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The *Mycobacterium tuberculosis pks2* Gene Encodes the Synthase for the Hepta- and Octamethyl-branched Fatty Acids Required for Sulfolipid Synthesis*

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Multidrug-resistant tuberculosis is a major global health emergency. Cell wall lipids of *Mycobacterium tuberculosis* can play crucial roles in the pathogenesis. The enzymes involved in their synthesis can be ideal new drug targets against tuberculosis, because many such lipids are unique to this pathogen. A variety of multiple methyl-branched fatty acids are among such unique lipids. We have identified seven genes highly homologous to the *mas* gene, which is known to be involved in the production of one class of such multiple methyl-branched fatty acids. One of these *mas*-like genes, *pks2*, was disrupted using a phage-mediated delivery of the disruption construct. Gene disruption by homologous recombination was confirmed by polymerase chain reaction analysis of the flanking regions of the introduced disrupted gene and by Southern analysis. Thin-layer and radio gas-chromatographic analyses of lipids derived from [1-^{14}C]propionic acid and gas chromatography/mass spectrometry analysis of the fatty acids and hydroxy fatty acids showed that the *pks2* mutant was incapable of producing hepta- and octamethyl phthioceranic acids and hydroxyphthioceranic acids that are the major acyl constituents of sulfolipids. Consequently, *pks2* mutant does not produce sulfolipids. Sulfolipid deficiency in *pks2* mutant was confirmed by two-dimensional thin-layer chromatographic analysis of lipids derived from [1-^{14}C]propionic acid and $^{35}SO_4^{-2}$. With this sulfolipid-deficient mutant, it should be possible to test for the postulated important roles for sulfolipids in the pathogenesis of *M. tuberculosis*.

Tuberculosis claims several million human lives each year, accounting for more than one quarter of all preventable adult deaths in the world (1). The causative agent, *Mycobacterium tuberculosis*, is an extremely difficult organism to combat, because it has many structural and functional features that allow it to evade the defense mechanisms of the host as well as antimicrobial drugs. Mycobacterial cell walls have a very high content (50–60%) of lipids that are uniquely complex, highly hydrophobic, and refractory to cellular hydrolytic enzymes (2–5). Thus, they constitute an effective permeability barrier to antimycobacterial therapies. Mycobacterial components also help the pathogen to enter macrophages (6) and evade the natural defense mechanisms of the host (7, 8), thus allowing the pathogen to grow within macrophages that normally phagocytose and destroy most other bacterial pathogens. Successful antimycobacterial therapies, used for almost half a century, involve the use of drugs that interfere with the biosynthesis of mycobacterial cell wall lipids together with antibiotics. With the advent of resistance to such drugs, it has become critical to develop new drugs targeted at other unique processes involved in cell wall lipid synthesis (9, 10).

A unique feature of mycobacterial cell wall lipids includes the presence of very long chain fatty acids with multiple methyl branches toward the carboxyl end of the carbon chain (see Fig. 1) (3, 4). For example, 2,4,6,8-tetramethyl C_{32} fatty acids and homologues, the mycocerosic acids (see Fig. 1*H*), are esterified to two long-chain diols, the phenolphthiocerols and phthiocerols. Dimycocerosyl lipids have been reported to play a key role in the host-pathogen interaction and pathogenesis (11–14). We have previously cloned and characterized mycocerosic acid synthase gene (*mas*) (15) and have identified a gene cluster involved in the synthesis of both phthiocerol and phenolphthiocerol (*pps*) (16). Disruption of *mas* and *pps* genes followed by analysis of the lipids generated by the mutants confirmed the biochemical functions of the products of these genes in *M. bovis* BCG[1] (16, 17). Scanning calorimetric examinations of the cell walls of these gene knock-out mutants showed that the physical properties of the walls were altered by the absence of the lipids generated by these gene products and these changes were reflected in the altered uptake of lipophilic chemicals by these mutants.[2] The ability of the mutants to grow in human peripheral blood monocytes[3] and in mice[4] (both spleen and lungs), was measurably decreased. More recently, transposon mutants of *M. tuberculosis* deficient in the dimycocerosyl phthiocerol family of lipids were found to have impaired ability to grow in the lungs (13, 14).

Another major class of multiple methyl-branched fatty acids is esterified to sulfated trehalose (3, 4, 18, 19). The principal

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[1] The abbreviations used are: BCG, bacillus Calmette-Guérin; *acoas*, acyl-CoA synthase gene; AT, acyl transferase; DH, dehydratase; ER, enoyl reductase; FAS, fatty acid synthase; GC/MS, gas-chromatography/mass spectrometry; KR, ketoreductase; KS, ketoacyl synthase; MAS, mycocerosic acid synthase; ORF, open reading frame; PCR, polymerase chain reaction; *pks*, polyketide synthase gene; *pps*, phenolphthiocerol/phthiocerol synthase gene cluster; bp, base pair(s); kb, kilobase(s); radio-GC, gas chromatography by radioactivity detection.

[2] C. E. Barry III, A. K. Azad, and P. E. Kolattukudy, unpublished results.

[3] R. Silver and P. E. Kolattukudy, unpublished results.

[4] M. H. Cynnamon and P. E. Kolattukudy, unpublished results.

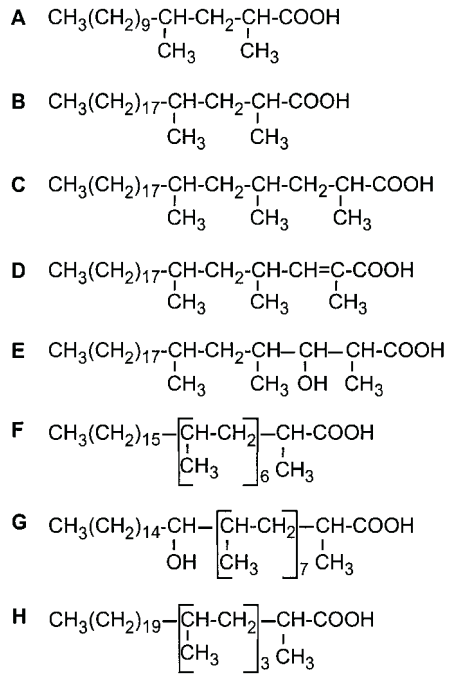

FIG. 1. **The major types of methyl-branched fatty acids found in *M. tuberculosis*.**

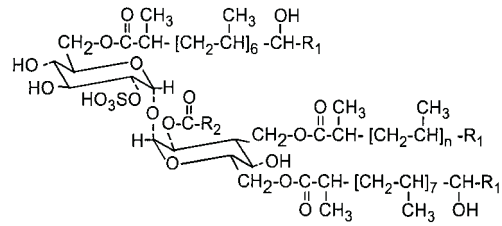

FIG. 2. **Structure of the major sulfolipid in *M. tuberculosis* R_1, $C_{15}H_{31}$, or $C_{17}H_{35}$; R_2, *n*-C_{16} to *n*-C_{28}; *n*, 6 or 7.**

sulfolipid in *M. tuberculosis* H37Rv is a 2,3,6,6′-tetraacyl-α,α′-D-trehalose-2′-sulfate (see Fig. 2). 2,4,6,8,10,12,14,16-Octamethyl-17-hydroxydotriacontanoate (hydroxyphthioceranic acid) (Fig. 1*G*) and its homologues constitute the major acyl groups with smaller amounts of nonhydroxylated multiple methyl-branched derivatives (phthioceranic acids) (Fig. 1*F*) as well as some *n*-fatty acids (Fig. 2) (18). Although nothing is known about the nature of the enzymes involved in the synthesis of such very long-chain multiple methyl-branched fatty acids, such enzymes must be multifunctional proteins encoded by genes with homology to *mas*. We have identified seven genes homologous to *mas* in the genome of *M. tuberculosis*. Disruption of such *mas*-like genes and determination of the biochemical consequences should help identify their functions. The sulfolipids, present uniquely in virulent strains of *M. tuberculosis* have been reported to play highly significant roles in the interaction of *M. tuberculosis* with its host (20–22). However, such roles have not been directly tested, because the genes involved in the synthesis of sulfolipids have not been identified and mutants deficient in sulfolipid synthesis have not been produced. Because hydroxyphthioceranic acids are the major acyl components of the sulfolipids, disruption of the gene encoding the enzyme responsible for the synthesis of these acids may produce a sulfolipid-deficient mutant. In this paper, we report the disruption of one of the *mas*-like genes, *pks2*, that is known to be expressed in *M. tuberculosis* growing in human macrophages (23). Disruption of this gene resulted in a mutant that is incapable of producing the hepta- and octamethyl fatty acids required for sulfolipid synthesis and thus is deficient in sulfolipids.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions—Escherichia coli DH5α and HB101 strains (Life Technologies, Gaithersburg, MD) were used for cloning and propagation of plasmids and phasmids. For selection of transformants, *E. coli* was grown in Luria-Bertani broth or agar containing 100 μg of ampicillin (Sigma Chemical Co., St. Louis, MO) or 150 μg of hygromycin B (Calbiochem, San Diego, CA) per milliliter. *M. smegmatis* mc^{2}155 (24) was grown in liquid Luria-Bertani medium with 0.5% Tween 80 for competent cell preparation and in Middlebrook 7H9 (Difco, Detroit, MI) broth with 0.05% Tween 80 for transduction. *M. tuberculosis* H37Rv (ATCC #25618) was obtained from the American Type Culture Collection and grown in Middlebrook 7H9 broth supplemented with 10% OADC enrichment (oleic acid, albumin (bovine, fraction V), dextrose, catalase (Difco) and 0.05% Tween 80 in plastic roller bottles at 3 rpm at 37 °C in a Roll-in rolling incubator (Bellco Glass, Vineland, NJ). Middlebrook 7H11 (Difco) supplemented with 10% OADC was used as an agar medium. Hygromycin B was used at a concentration of 50 μg/ml for *M. tuberculosis* transformants.

General DNA Techniques—Molecular cloning and restriction endonuclease digestions were performed by standard techniques (25). Methods used to generate disruption construct were essentially as recently described by K. H. Derbyshire and S. Bardarov (26). Cloning vectors used were pBluescript KS (−) (Stratagene, La Jolla, CA); pYUB572 and phAE 87[5] were kindly provided by Dr. Stoyan Bardarov, Albert Einstein College of Medicine, New York, NY.

Generation of pks2 Gene Disruption Construct—The region of *pks2* chosen for disruption was amplified from *M. tuberculosis* genomic DNA using the following primers: sense primer, 5′-GGATCCGATCCTCGACGAGTTGAT-3′(A), antisense primer, 5′-GAATTCAGCACGATCCGGCCGCAA-3′(B). The resulting 3159-bp PCR product, which contained *Bam*HI and *Eco*RI sites at the 5′- and 3′-ends, respectively, was cloned into pBS KS(−) plasmid. The disrupted copy of the gene was constructed by deleting a 1203-bp *Pst*I internal fragment and inserting the hygromycin gene (*hyg*) cassette from *Streptomyces hygroscopicus* amplified by PCR as a 1.67-kb *Pst*I fragment from plasmid pIJ963 (27) (a gift from Dr. John Hopwood, John Innes Center, Norwich, UK). The 3710-bp *Bam*HI-*Eco*RI fragment containing the disrupted *pks2* gene was excised from pBS-*pks2*::hyg plasmid, blunt-ended, and ligated to the 1647-bp *Bsp*HI fragment from pYUB572 containing a lambda *cos* site and a unique *Pac*I site. The resulting pYUB572-*pks2l*::hyg was digested with *Pac*I and ligated to phAE 87 that was self-ligated to form concatemers and digested with *Pac*I. The ligation mixture was transduced into λ-sensitive *E. coli* HB101 with the GigaPack Gold *in vitro* packaging kit (Stratagene, La Jolla, CA). The phasmid DNA extracted from pooled recombinant hygromycin-resistant clones was electroporated into *M. smegmatis* mc^2 155 and mycobacteriophage plaques were selected after 48 h of incubation at 30 °C. Individual plaques were tested for thermosensitivity, and phages yielding no visible plaques at 37 °C were amplified to 2×10^{10} plaque-forming units/ml.

Generation of M. tuberculosis pks2 Gene-disrupted Mutant—M. tuberculosis was grown to an A_{600} of 0.8–1.0 in Middlebrook 7H9-OADC without Tween 80. Cells from 10 ml of culture were collected by centrifugation, washed with Middlebrook 7H9-ADC with 0.3% added glycerol, and resuspended in 10 ml of Middlebrook 7H9-ADC. After overnight incubation at 37 °C, cells were collected by centrifugation and resuspended in 1 ml of Middlebrook 7H9-ADC. One milliliter of recombinant phage lysate (2×10^{10} plaque-forming unit/ml) was added to the cells to obtain a multiplicity of infection of 10. Infected cells were incubated for 4–6 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/ml hygromycin B. Hygromycin-resistant colonies were obtained after 3–4 weeks of incubation at 37 °C.

Genomic DNA Isolation and Southern Blotting—M. tuberculosis genomic DNA was isolated by the GTC method using guanidine thiocyanate, Tris-HCl, Sarcosyl solution (28). DNA samples were digested with appropriate restriction enzymes, transferred to Nylon membranes (Nytran Plus, Schleicher and Schuell, Keen, NH) and hybridized with [α-^{32}P]dCTP-labeled probes using the random prime labeling system, rediprime II (Amersham Pharmacia Biotech, UK).

PCR Analysis—To screen for disruption of the *pks2* gene, PCR amplification was performed directly on crude lysate obtained by boiling the cells. PCR amplification using standard protocols (25) and

[5] S. Bardarov, M. Larsen, M. Pavelka, S. S. Bardarov, and W. R. Jacobs, unpublished results.

Platinum *Taq* Polymerase (Life Technologies, Gaithersburg, MD) was performed using the following primer pairs: 5′-TGGTCGCAGGAGTGCTGTCAGCC-3′(C) and 5′-GGAACTGGCGCAGTTCCTCTGGGG-3′(H2) for amplification of the 5′-flanking region; 5′-TAGCACGCCTCGTACCGGAAGCCC-3′(D) and 5′-TGGACCTCGACGACCTGCAGGCAT-3′(H1) for amplification of the 3′-flanking region; 5′-AGCAGTCCGAAGTACGCGAC-3′(E) and 5′-CTTGATCGCGTAGCTGGTCG-3′(F) for amplification of the deleted internal fragment.

Biochemical Analysis of Cell Wall Lipids in the Wild Type and pks2-disrupted Mutant of M. tuberculosis—Sodium [1-^{14}C]propionate (25 μCi; specific activity, 55 Ci/mol) (American Radiolabeled Chemicals, St. Louis, MO) was added to 30 ml of 10-day-old cultures of *M. tuberculosis* H37Rv and the *pks2* mutant (A_{600} 1.7–1.8), and incubation was continued at 37 °C in roller bottles for a further 24 h. Cells were collected following centrifugation at 6000 × *g* for 10 min and autoclaved. The culture medium was passed through 0.22-μm filters. The cells were extracted with an excess of chloroform:methanol (2:1, v/v) with constant stirring at room temperature for several hours, and total cellular lipids were extracted by the Folch method (29) and assayed for total ^{14}C with Scintiverse BD scintillation fluid (Fisher Scientific, Pittsburgh, PA) in a Beckman LA3801 liquid scintillation counter. Media were also extracted with chloroform (twice), and the recovered lipids and the aqueous medium were assayed for ^{14}C. Total cellular lipids were separated on Silica Gel G plates (20 × 20 cm, 0.5 mm) using chloroform:methanol (9:1, v/v) as the developing solvent. The lipids were visualized by spraying chromatograms with 5% $K_2Cr_2O_7$ in 50% sulfuric acid and heating at 180 °C for 10 min or under UV after spraying chromatograms with 0.1% ethanolic solution of 2,7-dichlorofluorescein. 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 TLC plates, and the lipids were eluted with chloroform:methanol (2:1, v/v). The recovered lipids were subjected to alkaline hydrolysis in 2-methoxyethanol containing 12% H_20 and 5% KOH followed by methylation as described previously (17). The products recovered after methylation were subjected to TLC on Silica Gel G plates using *n*-hexane:diethyl ether (9:1, v/v) as the solvent. The hydroxy acid methyl esters were subjected to acetylation with acetic anhydride:pyridine (2:1, v/v) at room temperature overnight. The acetyl derivatives, isolated by TLC, with *n*-hexane:diethylether (9:1, v/v) as the developing solvent, were analyzed by radio-GC. The methyl esters of fatty acids and hydroxy fatty acids were recovered from the silica gel. The methyl esters of fatty acids were analyzed by radio-GC using a Varian model 3300 gas chromatograph with a coiled stainless steel column (3.2 mm × 2 m) packed with 3% OV-1 (w/w) on Chrom W-HP 80/100, and a Lablogic GC-RAM radioactivity monitor using Winflow (IN/US systems, Tampa, FL) software. For analysis of mycocerosic acids and related acids, a 180–300 °C program at 15 °C/min was used. For acetylated hydroxyphthioceranic acid methyl esters, isothermal analysis at 290 °C was done with a carrier gas (helium) flow of 30 ml/min.

For specific labeling of sulfolipids, $Na_2{}^{35}SO_4$ (specific activity ≈ 43 Ci/mg) was added to the wild type and *pks2* mutant cultures at A_{600} 0.8–1.0, and the cultures were incubated at 37 °C in a roller incubator for 6 days. The total lipids were extracted as described previously. For two-dimensional TLC analysis of the sulfolipids on Silica Gel G plates, chloroform:methanol:water (60:12:1, v/v) was used in the first dimension and chloroform:methanol:water (75:11:1, v/v) in the second dimension (30). The plates were sprayed with 0.02% cresyl violet reagent in 1% aqueous acetic acid and kept at room temperature for 5–10 min until a red-violet color of sulfolipids appeared against the blue-violet spots produced by other compounds. The plates were also subjected to autoradiography to compare the labeling pattern.

The lipids derived from [1-^{14}C]propionate in the wild type, but missing in the *pks2* mutant, were recovered from the silica gel from one-dimensional TLC with 10% methanol in chloroform and from two-dimensional TLC (corresponding to the sulfolipid staining) and subjected to alkaline hydrolysis in alkaline 2-methoxyethanol containing 12% H_2O (17). Two-thirds of the recovered hydrolysis products were methylated by refluxing with 14% BF_3 in methanol. One-half of the recovered methyl esters was acetylated with a 2:1 mixture of acetic anhydride and pyridine overnight at room temperature. The hydrolysis products, their methylation products, and the acetylated methyl esters were subjected to TLC with *n*-hexane:diethyl ether:formic acid (65:35:2, v/v) as the developing solvent. The methyl esters of the hydroxy acids were analyzed by combined gas-liquid chromatography/mass spectrometry (GC/MS) as their trimethylsilyl ethers prepared by treatment with bis (trimethylsilyl) trifluoroacetamide/dimethylformamide (1:1, v/v) for 10 min at room temperature. GC/MS analysis was carried out using a Hewlett-Packard 5890 gas chromatograph and a Hewlett-Packard 5972 mass spectrometer with a 30-m Hewlett-Packard 5-ms column. For the analysis of methyl esters of the nonhydroxy fatty acids esterified to sulfolipids, the column temperature was 150 °C for 2 min followed by a program to 240 °C at 20 °C/min. For the trimethylsilyl derivatives of the hydroxyphthioceranic acids, the column temperature was 240 °C for 2 min followed by a program to 310 °C at 30 °C/min.

RESULTS

Immunoblot analysis of the proteins produced by a *mas*-disrupted mutant of *M. bovis* BCG revealed the presence of protein(s) of approximately the same size as MAS that cross-reacted with the anti-MAS antibodies.[6] Amino acid sequence of the amino terminus and an internal proteolytically derived peptide showed the presence of protein(s) highly homologous to MAS (data not shown). Two-dimensional electrophoresis of the high molecular weight proteins of *M. bovis* BCG and immunoblot revealed at least five proteins of the same size as MAS that cross-reacted with anti-MAS antibodies[6] (data not shown). These observations showed that *M. bovis* BCG produces several MAS-like proteins. Because the ketoacyl synthase (KS) domain involved in branched fatty acid synthesis was distinctly different from the corresponding domains involved in *n*-fatty acid synthesis (15), we used a gene segment representing the KS domain as a hybridization probe to detect genes that are probably involved in branched fatty acid synthesis. This approach showed a highly homologous segment in a nonannotated cosmid clone y409 of *M. tuberculosis*. Because this segment did not show an uninterrupted open reading frame (ORF), we resequenced this cosmid and found a single ORF of 6378 bp with 60% identity to *mas*. This result was confirmed when the same sequence appeared in the *M. tuberculosis* H37Rv cosmid MTCY409 in the GenBank™. The same gene was later designated as *pks2* in the annotated mycobacterial genome sequence (31). The ORF of this *mas*-like gene would encode a protein that is highly homologous to MAS and contains all of the catalytic domains found in MAS in the same relative position. To determine the nature of the products synthesized by the enzyme encoded by the *pks2* gene, we proceeded to disrupt this gene using a recently developed phage-mediated system to deliver the knockout construct into *M. tuberculosis* cells.

Disruption of the pks2 Gene by Allelic Exchange—Recently, conditionally replicating bacteriophages have been used for the delivery of transposons and homologous DNA substrates for allelic exchange in *M. tuberculosis* (26, 28, 32, 33). A specialized transducing phage was thus constructed and used to disrupt *pks2*. A 3159-bp DNA fragment containing a segment of *pks2* ORF (encoding the domains of acyl transferase (AT), dehydratase (DH), enoyl reductase (ER), and ketoreductase (KR)) was subcloned into pBS KS(−) and disrupted by replacing a 1.2-kb *Pst*I fragment coding for part a of the DH domain with the hygromycin resistance gene (Fig. 3*A*). This *pks2*::*hyg* fragment was introduced into phAE 87 to generate phAE 87 *pks2*::*hyg*. The structure of this phasmid DNA was verified by restriction enzyme digestion, PCR analysis, and sequencing. After infecting *M. tuberculosis* H37Rv with the phasmid, the transductants were selected at 37 °C on Middlebrook 7H10 containing hygromycin and were screened by PCR using two sets of primers, each of which contained an *hyg* primer and a primer in the mycobacterial genome directly outside that used to make the disruption construct (Fig. 3*A*, primer pairs C and H2, D and H1). In the event of homologous recombination, primers C and H2 would generate a 1170-p product and primers D and H1 would generate an 1310-bp product. A total of 20 clones were analyzed using this PCR screening strategy, and

[6] A. K. Azad, T. Sirakova, and P. E. Kolattukudy, unpublished results.

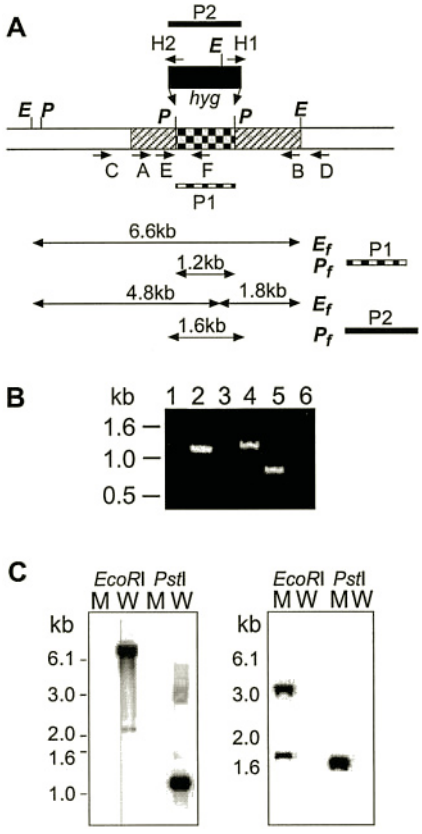

FIG. 3. *A*, schematic representation of the construct used to disrupt the *M. tuberculosis* H37Rv *pks2* locus by allelic exchange. *Hatched*, *checkered*, and *unshaded regions* represent *pks2* coding sequences, internal deletion region, and regions of the gene outside those used to make the disruption construct, respectively. *Black box*, *hyg* gene used for targeted disruption. Restriction enzyme sites: *E*, *Eco*RI; *P*, *Pst*I. Primer pair A/B was used to amplify the *pks2* segment used to generate the disruption construct. Primer pairs C/H2, D/H1, and E/F were used for PCR analysis of homologous recombination as described in the text. E_f and P_f, *Eco*RI and *Pst*I fragments from the wild type and *pks2* mutant expected to hybridize with probes P1 representing the fragment deleted in making the construct and P2 *hyg* gene, respectively. *P1* and *P2*, segments used as probes in Southern blot analysis. *B*, PCR analysis of internal and flanking regions of *pks2* locus showing products expected from homologous recombination. *Lanes 1*, *3*, and *5*, are wild type; *lanes 2*, *4*, and *6* are mutant. *Lanes 1* and *2*, 5′-flanking product with primers C/H2. *Lanes 3* and *4*, 3′-flanking product with primers D/H1. *Lanes 5* and *6*, internal deletion fragment with primers E/F. *C*, Southern blot analysis of *M. tuberculosis* H37Rv and mutant. Genomic DNA was digested with *Eco*RI and *Pst*I; *left*, hybridized with 1.2-kb fragment that was deleted in making the construct probe P_1; *right*, probed with P_2 (shown in *A*). *W*, wild type; *M*, mutant.

nine of them showed amplification products consistent with allelic exchange at the *pks2* locus (Fig. 3*B*). Disruption by gene replacement was confirmed by further PCR analysis using another set of primers: primer E located at 174-bp upstream of the *Pst*I site used for the deletion in the *pks2* sequence and primer F, located inside the deleted fragment. This pair of primers amplified a 900-bp product from the wild type *M. tuberculosis* and failed to amplify such a product from the *pks2*-disrupted mutant (Fig. 3*B*). By using primers specific to the *hyg* gene, the mutant was found to contain the *hyg* gene. The gene disruptant was further analyzed by Southern hybridization. Genomic DNA samples from the wild type and mutant strain were digested with *Eco*RI and *Pst*I. When the 1.2-kb internal segment of *pks2* gene, which was replaced by the *hyg* gene, was used as a probe the wild type showed the expected native 6.6- and 1.2-kb hybridization bands (Fig. 3, *A* and *C*). The *pks2* gene-disrupted mutant failed to show hybridization, confirming integration by double cross-over recombination.

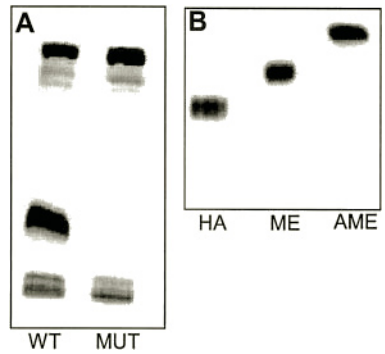

FIG. 4. **Autoradiograms showing the identity of the lipid that is missing in the mutant.** *A*, TLC of total lipids derived from [1-^{14}C]propionic acid in the wild type (*WT*) and *pks2* mutant (*MUT*) on silica gel with 10% methanol in chloroform as the solvent. *B*, TLC of the hydroxy acids (*HA*) generated by the hydrolysis of the wild type lipid (in *A*) that is missing in the *pks2* mutant, its methylation product (*ME*) and acetylated methylation product (*AME*), in *n*-hexane:diethyl ether:formic acid (65:35:2, v/v) as the developing solvent.

When the same blot was analyzed with the *hyg* gene as a probe, the mutant DNA samples yielded hybridization pattern in agreement with integration by allelic exchange (Fig. 3*C*). As expected, no hybridization was detected with the wild type DNA sample. These results confirm the integration of the *hyg*-disrupted *pks2* gene and replacement of the wild type allele in the mutant.

Biochemical Characterization of the Gene-disrupted Mutant—Because the *mas*-like *pks2* gene is thought to be involved in the biosynthesis of methyl-branched fatty acids, we tested whether the gene disruption altered incorporation of [1-^{14}C]propionate into lipids. Both the wild type and the *pks2* mutant incorporated similar amounts of [1-^{14}C]propionate (15–20% of administered ^{14}C) into total lipids. TLC analysis of the lipids, derived from this labeled precursor of branched acids in the wild type *M. tuberculosis* H37Rv, with 10% methanol in chloroform as the solvent, showed two major labeled fractions: one incompletely resolved group of nonpolar lipids near the solvent front and another at an R_F of about 0.3 (Fig. 4*A*). In the *pks2* mutant, this relatively polar component was missing. This polar component was found to be more polar than mycosides, because it stayed at or near the origin in 5% methanol in chloroform, a solvent that moves mycosides with an R_F of 0.4 (data not shown). About 60% of the label incorporated into lipids from the labeled propionate was found in the polar fraction and 40% in the nonpolar components in the wild type. On the other hand, virtually all of the label (>90%) incorporated into lipids by the mutant, was in the nonpolar components. Sulfuric acid/dichromate charring also showed that a chemical corresponding to the labeled polar component found in the wild type was missing in the mutant (data not shown).

To identify the lipid that was labeled in the wild type and missing in the mutant, this lipid was subjected to exhaustive alkaline hydrolysis, and the products were analyzed by TLC with *n*-hexane:diethyl ether:formic acid (65:35:2, v/v) as the solvent. The major part (80%) of the ^{14}C was found in a component with an R_F of 0.42 and the remaining part in a less polar fraction with an R_F of free fatty acids (data not shown). When the polar component (R_F 0.42) was methylated, the R_F increased to 0.58, indicating the presence of a free carboxyl group; when the methyl ester was acetylated, the R_F increased further to 0.72, showing the presence of an hydroxyl group (Fig. 4*B*). When the acetylated methyl ester was subjected to radio-GC, four very long-chain-labeled components were found (the results are not shown because they were very similar to those shown below in Fig. 7). Their retention times suggested that

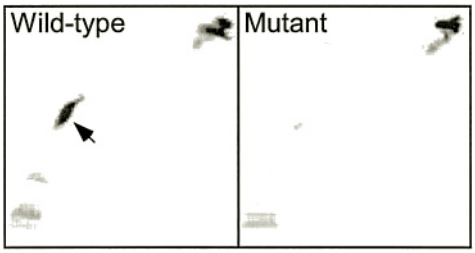

FIG. 5. **Autoradiograms of two-dimensional TLC of total cellular lipids derived from.** [1-^{14}C]Propionate in *M. tuberculosis* and its *pks2* mutant. The *arrow* indicates the presence of the major labeled sulfolipid in wild type (absent in the *pks2* gene-disrupted mutant).

they are hydroxylated phthioceranic acids that are known to be esterified to trehalose in the sulfolipids in *M. tuberculosis* H37Rv (18).

Two-dimensional TLC followed by autoradiography showed that the labeled lipid of the wild type, that was missing in the mutant, coincided exactly with the staining for sulfolipids. Comparison of the autoradiogram of the total lipids derived from [1-^{14}C]propionate in the wild type and the mutant showed that the strongly labeled lipid, which showed sulfolipid staining found in the wild type, was missing in the mutant (Fig. 5). Mass spectrometry of the hydroxyphthioceranic acid fraction, found only in the wild type but not in the *pks2*-disrupted mutant, confirmed their identity. GC/MS of the trimethylsilyl ether of the hydroxy acid methyl esters showed four major components that gave strong α-cleavage ions diagnostic of 15-hydroxy-2,4,6,8,10,12,14-heptamethyl triacontanoic acid (*m/e* 313 (base peak) and 441), 15-hydroxy-2,4,6,8,10,12,14-heptamethyldotriacontanoic acid (*m/e* 341 (base peak) and 441), 17-hydroxy-2,4,6,8,10,12,14,16-octamethyldotriacontanoic acid (*m/e* 313 (base peak) and 483), and 17-hydroxy-2,4,6,8,10,12,14,16-octamethyltetracontanoic acid (*m/e* 341 (base peak), 483) with some minor amounts of their homologues. GC/MS analysis of the nonhydroxy acid methyl esters showed that the major components were phthioceranic acids with some *n*-fatty acids, mainly C_{16}, C_{17}, and C_{18} and some longer *n*-fatty acids up to C_{28} (data not shown). The structure and composition of the fatty acids and the hydroxy acids of the lipids in the wild type that are missing in the mutant corresponded to the known composition of the fatty acids of sulfolipids (18).

To test for the possibility that the mutant can produce sulfolipids lacking the hydroxyphthioceranic acids, we used $^{35}SO_4^{-2}$ as the labeling agent. After 6 days of growth in a medium containing $^{35}SO_4^{-2}$, the total lipids from the wild type and the mutant were subjected to two-dimensional TLC followed by autoradiography. The results showed ^{35}S-labeled sulfolipids, appearing exactly as shown in Fig. 5, in the wild type, but the mutant lacked sulfolipids (data not shown).

To test whether the synthesis of phthioceranic acid is affected in the *pks2*-disrupted mutant that is deficient in hydroxyphthioceranic acids, we subjected the total lipids derived from [1-^{14}C]propionic acid to alkaline hydrolysis followed by methylation. Because phthioceranic acids are normally found only in sulfolipids, such a total lipid analysis was necessary to test whether such acids are present in some other lipids in the mutant that lacks sulfolipids. The total incorporation of ^{14}C from [1-^{14}C]propionic acid was similar in the wild type and the *pks2* mutant. TLC analysis with 10% ethyl ether in *n*-hexane showed that most of the label (86%) in the mutant was in the methyl ester fraction, whereas in the wild type only about 30% of the label was in the methyl ester fraction. Radio GC analysis of the methyl esters showed that the ^{14}C incorporated into lipids in the wild type was contained mainly in mycocerosic (short and long) acids and phthioceranic acids (Fig. 6), whereas no label was detected in phthioceranic acids in the *pks2* mutant. Radio GC analysis of the hydroxy acid methyl esters showed that the small amount of label incorporated into hydroxy acids by the mutant was contained in mycolipanolic acids that are much shorter than the phthioceranic acids (Fig. 7) whereas the major part of the label in the wild type was in hydroxyphthioceranic acids which were absent in the mutant.

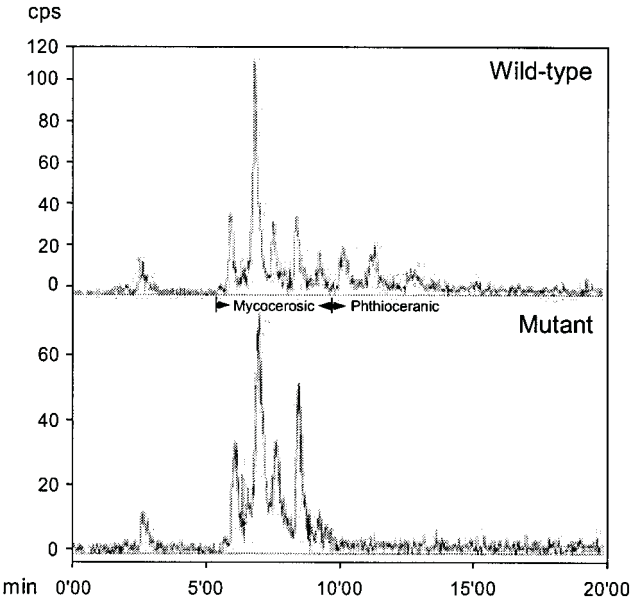

FIG. 6. **Radio-GC analysis of the total fatty acid methyl esters derived from.** [1-^{14}C]Propionate in *M. tuberculosis* H37Rv (*top*) and its *pks2* mutant (*bottom*). Elution times for mycocerosic acids and phthioceranic acids are indicated; the *pks2* mutant showed no phthioceranic acids.

A small variable portion of the [1-^{14}C]propionate incorporated into lipids was found in the medium. TLC analysis showed that this label was contained mainly in a nonpolar component that showed an R_F identical to dimycocerosylphthiocerol. Alkaline hydrolysis followed by methylation and TLC showed most of the label was contained in the fatty acid methyl ester fraction. Radio-GC of the methyl esters showed that the label was contained in mycocerosic acids (data not shown). The *pks2* mutant showed a higher level of label in this extracellular fraction when compared with the wild type.

DISCUSSION

M. tuberculosis contains a great diversity of complex lipids, particularly in the lipid-rich cell walls (2–4). The need to encode all of the large number of enzymes involved in the synthesis of such lipids is reflected in the fact that the mycobacterial genome contains an unusually high proportion of genes for lipid metabolism (31). Among these genes are a large number of *pks* genes that are probably involved in making aliphatic chains. *M. tuberculosis* contains *n*- and multiple methyl-branched carbon chains derived from malonyl-CoA and methylmalonyl-CoA, respectively. The AT and KS domains of the multifunctional *pks* genes probably have structural features that are selective for the use of malonyl-CoA or methylmalonyl-CoA as the substrate (15). In fact, such a selectivity of the AT and KS domains of methylmalonyl-CoA utilizing MAS has been demonstrated with expressed individual domains of *mas* (34). Homology of this gene to other *pks* genes in the genome indicates the presence of seven *mas*-like genes in the *M. tuberculosis* genome (Fig. 8). In some cases two adjacent ORFs together contain all of the domains that would be required for the synthesis of a branched fatty acid as shown by the domain organization indicated in Fig. 8. Therefore, we designate such a pair as a *mas*-like gene (*msl*). In one case, *msl6*(*pks12*), the

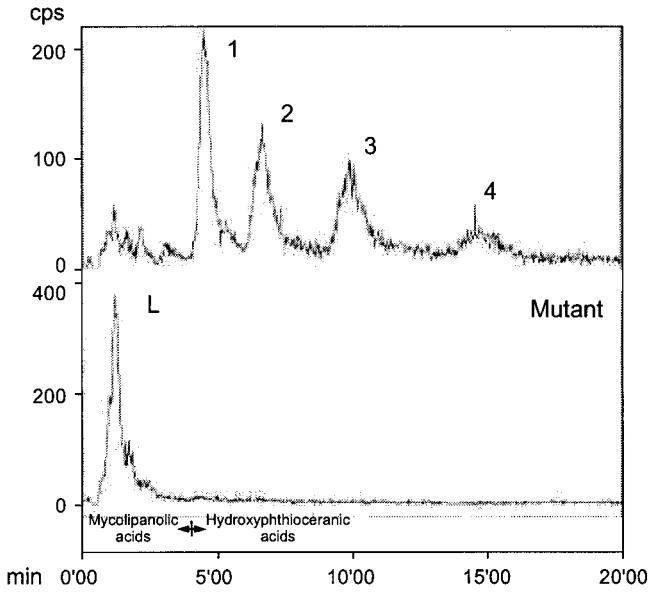

FIG. 7. **Radio-GC analysis of the total hydroxy fatty acid methyl esters derived from [1-^{14}C]propionate in *M. tuberculosis* H37Rv (*top*) and its *pks2* mutant (*bottom*).** *Peaks 1–4* represent hydroxyphthioceranic acids, heptamethyl C_{30}, heptamethyl C_{32}, octamethyl C_{32}, and octamethyl C_{34} acids, respectively; *peak L*, unresolved mycolipanolic acids. The hydroxy acids were acetylated before GC. GC conditions are given in the text.

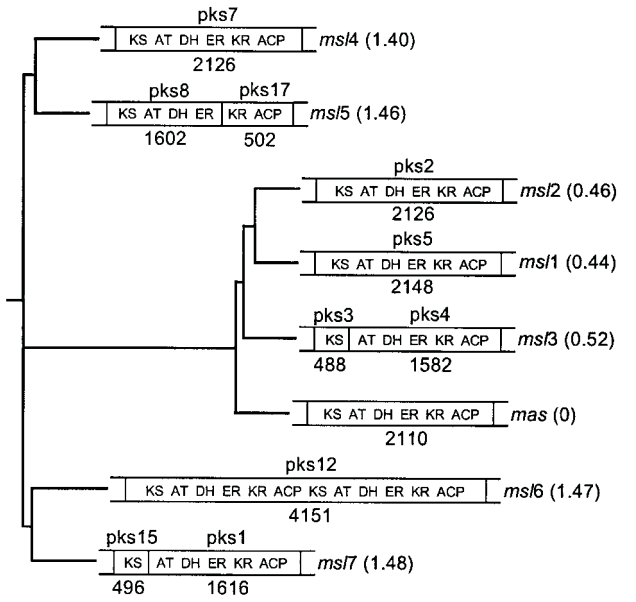

FIG. 8. **Dendrogram showing amino acid sequence homology relationships among the *mas*-like (*msl*) genes and their domain organization in *M. tuberculosis*.** The *length of each branch* represents the genetic distance between the different genes. The *numbers in parentheses* indicate phylogenetic distance from *mas*.

ORF contains two sets of catalytic domains with an expected protein of twice the size of the other synthases, representing the largest ORF in the genome of *M. tuberculosis*. The seven *mas*-like genes include *msl1*(*pks5*), *msl2*(*pks2*), and *msl3*(*pks3+4*), which have a higher degree of homology to each other and to *mas*, when compared with the other *mas*-like genes: *msl4*(*pks7*), *msl5*(*pks8+17*), *msl6*(*pks12*), and *msl7*(*pks1+15*) as indicated by the phylogenetic relationships indicated in Fig. 8. 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* (Fig. 1).

If the *pks* pairs jointly produce branched acids, as we postulate, that would be reminiscent of the yeast fatty acid synthase (FAS) where two multifunctional peptides jointly produce a fatty acid. Mycobacteria are known to contain a bacterial type fatty acid synthase complex composed of separate proteins for each catalytic domain (FAS2) and the vertebrate type multifunctional synthases (FAS1) (3, 4, 35, 36). The presence of the yeast type dual peptide synthase, in addition to the other two types, constitutes a unique combination of strategies for the synthesis of fatty acids in this organism. Such different strategies may be necessary for the production of the large diversity of fatty acids that are produced by this organism (Fig. 1). If the unique branched mycobacterial lipids are essential for the survival of the organism in the host, the enzymes that catalyze the synthesis of such lipids can be suitable targets for new antimycobacterial drugs. In the absence of any direct information about the nature of such enzymes, one way to seek the identity of the genes that encode the enzymes specifically involved in the synthesis of such lipids is to disrupt *mas*-like genes and identify the lipids missing in the mutants. The disruption of one such gene described here illustrates this approach by identifying the lipids missing in the *pks2* gene-disrupted mutant.

Allelic exchange in slow growing mycobacteria has been successfully achieved, but efficient generation of such gene-targeted disruption mutants remains a challenge. We have disrupted the *mas* gene (17), the *acoas* gene (37), and the *pps* cluster (16) in *M. bovis* BCG using a suicide vector approach, but this method gives a high frequency of integration by nonhomologous recombination leading to the need to screen a high number of transformants. Recently, an improved one-step gene replacement method that uses *in vitro* generated specialized transducing mycobacteriophages was described (26). This method takes advantage of the unique ability of phages to efficiently deliver foreign DNA into every cell of the bacterial population and thus substantially increases the chances of detecting rare recombination events such as targeted double cross-over. This method that we used for the disruption of the *pks2* gene, gave a high efficiency of allele-specific replacement (~50% of the analyzed clones), much higher than that observed when using a suicide vector for delivery of the substrate for allelic exchange. Analysis of the gene-disrupted mutant, clearly showed homologous recombination by double cross-over causing replacement of the native gene by the disrupted copy of the gene. Because the knock-out strategy is to prevent the synthesis of the intact multifunctional enzyme encoded by the *pks2* gene, the substitution of parts of two domains (DH and ER) of the enzyme by the hygromycin resistance gene would prevent the formation of a functional enzyme. The biochemical consequences we observed confirm this expectation.

Because the *pks2* disruption totally abolished the synthesis of the hepta- and octamethyl hydroxyphthioceranic acids, the major acyl components of the sulfolipids in *M. tuberculosis* (18), *pks2* probably encodes the hydroxyphthioceranic acid synthase. This synthesis would require all of the steps involved in mycocerosic acid synthesis with one significant difference. During the first cycle the ketoacid generated by condensation of methylmalonyl-CoA with an *n*-fatty acyl moiety is reduced to the hydroxy acid but not dehydrated, leaving the hydroxyl group in the final heptamethyl- and octamethyl-branched products. Our mass spectrometric analysis showed that both *n*-C_{16} and *n*-C_{18} acyl groups participate as starting primers, yielding the final products that carry an hydroxyl group at the position corresponding to C-1 of both of these starting *n*-fatty acids. The mass spectral analysis also showed that both C_{16} and C_{18} primers generate hydroxy products expected from elongation with 7- and 8-methylmalonyl-CoA units. There is no direct proof for the

above hypothesis about the synthesis of the hydroxyphthioceranic acids, and therefore, introduction of the hydroxyl group into preformed heptamethyl and octamethyl phthioceranic acids cannot be ruled out, especially in view of the fact that the nonhydroxylated phthioceranic acids are present in *M. tuberculosis* (18). However, based on the biosynthetic strategies used in the synthesis of polyketide natural products in general, we think that it is likely that phthioceranic acids and hydroxyphthioceranic acids are generated by two different synthases, one that completely reduces the carbon chains and the other that leaves the C-1 of the starting *n*-fatty acid as an hydroxyl group.

If *pks2* were to encode phthioceranic acid synthase that generates the acids into which another enzyme introduces an hydroxyl group, *pks2* disruption would be expected to prevent the synthesis of both families of acids. Another possibility that could explain the absence of both phthioceranic and hydroxyphthioceranic acids is that *pks2* encodes the enzyme that synthesizes hydroxyphthioceranic acids, which constitute the major acyl groups in the sulfolipids and the absence of these acids prevents sulfolipid synthesis. In the absence of sulfolipids there are no sites for esterification of phthioceranic acids, and therefore, phthioceranic acids are not found in the mutant. Previously we have found that in the absence of phthiocerol and phenolphthiocerol, the esterification sites for mycocerosic acids, these acids were not found in the *pps*-disrupted mutant of *M. bovis* BCG, even though enzymatically active mycocerosic acid synthase was present in the mutant (16).

The relative amounts of [1-^{14}C]propionic acid incorporated into the mycocerosic family of acids was much higher in the mutant. The total amount of ^{14}C incorporated into lipids in the mutant was about the same as that in the wild type, although phthioceranic acids and hydroxyphthioceranic acids were not produced in the mutant. Obviously, methylmalonyl-CoA generated from propionate was channeled into the mycocerosic family of acids (*e.g.* Fig. 1*H*, and its shorter homologues also called mycolipanoic acids, Fig. 1*C*) because of the absence of the hepta- and octamethyl-branched acid synthesis that would have normally consumed a large proportion of this substrate in the presence of a functional *pks2*. The [^{14}C]propionate labeling observed in the *M. tuberculosis* H37Rv is quite different from that observed in *M. bovis* BCG. The major difference is the incorporation of a large proportion of ^{14}C-labeled propionate in *M. tuberculosis* into the hepta- and octamethyl-branched acids attached to the sulfolipids and smaller amounts in the mycocerosic acids esterified to phthiocerol and less into mycolipanolic acids (Fig. 1*E*) found in the acyltrehaloses. In *M. tuberculosis*, very little label is found in mycosides. On the other hand, in *M. bovis* BCG most of the [1-^{14}C]propionate was incorporated into the mycocerosic acids in the mycosides with less in the other esters of mycocerosic acids and very little esterified to trehalose (17).

Sulfolipids have been postulated to play significant roles in the interaction of *M. tuberculosis* with its host. Their role in the prevention of phagosome-lysosome fusion (38) has been questioned (39). However, there is evidence that the sulfolipids of *M. tuberculosis* inhibit priming of monocytes with bacterial cell components (*e.g.* lipopolysaccharides) or cytokines induced in the monocytes (*e.g.* interferon-γ) (20, 22). Such suppression of reactive oxygen formation by monocytes could help weaken the antibacterial capacity of the monocytes/macrophages. It is possible that the enhanced expression of *pks2* noted in response to phagocytosis of *M. tuberculosis* by cultured human primary macrophages (23) represents an effort by the pathogen to lessen the effectiveness of the activation of macrophages to allow increased survival of the pathogens in the host by increasing sulfolipid synthesis. The availability of the sulfolipid-deficient mutant of *M. tuberculosis* should allow direct tests for the roles in pathogenesis postulated for this class of lipids that are found only in virulent strains.

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