biosynthesis of the unique cell wall lipids of mycobacteria started with the cloning of the mycocerosic acid synthase (*mas*) gene (17). In this case, the cloning was done based on the amino acid sequence of the purified multifunctional enzyme that catalyzes the elongation of *n*-C_{20} fatty acid (and homologues) to generate a major class of fatty acids called mycocerosic acids (20). These acids are esterified to a long-chain diol, phthiocerol, to generate dimycocerosyl phthiocerol (DIM), which has been found to be a virulence factor (5, 8, 22). In some mycobacterial species, including some clinical isolates of *M. tuberculosis*, they are also esterified to phenolphthiocerol, which is glycosylated to produce mycosides (9, 15) (Fig. 1).

A plausible pathway for the biosynthesis of phthiocerol has been postulated on the basis of biochemical analogies. Based on the nature of the reactions that must be involved in this synthesis, a set of multifunctional proteins have been postulated to be involved in this process (15). On the basis of the catalytic domains required to catalyze such a sequence of reactions, a segment of mycobacterial genome that could encode such proteins was found. Strong support for the involvement of this segment of the genome in DIM synthesis was provided when it was demonstrated that disruption of this gene segment in *Mycobacterium bovis* BCG in fact prevented the synthesis of DIM (2). Transposon mutagenesis confirmed this conclusion also for *M. tuberculosis* (5, 8). However, the enzymology of biosynthesis of DIM has not been elucidated, and not all of the genes involved in the synthesis of DIM have been identified.

The mycobacterial genome contains a large number of *pks* genes, including *mas*-like genes (*msl*). It also contains several chalcone synthase-like genes that have been found only in plants until the recent discovery of bacterial homologues (7, 13, 19). *pks10* is one such gene found in *M. tuberculosis*. To examine the possible biological functions of *pks1*/*pks15* and *pks10*, we used a gene disruption approach. In this paper, we report that disruptions of *msl7* (*pks1* and *pks15*) and a chalcone synthase-like gene (*pks10*) in *M. tuberculosis* results in DIM deficiency and attenuation.

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MATERIALS AND METHODS

Bacterial strains and culture conditions. *M. tuberculosis* H37Rv (ATCC 25618) was grown either in Middlebrook 7H9 broth supplemented with 10%

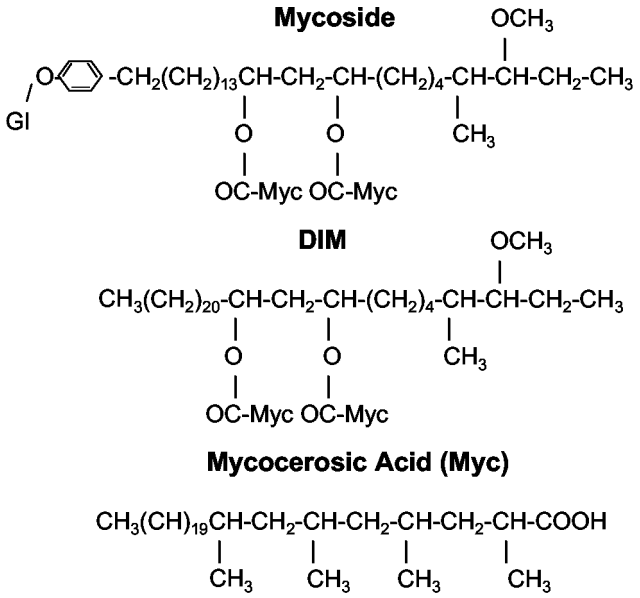

FIG. 1. Structure of dimycocerosyl esters of glycosylated (Gl) phenolphthiocerol (mycoside) and phthiocerol (DIM).

OADC (oleic acid, albumin, and dextrose; 7H9-OADC; BBL) enrichment plus 0.2% glucose and 0.05% Tween 80 at 37°C in roller bottles or on Middlebrook 7H10-OADC agar plates incubated at 37°C in sealed plastic bags. *Mycobacterium smegmatis* mc^{2}155 was grown in liquid Luria-Bertani (LB) medium with 0.05% Tween 80 for competent cell preparation and in Middlebrook 7H9 broth (Difco) with 0.05% Tween 80 for transduction. *Escherichia coli* DH5α (Life Technology) and HB101 were used as host strains for cloning experiments and were grown on Luria-Bertani broth or agar. When required, antibiotics were added to the culture media at the following concentrations: ampicillin, 100 μg/ml for *E. coli*; hygromycin B, 150 μg/ml for *E. coli* and 50 μg/ml for *M. tuberculosis*.

General DNA techniques. All recombinant DNA techniques were performed according to standard procedures (21). DNA restriction and modifying enzymes were obtained from New England Biolabs (Beverly, Mass.) and used according to the manufacturer's suggestions.

Generation of disruption constructs for *msl7* and a chalcone synthase-like gene (*pks10*). The general strategy used for gene disruption with the specialized transducing phage system (10) was similar to that used previously for other *msl* genes (11, 22).

In order to make a disruption construct for *msl7* (*pks1* [Rv2946c] plus *pks15* [Rv2947c]), a 2,937-bp PCR product containing part of the *pks1* gene (bp 1231 to 4168 of the *pks1* coding region, including the dehydratase, enoyl reductase, and ketoreductase domains) was amplified from *M. tuberculosis* genomic DNA, introducing *Bam*HI sites at the 5′ and 3′ ends of the sequence (sense primer A, 5′-GGATCCGTGCAGCTGCCCACGTATGC-3′; antisense primer B, 5′-GGATCCGAGCGGCCAACCCGTCCA-3′). This product was subcloned into *Bam*HI-digested pUC19 vector, and a 779-bp internal *Nco*I fragment (containing the enoyl reductase domain) was replaced with the *hyg* gene and used to generate recombinant phages containing the disrupted copy of *msl7*.

For *pks10* (*chs* [Rv1660]) disruption, a 2,337-bp region including the total open reading frame of *pks10* as well as flanking regions (bp 29760 to 32097 in MTCY06H11, accession number Z85982.1) was amplified by PCR from *M. tuberculosis* H37Rv genomic DNA with the following primers: sense primer A (5′-GGGAAGCTTGTCACGTTGCATGAC-3′) and antisense primer B (5′-GGGTCTAGAATACGAGCCGTGGAA-3′), introducing *Hin*dIII and *Xba*I restriction sites, respectively, and cloned into pBluescript SK(+) vector. A disrupted copy of *pks10* was prepared by replacing a 333-bp *Nco*I internal fragment of the cloned gene with the *hyg* gene cassette. The disrupted *pks10*-containing fragment was used to generate specialized transducing phages.

Generation of *M. tuberculosis* gene-disrupted mutants. *M. tuberculosis* H37Rv was grown to an optical density at 600 nm of 1.0 in Middlebrook 7H9-OADC without Tween 80. Cells from a 10-ml culture were collected by centrifugation, washed with Middlebrook 7H9-OADC with 0.3% added glycerol, and resuspended in 10 ml of Middlebrook 7H9-OADC. After overnight incubation at 37°C, cells were collected by centrifugation and resuspended in 1 ml of Middlebrook 7H9-OADC. One milliliter of lysate from *msl7* and *pks10* recombinant phages (2×10^{10} PFU/ml) was added to the cells to obtain a multiplicity of infection of 10. Infected cells were incubated for 4 to 6 h at 37°C, collected by centrifugation, resuspended in 1 ml of Middlebrook 7H9-OADC, and plated on Middlebrook 7H10 agar medium supplemented with 10% OADC and 50 μg of hygromycin B per ml. Hygromycin-resistant colonies were obtained after 3 to 4 weeks of incubation at 37°C.

PCR analysis. PCR screening for disruption was performed directly on crude lysate obtained by boiling the cells by a standard protocol (21) and Platinum *Taq* polymerase (Life Technologies, Gaithersburg, Md.). PCR screening of *msl7* transductants was carried out with a set of primers specific for the deleted segment of *msl7*: sense primer E, located upstream and just outside of the deleted segment, 5′-GTATGCGGCCACCCACAC-3′; and antisense primer F, located inside the deleted segment, 5′-CGCGCAGCGTGTCCCAC-3′. PCR analysis of the flanking regions was performed with sense primer C, 5′-TGAGGCGATGGTGGTGTCGATGCT-3′, and antisense primer H_1, 5′-TGGACCTCGACGACCTGCAGGCAT-3′, for the 5′-flanking region and with sense primer H_2, 5′-GGAACTGGCGCAGTTCCTCTGGGG-3′, and antisense primer D, 5′-ATGCGGGCCAGATCTCGGCTGCTC-3′, for the 3′-flanking region,.

pks10 mutants were screened for disruption by PCR analysis with the following primer pairs: sense primer E, 5′-GCCGTGGTCGAGCAGTATCTG-3′, and antisense primer F, 5′-CACATGCAGCACCGACGCTGA-3′, for the internal deleted segment; sense primer C, 5′-GTGTTCGCCATGATCGCCGTCGAC-3′, and antisense primer H1 for the 5′-flanking region; and sense primer H2 and antisense primer D, 5′-GTGTTCGCCATGATCGCCGTCGAC-3′, for the 3′-flanking region.

Genomic DNA isolation and Southern blotting. *M. tuberculosis* genomic DNA was isolated by the GTC method with guanidine thiocyanate, Tris-HCl, and Sarcosyl solution (10). DNA samples were digested with appropriate restriction enzymes, subjected to 1% agarose gel electrophoresis, transferred to nylon membranes (Nytran Plus; Schleicher and Schuell, Keene, N.H.), and hybridized with [α-^{32}P]dCTP-labeled probes with the random prime labeling system (Rediprime II; Amersham Pharmacia Biotech).

Test for expression of *msl7* and *pks10*. RNA was isolated from *M. tuberculosis* cells grown to the mid-exponential phase. Chilled cells isolated by centrifugation were resuspended in RNeasy lysis buffer (Qiagen), transferred to a 2-ml tube containing ceramic and silica beads (FastRNA Blue), and disrupted with a FastPrep F120 instrument (Q. Biogene). The extract collected by centrifugation was used to isolate total RNA with the RNeasy kit (Qiagen) according to the protocol provided by the manufacturer. Reverse transcription was performed with random primers and SuperScript RNase H reverse transcriptase (Life Technologies). PCR on the cDNA was done with Platinum *Taq* DNA polymerase (Invitrogen) and primers E and F used to test *msl7* and *pks10* disruptions described for PCR analysis. A control without the reverse transcriptase verified the absence of DNA contamination.

Biochemical analysis of cell wall lipids in wild type and *pks* gene-disrupted mutants of *M. tuberculosis*. Sodium [1-^{14}C]propionate (50 μCi; specific activity, 55 Ci/mol) (American Radiolabeled Chemicals, St. Louis, Mo.) was added to 30 ml of 12-day-old cultures of *M. tuberculosis* H37Rv and its *pks* gene-disrupted mutants (optical density at 600 nm of 1.6 to 1.8), and incubation was continued at 37°C in roller bottles for a further 24 h. Cells were collected by centrifugation at 6,000 × *g* for 10 min and autoclaved. The cells were extracted with an excess of chloroform- methanol (2:1) with constant stirring at room temperature for several hours, and total lipids were extracted and assayed for total ^{14}C with Scintiverse BD scintillation fluid (Fisher Scientific, Pittsburgh, Pa.) in a Beckman LA3801 liquid scintillation counter (22).

The lipids in the medium were also extracted and assayed for ^{14}C. Total lipids were separated on silica gel G plates (20 by 20 cm, 0.5 mm) with hexane-ethyl ether (9:1, vol/vol), and the polar lipids that remained at the origin in this solvent system were recovered from the silica gel with chloroform-methanol (2:1, vol/vol) and subjected to thin-layer chromatography (TLC) with chloroform-methanol (9:1, vol/vol) as the developing solvent. The lipids were visualized under ultraviolet light after spraying the plates with a 0.1% ethanolic solution of 2,7-dichlorofluoresein or by spraying the plate with 5% $K_2Cr_2O_7$ in 50% sulfuric acid, followed by heating at 180°C for 10 min. The ^{14}C-labeled lipids were detected by scanning chromatograms in a Berthold Tracemaster 20 automatic TLC linear analyzer and by autoradiography.

Silica gel containing labeled material was scraped from the TLC plates, and the lipids were eluted with chloroform-methanol (2:1, vol/vol). The recovered lipids were subjected to alkaline hydrolysis followed by methylation as described previously (22). The products recovered after methylation were subjected to TLC

on silica gel G plates with hexane-ethyl ether (9:1, vol/vol) as the developing solvent. The hydroxy fatty acid methyl esters were subjected to acetylation with acetic anhydride-pyridine (2:1) at room temperature overnight. The acetyl derivatives, isolated by TLC with hexane-ethyl ether (9:1, vol/vol) as the developing solvent, and the fatty acid methyl esters were analyzed by radio-gas chromatography (radio-GC).

To examine the composition of the fatty acids from the total lipids, these lipids were subjected to exhaustive alkaline hydrolysis, followed by methylation with BF_3 in methanol (22). The methyl esters of fatty acids and hydroxy fatty acids were isolated by TLC and analyzed by radio-GC. The total methyl esters of fatty acids from both the wild type and the mutants were also separated by argentation-TLC (5% $AgNO_3$ in silica gel) with hexane-ethyl ether (9:1, vol/vol) as the developing solvent (11), and each separated fatty acid fraction was analyzed by radio-GC with a Varian model 3300 gas chromatograph with a coiled stainless steel column (3.2 mm by 2 m) packed with 3% OV-1 (wt/wt) on Chrom W-HP 80/100 and a Lablogic GC-RAM radioactivity monitor with Winflow (IN/US Systems, Tampa, Fla.) software. For analysis of fatty acid methyl esters and acetylated mycolipanolic acid methyl esters, a 180 to 300°C program increasing at 15°C/min was used. For acetylated hydroxyphthioceranic acid methyl esters, isothermal analysis at 290°C was done with a helium carrier gas flow of 30 ml/min.

Bacterial growth in alveolar macrophage cell line. Mouse alveolar macrophage cell line MH-S (ATCC CRL-2019) was obtained from the American Type Culture Collection and propagated in RPMI 1640 medium (Gibco-BRL) supplemented with 10% heat-inactivated fetal bovine serum. Bacteria were grown in Middlebrook 7H9 medium to an optical density at 600 nm of 0.4 and diluted 1:400 in RPMI plus fetal bovine serum.

Two days before infection, 24-well plates were seeded with 2×10^5 MH-S cells, and cultures were infected in triplicate at 37°C with 0.1 ml of a single-cell suspension of *M. tuberculosis* H37Rv or its *msl7* and *pks10* gene-disrupted mutants (0.5 bacterial cell/macrophage; multiplicity of infection, 0.5). The bacteria were allowed to infect for 4 h, and extracellular bacteria were removed by four successive washes with warm RPMI. The infected cells were lysed in 200 ml of 0.067% sodium dodecyl sulfate in 7H9 medium for 30 min at 37°C. Serial 10-fold dilutions of the lysates were plated in triplicate on 7H10 Middlebrook agar medium supplemented with 10% OADC, 0.5% Tween 80, and hygromycin when needed. Colonies were counted after 4 weeks of incubation at 37°C. The experimental values and standard deviations were calculated from the results of three independent experiments.

Assay for virulence in murine model. Aliquots of *M. tuberculosis* strain H37Rv and its mutant strains were grown in modified 7H10 liquid medium (7H10 agar formulation with agar and malachite green omitted) supplemented with 10% OADC for 1 week on a 37°C rotary shaker. Media for the mutant strains were supplemented with 50 μg of hygromycin per ml. At the end of the incubation period, culture growth was measured with a Klett-Summerson colorimeter (Klett Manufacturing, Brooklyn, N.Y.) and diluted to yield a final concentration of 1 Klett unit/ml (5×10^5 CFU/ml). The inoculum size was determined by titration in triplicate on Middlebrook 7H10 (Difco Laboratories, Detroit, Mich.) agar plates with 10% OADC enrichment.

Female C57BL/6J mice (Jackson Laboratories, Bar Harbor, Maine) were purchased at 6 weeks of age and allowed to acclimate in the facility for 1 week. Animals were housed in microisolator cages (Lab Products, Maywood, N.J.) and maintained with water and Prolab RMH 3000 rodent chow (Purina, St. Louis, Mo.) in a biosafety level 3 animal facility. Mice were randomly assigned to groups on day 1 postinfection (four mice) and day 20 postinfection (four mice). Mice anesthetized with telazol and xylazine were infected intranasally with 20 μl of a suspension containing 10^4 CFU. At the designated time points, mice were euthanized by CO_2 asphyxiation, and their right lungs were sterilely removed. Lungs were homogenized in a 1-ml volume contained in an aerosol-resistant grinding assembly (Idea Works Laboratory Devices, Syracuse, N.Y.). Aliquots of the homogenate were serially diluted and titrated on 7H10 agar plates. Agar plates were incubated at 37°C in ambient air for 4 weeks, and viable colonies were enumerated.

RESULTS

Disruption of *msl7* (*pks15* and *pks1*) and chalcone synthase-like (*pks10*) genes in *M. tuberculosis*. To investigate the function of *msl7* (*pks15* and *pks1*) and *pks10* in *M. tuberculosis*, the corresponding open reading frames were disrupted by allelic exchange, with a phage-mediated system used to deliver the disrupted copy of each gene.

The *msl7* gene is located downstream of *mas* and consists of two overlapping open reading frames, *pks15*, encoding a 496-amino-acid protein containing a β-ketoacyl synthase domain, overlapping *pks1* by 3 bp, that would encode a protein of 1,616 amino acid containing acyl transferase, dehydratase, enoyl reductase, ketoreductase, and acyl carrier protein domains. The *msl7* gene showed the highest homology to a putative *pks* from *Mycobacterium leprae* (accession number NP002677). Among the *M. tuberculosis pks* genes, *msl7* shows the most similarity to *pks8* (59% identity and 71% similarity to *pks1* over 1,144 amino acids and 73% identity and 83% similarity to *pks15* over 445 amino acids).

In order to make a disruption construct for *msl7*, a 2,937-bp PCR product containing part of the *pks1* gene (bp 1231 to 4168 of *pks1* coding region, including the dehydratase, enoyl reductase, and ketoreductase domains) was amplified from the *M. tuberculosis* genomic DNA template. A 779-bp internal *Nco*I fragment (containing the enoyl reductase domain) was replaced with the *hyg* gene and used to generate recombinant phages containing the disrupted copy of *msl7* (Fig. 2A). PCR screening of the hygromycin-resistant transductants with a set of primers specific for the deleted segment of *msl7* (sense primer E, located upstream and just outside of the deleted segment, and antisense primer F, located inside the deleted segment) identified two disruption mutants that failed to amplify the expected 860-bp fragment obtained with the wild-type *M. tuberculosis* DNA (Fig. 2B).

Disruption of *msl7* by homologous recombination was confirmed by further PCR analysis of the flanking regions, which yielded the expected 1,382-bp 5′-flanking region and 1,062-bp 3′-flanking region (Fig. 2B). Southern blot analysis of *M. tuberculosis* H37Rv and the *msl7* mutant along with the restriction sites used to confirm the mutant genotype are shown (Fig. 2A and C). The gene-specific probe (P_1) consisting of the deleted *pks1* segment that was replaced by the *hyg* gene hybridized to a 5.4-kb genomic fragment which was lost in the gene-disrupted mutant. The fainter hybridization signals at 4 and 2.9 kb that appeared in both the wild-type and mutant samples in Fig. 2C (left) reflect the hybridization of the gene-specific probe to homologous regions in other *pks* genes known to be present in the genome of *M. tuberculosis*. Probing with the *hyg* gene yielded a single shorter, 2.9-kb hybridization band in the mutant due to the *Pst*I site present in the *hyg* gene cassette (Fig. 2A and C).

Recently, it was noted that *pks15/1* in *M. bovis* BCG constitutes a single open reading frame, whereas in *M. tuberculosis* H37Rv, there is a single nucleotide insertion and this frameshift mutation was postulated to inactivate *pks1* (6). Therefore, we tested whether the *pks1* segment of *msl7* is transcribed in *M. tuberculosis* H37Rv. Reverse transcription-PCR clearly demonstrated the presence of *pks1* transcripts in the wild type, but, as expected, it was absent in the *msl7* mutant (Fig. 2D). The control without reverse transcriptase did not give any product, ensuring that the PCR product was not due to DNA contamination.

M. tuberculosis genome contains a chalcone synthase-like gene, *chs* (*pks10*), located within a cluster of *pks* genes, directly upstream of *pks7* (Rv1661) (*msl4*). *pks10* would encode a 353-

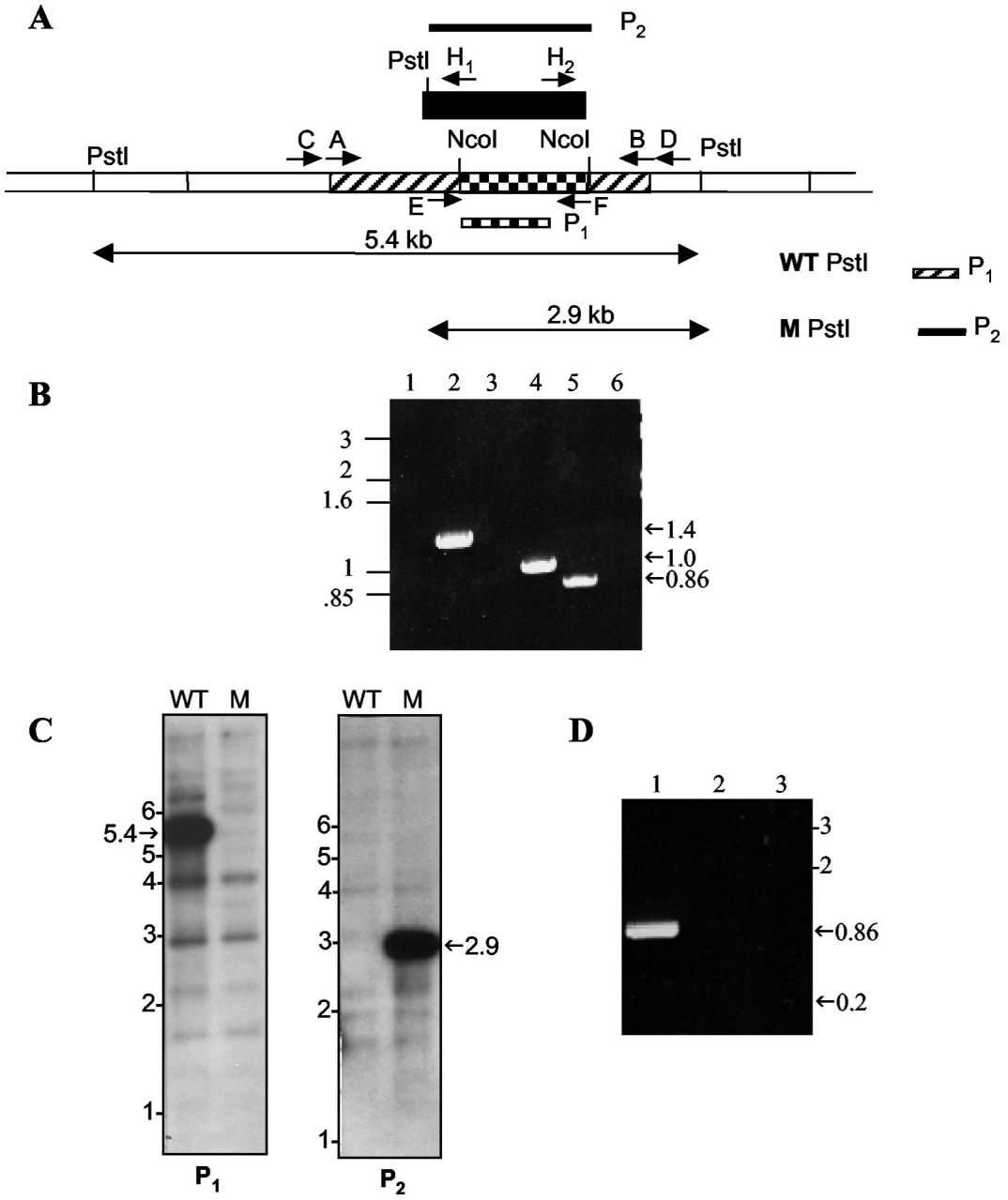

FIG. 2. Disruption strategy for *msl7* and evidence for gene replacement in the *M. tuberculosis* H37Rv mutant used for making the disruption construct. (A) Schematic representation of the construct used for disruption. Hatched, coding sequence; checkered, internal segment that was replaced with *hyg* gene cassette (black box); unshaded, regions of the gene outside those used to make the disruption construct. Primers A and B were used to amplify the DNA segment used to generate the disruption construct. WT *Pst*I, *Pst*I fragment from the wild type, expected to hybridize with probe P_1, representing the fragment deleted in making the construct. M *Pst*I, *Pst*I fragment form the *msl7* mutant, expected to hybridize with probe P_2 (*hyg* gene). (B) PCR analysis of *msl7* mutant. Lanes 1 and 2, PCR analysis of the 5′-flanking region, showing the product expected from the gene replacement mutant in lane 2 but not in the wild type (lane 1); primers C and H1. Lanes 3 and 4, PCR analysis of the 3′-flanking region, showing the expected product from the *msl7* mutant (lane 4) but not from the wild type (lane 3); primers H2 and D. Lanes 5 and 6 show the PCR product representing the internal segment replaced by *hyg* from the wild type (lane 5) but not from the *msl7* mutant (lane 6); primers E and F. (C) Southern blot analysis of *M. tuberculosis* H37Rv and the *msl7* mutant. Genomic DNA was digested with *Pst*I and hybridized with probe P_1 (left) or P_2 (right). WT, wild type; M, mutant. (D) Reverse transcription-PCR showing the presence of transcripts containing the *pks1* segment of *msl7* in *M. tuberculosis* H37Rv. For lanes 1, 2, and 3, primers E and F were used. Lane 1, wild type; lane 2, wild type without reverse transcriptase; lane 3, *msl7* mutant. In all panels, sizes are shown in kilobases.

amino-acid protein with a deduced molecular mass of 37.09 kDa which shows 74% identity and 85% similarity to *M. tuberculosis pks11* (Rv1665), a second chalcone synthase-like gene located in the same region, at the end of the *pks* cluster. A third chalcone synthase-like gene annotated, *pks18* (Rv1372), shows less homology to *pks10* (28% identity and 45% similarity). Whether these *chs* genes are expressed in *M. tuberculosis* and, if they are, what roles they play remain unknown.

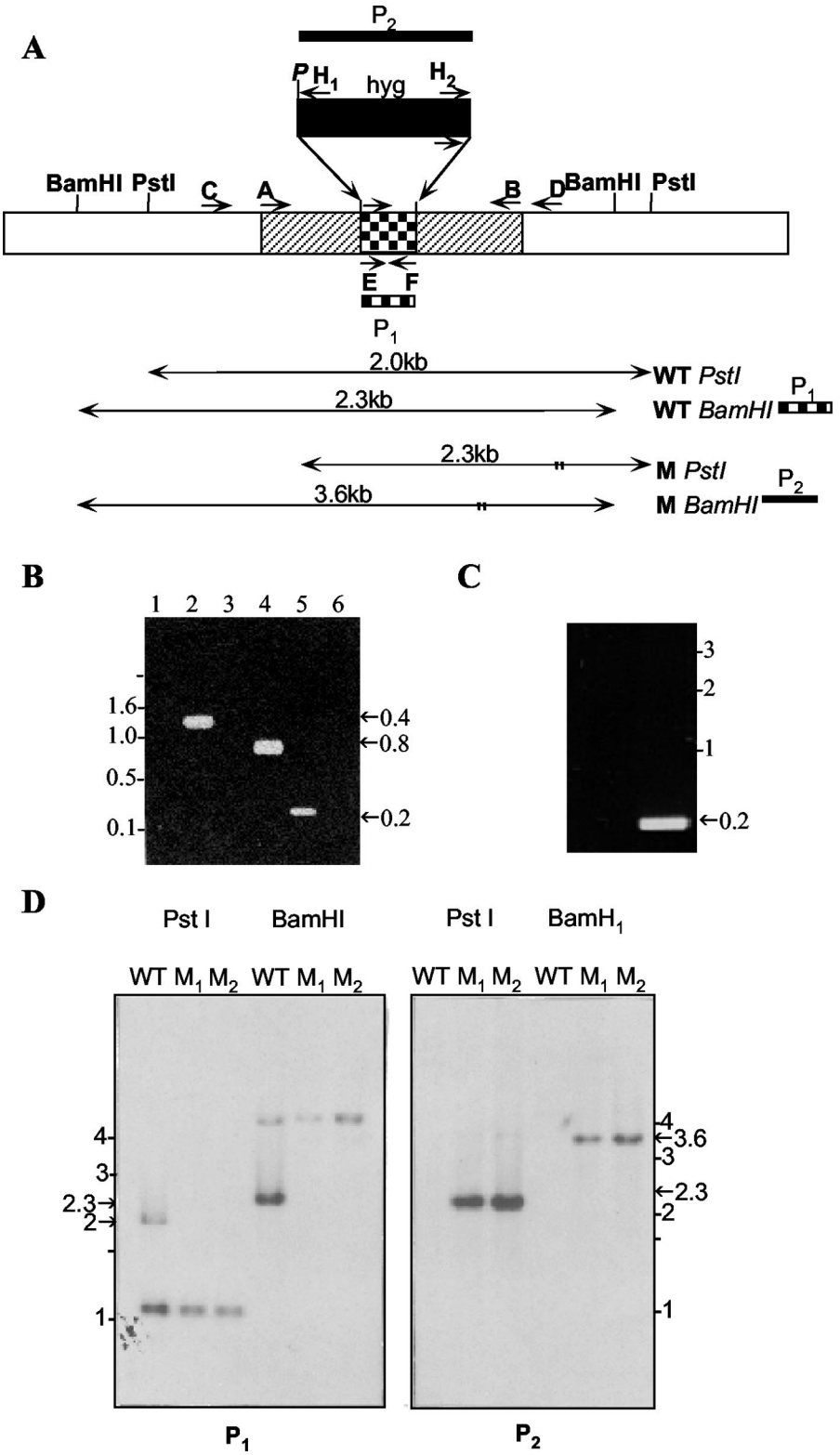

FIG. 3. Disruption strategy for *pks10* and evidence for *pks10* gene replacement in *M. tuberculosis* H37Rv. (A) Schematic representation of the disruption construct. Hatched and checkered regions represent the region used to make the disruption construct (amplified with primers A and B). The checkered segment was replaced with the *hyg* gene cassette (black box). Primer pairs C/H1, D/H2, and E/F were used for PCR analysis of homologous recombination as described in the text. WT *Pst*I, WT *Bam*HI, M *Pst*I, and M *Bam*HI are *Pst*I and *Bam*HI fragments from the wild-type (WT) and *pks10* mutant (M) that are expected to hybridize with probes P1, representing the fragment deleted in making the construct, and P2, the hygromycin resistance gene cassette, respectively. (B) PCR analysis of 5′-flanking (lanes 1 and 2, primers C and H_1), 3′-flanking (lanes 3 and 4, primers H_2 and D), and the segment replaced by *hyg* (lane 5 and 6, primers E and F), demonstrating homologous recombination. Lanes 1, 3, and 5, wild type; lanes 2, 4, and 6, mutant. (C) Reverse transcription-PCR analysis showing expression of *pks10* in the wild type (right) but not in the *pks10* mutant (left). Primers, E and F. (D) Southern blot analysis of *M. tuberculosis* H37Rv and *pks10* mutants. Genomic DNA was digested with *Pst*I and *Bam*HI. Left, hybridized with *pks10* segment replaced by *hyg* (probe P_1); right, probed with *hyg* gene (probe P_2). WT, wild type; M_1 and M_2, mutants.

For *pks10* disruption, a 2,337-bp region including the entire open reading frame of *pks10* as well as 708 bp of the 5′-flanking and 568 bp of the 3′-flanking regions was amplified by PCR from *M. tuberculosis* H37Rv genomic DNA. A 333-bp *Nco*I internal fragment of *pks10* was replaced with the hygromycin resistance gene cassette (*hyg*) (Fig. 3A). This *pks10*::*hyg* fragment was introduced into phAE87 to generate phAE87-*pks10*::*hyg*. The phasmid DNA was verified by restriction enzyme analysis and PCR. After infecting *M. tuberculosis* H37Rv with the recombinant phages, the transductants were screened by PCR with two sets of unique primers that would amplify the flanking regions (Fig. 3A). The expected 1.4-kb and a 0.8-kb 5′- and 3′-flanking product, respectively, were generated from the mutants (Fig. 3B, lanes 1 to 4). Gene disruption was further supported by the fact that the primers representing the deleted internal segment (primer pair E/F) did not yield the 0.2-kb DNA fragment that was seen with the wild type (Fig. 3B, lanes 5 and 6). Disruption of the *pks10* gene was verified by Southern blot analysis with *Bam*HI and *Pst*I as the restriction enzymes and two probes: P_1, the deleted 333-bp *Nco*I segment of *pks10* that was replaced with the *hyg* gene, and P_2, the hygromycin resistance gene. The hybridization bands expected from homologous recombination were found (Fig. 3D). Reverse transcription-PCR analysis showed that the *pks10* transcript found in the wild type was not present in the *pks10* mutant (Fig. 3C).

Analysis of lipids derived from [1-^{14}C]propionic acid. To elucidate the biosynthetic defects caused by the absence of functional *msl7* and *pks10* genes, we administered [1-^{14}C]propionate as a radiotracer to label the lipids in the wild type and the mutants. The wild type and the gene-disrupted mutants incorporated similar amounts (20 to 25%) of the label into lipids. We carried out detailed analysis of the lipid classes and fatty acids in each lipid class. TLC of total lipids with 10% ethyl ether in hexane showed the absence of label in the dimycocerosyl phthiocerol (DIM) fraction in the two mutants, whereas in the wild type, 30% of the total label was contained in this fraction (Table 1, Fig. 4). The absence of DIM in these mutants was further confirmed by treatment of the plate with dichromate-sulfuric acid, followed by heating; the charred band at R_f 0.6, corresponding to DIM found in the wild type, was absent in the mutants (data not shown).

Radio-GC analysis of the methyl esters of fatty acids from the total lipids showed that the two mutants generated all the classes of fatty acids that were produced by the wild type. However, labeling of mycocerosic acids was much less in the mutants (Fig. 5). Argentation-TLC of fatty acids (as methyl esters) from the total lipids and radio-GC analysis of each fraction showed that these mutants generated all the classes of methyl-branched fatty acids except that the labeling of the mycocerosic acid-containing fraction was much less in these mutants compared to the wild type (data not shown). That the mutants had the ability to synthesize the acyl portion of DIM was clearly indicated by radio-GC analysis of the fatty acids contained in the small amounts of labeled lipids recovered from the apolar lipids that migrated near DIM. Labeled mycocerosic acids were detected. Furthermore, radio-GC of the methyl esters from the sulfolipids in all these mutants also showed detectable levels of mycocerosic acids. Thus, these gene-disrupted mutants were found to be capable of generating mycocerosic acids. Consistent with this observation was our finding that sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) revealed the presence of mycocerosic acid synthase, and immunoblot analysis showed cross-reactivity only with this band (data not shown). However, these mutants, which did not show a deficiency in mycocerosic acid synthesis, could not produce DIM. There was also no DIM in the medium.

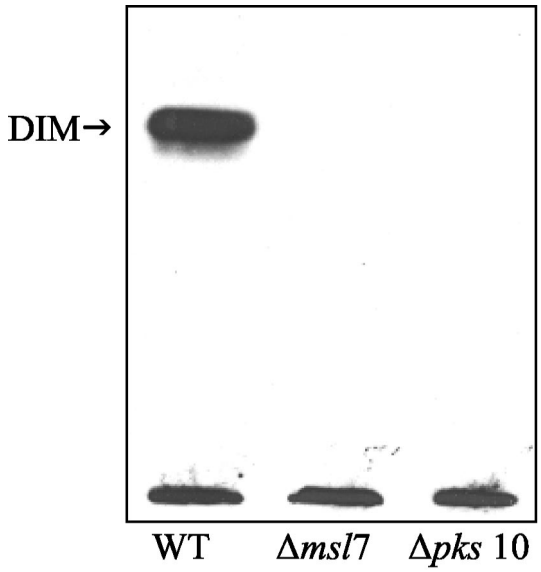

FIG. 4. Autoradiogram of thin-layer chromatogram of lipids derived from sodium [1-^{14}C]propionate in *M. tuberculosis* H37Rv (wild type [WT]), *msl7* mutant, and the *pks10* mutant. Total lipids were subjected to TLC on silica gel G with 10% ethyl ether in *n*-hexane as the solvent. A similar amount of radioactivity was used in each case. DIM, dimycocerosyl phthiocerol.

A detailed analysis of the more polar lipids labeled with sodium [1-^{14}C]propionate showed that the distribution of ^{14}C in lipids other than DIM was not affected in the *msl7* and *pks10* mutants except for an increase in the amount of ^{14}C found in the sulfolipids (Table 1). The polar lipids that remained at the origin on TLC plate developed in hexane-ethyl ether (9:1, vol/vol) as the solvent system were recovered and separated by TLC with 10% methanol in chloroform as the developing solvent. The distribution pattern of ^{14}C among the polyacyl trehaloses, sulfolipids, and diacyltrehaloses was similar among the wild type and its *pks* mutants except that there was a 55 to 60% increase in the labeling of sulfolipids in the mutants compared to the wild type (Table 1).

A detailed analysis of the fatty acids present in individual lipid fractions was done by a combination of argentation-TLC and radio-GC analysis of each fraction. The hydroxy fatty acids were also analyzed by radio-GC as their acetylated methyl esters. The high content of ^{14}C found in hydroxyphthioceranic acids explains the increased labeling of sulfolipids in the mutants (Table 1). Radio-GC analysis of each fatty acid methyl ester fraction separated by argentation-TLC showed no significant differences in the fatty acid composition in individual lipids between the wild type and the two mutants. Such detailed analyses revealed identical distribution of ^{14}C among all classes of lipids and fatty acids (data not shown).

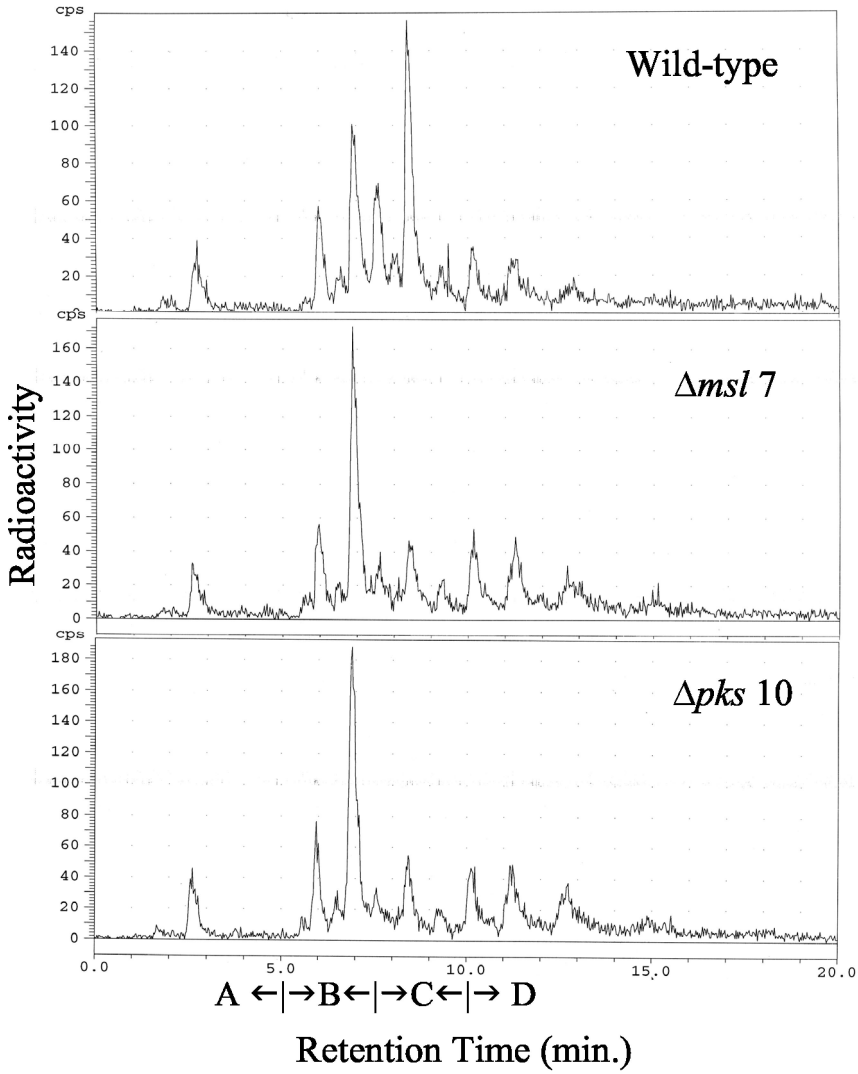

FIG. 5. Radio-GC analysis of total fatty acid methyl esters derived from sodium [1-^{14}C]propionate in *M. tuberculosis* H37Rv (wild type), the *msl7* mutant, and the *pks10* mutant. Retention time ranges for branched short-chain fatty acids (A), mycolipanoic and mycolipenic acids (B), mycocerosic acids (C), and phthioceranic acids (D) are indicated.

Virulence. To determine whether the products of the *msl7* and *pks10* genes affect virulence, we compared the ability of the wild type and the mutants to grow in a murine alveolar macrophage cell line, MH-S. Starting with $2.3 \times 10^4 \pm 0.4 \times 10^4$ CFU, the wild type reached $4.3 \times 10^5 \pm 0.4 \times 10^5$ CFU in 5 days, whereas the *msl7* mutant, starting with $2.3 \times 10^4 \pm 0.4 \times 10^4$ CFU, reached $2.6 \times 10^5 \pm 0.6 \times 10^5$ CFU, and the *pks10* mutant, starting with $2.0 \times 10^4 \pm 0.6 \times 10^4$ CFU, reached $2.3 \times 10^5 \pm 0.3 \times 10^5$ CFU. Thus, in a 5-day growth period, both mutants showed 50% lower growth. We also tested virulence in mice in vivo after intranasal inoculation. Growth of the pathogen and its gene-disrupted mutants in murine lungs was measured over time (Fig. 6). The *msl7* and *pks10* mutants showed major attenuation, showing several orders of magnitude less growth compared to the wild type. The levels of growth of the wild type found in these experiments were similar to those published previously (12). At 10^9 CFU, the animals appeared sick, but there were no fatalities during the 20-day growth period. The growth rates of the mutants in vitro were identical to that of the wild type (data not shown).

DISCUSSION

The variety of multiple methyl-branched fatty acids found in *M. tuberculosis* is probably produced by unique synthases encoded by some of the many *pks* genes found in the mycobacterial genome (15). Since the multifunctional synthases encoded by these genes are large proteins similar in size and properties, isolation and characterization of these individual synthases is not a convenient way to determine their biosynthetic function. Therefore, a genetic approach was taken in which identification of the lipid that is missing in targeted gene disruption mutants was used to elucidate the biosynthetic function of the gene product (1, 2, 6, 11, 22).

In the present case, disruption of two different genes caused abolition of the production of DIM without causing any other

TABLE 1. Relative distribution of ^{14}C among the lipid classes derived from sodium [1 − ^{14}C]propionic acid in *M. tuberculosis* and its *msl7* and *pks10* mutants

Class[a]	Relative distribution of ^{14}C (%)		
	Wild type	*msl7* mutant	*pks10* mutant
DIM	30	0	0
Fatty acids	28	0	0
Hydroxy fatty acids	2	0	0
PAT-I	16	18	20
Fatty acids	14	14	16
Mycolipanolic acids	1.7	3	3
Hydroxyphthioceranic acids	0.3	1	1
PAT-II	4	3	4
Fatty acids	1	1	1
Mycolipanolic acids	1	0.6	1
Hydroxyphthioceranic acids	2	1.4	2
Sulfolipids	45	73	70
Fatty acids	10	10	10
Mycolipanolic acids	4	10	9
Hydroxyphthioceranic acids	31	53	51
Very polar lipids	5	6	6
Fatty acids	0.5	1	1
Mycolipanolic acids	0.3	0.5	0.6
Hydroxyphthioceranic acids	4.2	4.5	4.4

[a] PAT, polyacyl trehaloses; DIM, dimycocerosyl phthiocerols. Very polar lipids (including diacyl trehalose) remained at and near the origin on TLC with 10% methanol in chloroform as the solvent system.

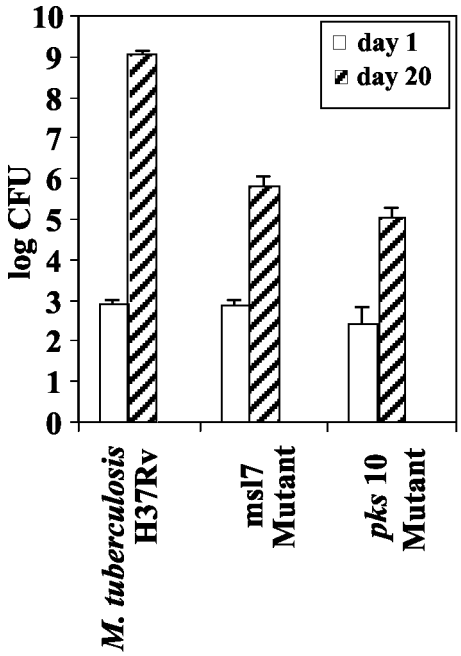

FIG. 6. Growth of intranasally administered *M. tuberculosis* H37Rv and its *msl7* and *pks10* gene-disrupted mutants in the lungs of C57BL/6J mice. The experimental details are in the text.

qualitative change in lipid synthesis. Detailed analysis of all of the major classes of lipids and detailed analysis of fatty acids in each class derived from sodium [1-^{14}C]propionate showed no alteration except for a substantial increase in labeling of sulfolipids. Since the major metabolic fate of methylmalonyl-coenzyme A is incorporation into mycocerosic acids (in DIM) and phthioceranic and hydroxyphthioceranic acids (in sulfolipids), blockage of DIM synthesis causes channeling of methylmalonyl-coenzyme A into sulfolipids. The most dramatic consequence of the absence of a functional *msl7* and *pks10* is the abolition of DIM synthesis.

One of the most unusual lipids in the cell walls of pathogenic mycobacteria is DIM, which is composed of a long-chain diol esterified to multiple methyl-branched fatty acids called mycocerosic acids (9, 15, 18). In some species within the tuberculosis complex, such as *M. bovis* and some strains of *M. tuberculosis* such as Canetti, mycocerosic acids are esterified to a structurally analogous diol called phenolphthiocerol, in which there is a hydroxyphenyl group at the end of the alkyl chain (Fig. 1). The exact mechanism of biosynthesis of these diols is not known. Based on the structural features of this diol, a plausible biosynthetic pathway has been postulated (15).

Based on the type of the postulated reactions, the domains required for the catalysis of each step and the likely organization of these catalytic domains, and the probable organization of the genes that would encode such proteins, a set of open reading frames probably involved in this process were identified in the genome. Gene disruption provided evidence for the involvement of this cluster of open reading frames in the biosynthesis of these diols (2). However, this cluster is probably involved in synthesis of the diols from already elongated precursors (Fig. 7). The genes that encode the enzymes involved in the synthesis of these precursors remain unknown. Most probably, several *pks* genes are involved in producing these elongated precursors of the diols. In the present paper, we report that disruption of two *pks* genes, *msl7* and *pks10*, blocks DIM synthesis, although the mutants can produce mycocerosic acids. Thus, the disruption of these *pks* genes probably prevents the synthesis of the diol. Since *M. tuberculosis* H37Rv produces only phthiocerol, not phenolphthiocerol, we cannot determine whether *msl7* and *pks10* are involved uniquely in phthiocerol synthesis.

Comparison of the sequence of *msl7* in *M. bovis* BCG with its homologue in *M. tuberculosis* H37Rv showed the insertion of a nucleotide in the *pks15* part of the latter, and this frameshift is thought to inactivate *pks1* in this organism (6). However, this shift in the open reading frame creates a GTG start codon that could allow translation of the open reading frame of *pks1*. Our reverse transcription-PCR results clearly showed that *pks1* is transcribed in *M. tuberculosis* H37Rv, although we do not have direct evidence for the translation of the open reading frame of the *pks1* product. Our finding that *pks1* disruption prevents the synthesis of DIM implies that the *pks1* product is translated in *M. tuberculosis* H37Rv. In *M. bovis* BCG, disruption of *pks15/1* was reported to cause selective abolition of phenolphthiocerol synthesis without affecting DIM synthesis (6). On this basis, it was postulated that *pks15/1* is involved in the elongation of *p*-hydroxybenzoic acid to the *p*-hydroxyphenylalkanoic acid precursor required for synthesis of the phenolphthiocerol catalyzed by the products of the *pps* cluster in *M. bovis* BCG (Fig. 7). However, our results that

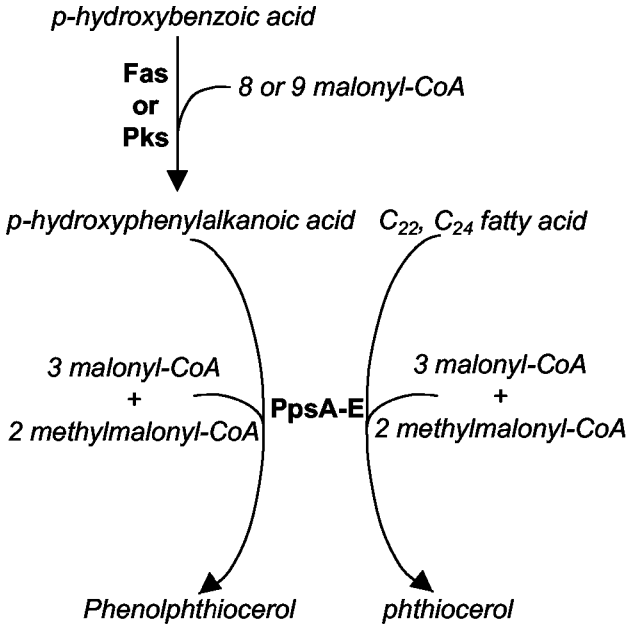

FIG. 7. Major stages involved in the biosynthesis of phenolphthiocerol and phthiocerol. CoA, coenzyme A.

disruption of *pks1* blocks DIM synthesis in *M. tuberculosis* H37Rv, which does not produce phenolphthiocerol, are not consistent with the postulated role of *pks15/1* uniquely in the synthesis of phenolphthiocerol; *msl7* is probably involved in generating the precursor for phthiocerol synthesis in *M. tuberculosis* H37Rv.

Among the large number of *pks* genes present in the *M. tuberculosis* genome, we identified seven genes homologous to *mas* (22). The enzymes encoded by these *mas*-like genes probably catalyze the synthesis of the different classes of multiple methyl-branched fatty acids found in *M. tuberculosis*. The two gene-disrupted mutants presented here showed attenuation in the murine model, and they were both defective in the synthesis of DIM. Our results support previous observations that suggested that DIM is involved in virulence (5, 8).

pks10 of *M. tuberculosis* would encode a chalcone synthase-like protein found only in plants until recently. In recent years, more than eight bacterial homologues have been detected (7, 13, 19), and *pks10* is one of them. This family of proteins catalyze the synthesis of carbon chains from coenzyme A esters without the involvement of acyl carrier protein. Although in plants such proteins use phenylpropanoyl-coenzyme A as the starting material to produce flavanoids, the recently discovered homologues, particularly the bacterial *chs* products, show the use of an expanding array of starter units. However, the nature of the substrates used by *pks10* in *M. tuberculosis* H37Rv is not known. Our finding that disruption of this gene causes DIM deficiency implies that it is involved in the biosynthesis of this diol.

It is possible that *p*-hydroxybenzoyl-coenzyme A elongation, which is involved in the biosynthesis of phenolphthiocerol, is catalyzed by this gene product. However, DIM synthesis would require only aliphatic starter molecules, and therefore how the *pks10* product may be involved in generating such starters is not clear. If the *pks10* product catalyzes the elongation of hydroxybenzoyl-coenzyme A as well as elongation of the aliphatic precursor involved in the synthesis of DIM, its disruption could abolish DIM synthesis as well as phenolphthiocerol synthesis. However, *pks10* has not been disrupted in an organism that produces both. If the many enzymes involved in the production of DIM and phenolphthiocerol function as a multienzyme complex that would accept precursors of phthiocerol and phenolphthiocerol, the absence of any one of the component enzymes, such as the *pks10* product, may block the function of the complex and thus cause DIM deficiency. An indirect polar effect of the *pks10* mutation on another *pks* such as *pks7*, which is 83 bp away, is highly unlikely, since reverse transcription-PCR analysis showed that the *pks10* mutation did not affect the *pks7* transcript level (data not shown).

In view of the increasing importance of finding new targets for antimycobacterial drugs, genes that are expressed during growth of the pathogen in the host would be appropriate candidates as new drug targets. By selective capture of transcribed sequences, *pks10* has been found to be expressed by *M. avium* 48 h after infection of human macrophages (14). *pks10* has also been found to be upregulated in *M. tuberculosis* during nutrient starvation, which mimics some of the features of persistence (3). Whether *pks15/1* and *pks10* are expressed in *M. tuberculosis* in the human host is not known. However, there is mounting evidence that DIM is a virulence factor, and our finding that disruption of *pks1* and *pks10* causes DIM deficiency suggests that these genes might be suitable targets for new antimycobacterial drugs. Since DIM synthesis involves many enzymes, it may be possible to find many different drugs that can intervene in the production of DIM, making it an attractive target for investigation.

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