**Analysis of the Phthiocerol Dimycocerosate Locus of Mycobacterium tuberculosis**

**EVIDENCE THAT THIS LIPID IS INVOLVED IN THE CELL WALL PERMEABILITY BARRIER**

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Among the few characterized genes that have products involved in the pathogenicity of *Mycobacterium tuberculosis*, the etiological agent of tuberculosis, are those of the phthiocerol dimycocerosate (DIM) locus. Genes involved in biosynthesis of these compounds are grouped on a 50-kilobase fragment of the chromosome containing 13 genes. Analysis of mRNA produced from this 50-kilobase fragment in the wild type strain showed that this region is subdivided into three transcriptional units. Biochemical characterization of five mutants with transposon insertions in this region demonstrated that (i) the complete DIM molecules are synthesized in the cytoplasm of *M. tuberculosis* before being translocated into the cell wall; (ii) the genes *fadD26* and *fadD28* are directly involved in their biosynthesis; and (iii) both the *drrC* and *mmpL7* genes are necessary for the proper localization of DIMs. Insertional mutants unable to synthesize or translocate DIMs exhibit higher cell wall permeability and are more sensitive to detergent than the wild type strain, indicating for the first time that, in addition to being important virulence factors, extractable lipids of *M. tuberculosis* play a role in the cell envelope architecture and permeability. This function may represent one of the molecular mechanisms by which DIMs are involved in the virulence of *M. tuberculosis*.

*Mycobacterium tuberculosis*, the etiological agent of tuberculosis, is an intracellular pathogen that causes more human deaths than any other single infectious agent. Despite its tremendous importance as a public health problem, the molecules involved in the pathogenicity of the tubercle bacillus remain largely unknown. The mycobacterial cell envelope has long been thought to be involved in both the pathogenicity of these bacteria and their resistance to hostile environments and antibiotics. In addition to its postulated passive role through a strong resistance to degradation by host enzymes, impermeability to toxic macromolecules, and inactivation of small reactive molecules, such as reactive oxygen and nitrogen derivatives, the mycobacterial cell envelope may exert a more active role, notably by interacting with host cell receptors to facilitate uptake of the bacterium and by modulating the immune response (1).

The mycobacterial envelope is unique, both in molecular composition and in the architectural arrangement of its constituents. From the cytoplasm to the external side of the bacterium, the cell envelope is composed of: (i) a plasma membrane; (ii) a cell wall consisting of a peptidoglycan covalently attached to the heteropolysaccharide arabinogalactan, which is in turn esterified by very long chain (C60–C90) fatty acids called mycoceric acids and various noncovalently attached lipids and glycolipids; and (iii) a capsule of polysaccharides, proteins, and lipids (1). In the last 50 years, considerable effort has been devoted to searching for putative virulence factors among constituents of the mycobacterial cell envelope. Two structurally related families of noncovalently attached cell wall and capsular lipids, phthiocerol and phenolphthiocerol diesters (Fig. 1), have retained special attention. These complex lipids are composed of a mixture of long chain β-diols, which are esterified by multimethyl-branched fatty acids. Depending on the stereo-chemistry of the chiral centers bearing the methyl branches, the fatty acids are called mycogenic or phthioceranic acids (2, 3). Phthiocerol dimycocerosates (DIM)1 and diphthioceranates have been identified to date in eight *Mycobacterium* species. DIM have been found in *M. tuberculosis*, *Mycobacterium bovis*, *Mycobacterium africanum*, *Mycobacterium leprae*, *Mycobacterium gastri*, and *Mycobacterium kansasi*, and phthiocerol diphthioceranates have been found in *Mycobacterium marinum* and *Mycobacterium ulcerans*. With the exception of *M. gastri*, all of the DIM- or phthiocerol diphthioceranate-containing species are mycobacterial pathogens (3). In addition, a DIM-less *H37Rv*-derived strain of *M. tuberculosis* has been shown to be attenuated in the guinea pig model in comparison with the DIM-producing *H37Rv* strain (4). Furthermore, an avirulent strain of *M. tuberculosis* coated with a mixture of DIM and cholesteryl oleate has been shown to persist longer than the uncoated strain in the spleen and lungs of infected

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1 The abbreviations used are: DIM, phthiocerol dimycocerosate(s); kb, kilobase(s); ORF, open reading frame(s); bp, base pair(s); PCR, polymerase chain reaction; RT, reverse transcriptase; DIMA, dimycocerosate(s) of phthiocerol A; DIMA, dimycocerosate(s) of phthiocerol B; DMB, dimycocerosate(s) of phthiodiolone; GC, gas chromatography; MS, mass spectrometry; ICD, isocitrate dehydrogenase; RNI, reactive nitrogen intermediate(s); BCG, bacillus Calmette-Guerin; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight.

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Phthiocerol Dimycocerosate in M. tuberculosis

EXPERIMENTAL PROCEDURES

Bacterial Strains and Culture Conditions—M. tuberculosis Mt103, the wild type strain used in this study, was isolated from an extensively drug-resistant tuberculosis patient (14). Strains MYC2251 to MYC2261 were isolated using the Signature-tagged transposon mutagenesis procedure, as previously described (12). MYC2251 contains an IS1096::km insertion 113 bp upstream from the predicted start codon of fadD26. MYC2253, MYC2260, MYC2261, and MYC2267 harbor insertions within the fadD26, mmpL7, drrC, and fadD28 genes, respectively. Strain MYC2267 was obtained by PCR screening of our 6912 insertional mutant library as described by Jackson et al. (15). The occurrence of an IS1096::km insertion within fadD28 in MYC2267 was confirmed by PCR using primers fadD28C3 and fadD28S3, which are specific for fadD28, and primers IS1 and IS2, which are specific for IS1096::km (Table I). The use of the two fadD28-specific primers in a PCR reaction gave a 1882-bp fragment with the wild type strain Mt103, whereas no PCR fragment was obtained with MYC2267. In contrast, DNA fragments of the expected size were amplified if IS1 and fadD28C3 or IS2 and fadD28S3 were used (data not shown). The IS1096::km insertion site was sequenced and found to be located 460 bp downstream from the predicted start codon of the fadD28 gene.

Strains were grown on Sauton medium as surface pellicles, Middlebrook 7H9 medium (Difco) supplemented with ADC (0.005% oleic acid, 0.2% dextrose, 0.05% Tween 80 where indicated or on solid Middlebrook 7H11 medium (Difco) supplemented with ADC (0.2% dextrose, 0.05% bovine serum albumin fraction V, 0.0005% NaCl, 0.0003% beef catalase). Kanamycin was added when required at a concentration of 20 μg/ml.

RNA Isolation and RT-PCR Assays—M. tuberculosis cultures (10 ml) were grown to mid-exponential growth phase. Bacterial cells were pelleted by centrifugation for 10 min at 4000 × g, resuspended in 1 ml of TE (10 mM Tris-Cl, 1 mM EDTA, pH 8) containing lysozyme (5 mg/ml), and incubated for 20–30 min at 37 °C. Cells were disrupted by adding 500 μl of mini glass beads (0.1 mm in size; PolyLabo) and vigorous shaking for 3 min using a mini bead beater. Total RNA was then extracted using the RNAeasy total RNA kit (Qiagen). Contaminating DNA was removed by digestion with DNase I according to the manufacturer’s instructions (Roche Molecular Biochemicals). This enzyme was removed by extractions with two chloroform/isomyl alcohol followed by ethanol precipitation. RNA (1 μg) and oligonucleotide primer were denatured by heating to 65 °C for 10 min. Reverse transcription was performed with the Expand<sup>TM</sup> reverse transcriptase (Roche Molecular Biochemicals) in a final volume of 20 μl containing 10 mM dithiothreitol, 1 mM dNTP (Amersham Pharmacia Biotech), 1 μl of RNase inhibitor (Amersham Pharmacia Biotech), 50 units of reverse transcriptase, and the manufacturer’s buffer (provided with the enzyme). This mixture was incubated for 5 min at 43 °C, and the enzyme was inactivated by heating for 2 min at 95 °C. A control reaction containing the RNA components but no reverse transcriptase was included to check for DNA contamination. The cDNA products (2 μl) were then used in a PCR reaction performed in a final volume of 50 μl containing 2 units of AmpliTaq Gold polymerase (PerkinElmer Life Sciences), 10% Me<sub>2</sub>SO, and 15 pmol of each primer. A positive control in which M. tuberculosis chromosomal DNA was used as a template for the PCR reaction was included. The amplification program consisted of one cycle

A

CH₃-(CH₂)₇-O-CH₂-CH₂-CH₂-CH₂-CH₂-CH₂-CH₂-CH₃

CH₃

B

CH₃-(CH₂)₇-O-CH₂-CH₂-CH₂-CH₂-CH₂-CH₂-CH₂-CH₃

CH₃

FIG. 1. Structures of diesters of phthiocerol and related compounds. The long chain β-diol (phthiocerol moiety) is esterified by multimethyl-branched fatty acids (mycocerosic or phthioceranic acids). In members of the M. tuberculosis complex mycocerosic acids esterify phthiocerols to yield phthiocerol DIM in which m = 20, 22, n₉ = 16, 18; and p₉ = 2–5. A, DIMA and DIMB contain phthiocerol A and B where R = –CH₂-CH₃ and -CH₃, respectively. B, DIMB corresponds to dimycocerosates of phthiodiolone. The other letters (a–g) correspond to protons with resonances that are visible in Fig. 4 and described in the text.
Oligonucleotide primers used for MYC2267 characterization, RT-PCR analysis, and construction of pLCF26, pLCF28, and pLCD

| Primer          | Nucleotide sequence (5′ → 3′)          | Annealing temperature used (°C) |
|-----------------|---------------------------------------|---------------------------------|
| **Transcriptional coupling** |                                       |                                 |
| f2C16           | CGCAGTGCCCCGACGATATCA                 | 65                              |
| fD26            | GTCGAGTGGCCGTAACGC                   | 65                              |
| ppsA1           | GCCGAGGCTGGTGTGATGC                  | 55                              |
| ppsB1           | AGCATTCGCTGCTGGGTC                   | 55                              |
| ppsB7           | ATGACGCGCGACGACGTGCTGATC             | 55                              |
| ppsC1           | TGGCGGCCGCCGGACGACGTGCTGATC          | 55                              |
| ppsC2           | ATGACGCGCGACGACGTGCTGATC             | 55                              |
| ppsD5           | TCCTGCGTCTGAGTAGGGTCGAT              | 55                              |
| ppsD11          | CCGGGCGCTGGCAGCC                    | 66                              |
| ppsE12          | CGGCCGCCGCTCCACAC                   | 66                              |
| ppsE1           | TACAGTTCGGACTGCGATGAC                | 60                              |
| draR1           | GACCTGGCTGACTGGCCAGA                 | 60                              |
| draR2           | GTGCTCCGGCGATCTGAGGGATCTG            | 60                              |
| draR2           | CCATCGGCTGCTGACAAACG                | 60                              |
| drrC3           | ATGAGTGGACCCATGGGACGGCC             | 60                              |
| pap1            | GATGCTCCGGACGCGGAC                    | 60                             |
| pap2            | TCCAGAAGTGACAGAGGTGTCGT              | 60                              |
| mpL7.3          | CCCAGCTGGCGGCGGATGTGGCCG             | 68                              |
| Characterization of MYC2267 |                                       |                                 |
| IS1             | CTCTTGACGCAACCACGCGCCGACTCA          | 60                              |
| IS2             | GAGGGCCGCAAGAATGCTGACGATG            | 60                              |
| fadD28S3        | ATGGTGACCCATGGGACGC                  | 60                              |
| fadD28C3        | GACTAGTTCGACTCCGATCAA                | 60                              |
| Construction of pLCF26 |                                       |                                 |
| f26C1           | GACTAGTTCGGCCTGCAACAG                | 60                              |
| f26C2           | GACTAGTTCGGCCTGCAACAG                | 60                              |
| Construction of pLCF28 |                                       |                                 |
| f28C1           | GACTAGTTCGGATTTACCGAC                | 62                              |
| f28C3           | GACTAGTTCGGATTTACCGAC                | 62                              |
| Construction of pLCDc |                                       |                                 |
| drrC1           | CGGGATCCATGATCACGACGACAGGT           | 63                              |
| drrC41          | GTCTGCGAGATCCGGGCTGTCGCCC            | 63                              |

of 10 min at 95 °C followed by 35 cycles of 1 min at 95 °C, 1 min at annealing temperature (depending on the primer used (Table I), 1 min at 72 °C, and a final 10 min at 72 °C. The PCR products were then analyzed by electrophoresis in 0.8% agarose gels. The identities of the PCR products were confirmed by sequencing.

Complementation of M. tuberculosis Mutants—Plasmid pMIP12 is an Escherichia coli/mycobacteria shuttle vector derived from pAL5000. It contains a mycobacterial promoter, pBlaF, upstream from a multicloning site followed by a transcription terminator. A 1.6-kb BamHI fragment containing the hygromycin resistance gene (hyg) was blunt-ended and inserted into the blunt-ended NsiI + DraI-digested pMIP12 vector to give rise to pMIP12H. The drrC gene was amplified from cosmid MTCY19H9 (11) with the drrC1 and drrC4 primers (Table I) using the Expand High Fidelity PCR system (Roche Molecular Biochemicals) according to the manufacturer’s instructions. The 830-bp PCR product was digested with BamHI and PsfI and inserted into the BamHI + PsfI-cut pMIP12H vector leading to give pLCF26. In this construct, the predicted start codon of drrC was located 6 bp downstream from a Shine-Dalgarno sequence and is under the control of the mycobacterial promoter pBlaF.

Plasmid pOIP23H is an integrative mycobacterial vector containing the integrase gene, SpeI and the 287-bp sequence located upstream from drrC. A 1740-nucleotide and the intergenic sequence upstream from fadD28 (620 nucleotides) were amplified from cosmid MTCU24G1 using primers f28C1 and f28C3. The amplicon was digested with SpeI and cloned into pOIP23H to give pLCF28. The integrity of genes drrC, fadD26, and fadD28 was checked by sequencing.

**DIM Purification**—Bacterial pellets from 200 ml of 20-day cultures in Sauton medium were used for the isolation of DIM for structural analysis. Cells were collected by pouring off the medium and were inactivated by heating at 95 °C for 2 h. Bacterial pellets were left in CHCl3/CH3OH (2:1 v/v) at room temperature overnight, and lipids were inactivated by heating at 95 °C for 2 h. Bacterial pellets were left in CHCl3/CH3OH (2:1 v/v) at room temperature overnight, and lipids were extracted twice with CHCl3/CH3OH (1:1 v/v), concentrated under vacuum, washed three times with water, and dried. This crude lipid extract was subjected to chromatography on a Florisil (60–100 mesh) column and was eluted with increasing concentrations of diethyl ether (0, 10, 20, 30, 50, and 100%) in petroleum ether. The DIM content of each fraction was determined by TLC on silica gel G 60 plates (20 × 20 cm; Merck) using petroleum ether/diethyl ether (9:1 v/v) as the eluent. Lipid compounds were visualized by spraying the plates with 10% phosphomolybdate acid in ethanol and heating. The DIM-containing fractions (10% diethyl ether in petroleum ether fractions) were pooled, dried, and subjected to chromatography on another Florisil column. Increasing concentrations of diethyl ether (0, 1, 2, 3, 5, 8, 10, and 50%) in petroleum ether were used as eluents to obtain the various members of the DIM family (e.g., dimycocerosates of phthiocerol A (DIMA), dimycocerosates of phthiocerol B (DIMB), and dimycocerosates of phthiodiolone (DIMD); Fig. 1).

**Structural Analysis of DIM**—Samples of purified DIM (2 mg) were analyzed by NMR spectroscopy. Spectra were recorded on a Bruker AMX-500 spectrometer equipped with an Aspect X32 computer. The samples were dissolved in CDCl3 (99.96 atom % D) and analyzed in 200 × 5-mm 535-PP NMR tubes. One-dimensional 1H spectra were recorded at 295 K; 13C chemical shifts were expressed with respect to the internal CDCl3 (7.27 ppm).

Purified DIM samples were also analyzed by mass spectrometry with a linear mode of detection using a VOYAGER DE-STR MALDI-

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Phthiocerol Dimyocerocesate in M. tuberculosis

TOF instrument (PerSeptive Biosystems, Framingham, MA). DIM (1 μl of a 1 mg/ml solution) was mixed with 0.5 μl of the matrix solution. The mass spectra were mass assigned using an external calibration. The matrix used was 2,5-dihydroxybenzoic acid (10 mg/ml) in CHCl3/CH3OH (1:1 v/v).

The constituents of DIM, mycocerosic acid residues and phthiocerol and related substances, were structurally characterized as their methyl and O-methylated derivatives, respectively, using the conventional Hakomori procedure. Briefly, 200 μg of DIMs were dissolved in dimethysulphinyl potassium in dimethyl sulphoxide (200 μl), and the mixture was stirred at room temperature for 4 h. A large excess of CDI (100 μl) was then added, and the reaction was left for 2 h at room temperature by adding 1 ml of H2O and sodium thiosulfate. Fatty acid trideuteriotmethylyl esters and per-O-deuteriotmethylylated substances of the phthiocerol family were extracted with CHCl3, washed with water, dried under nitrogen, and dissolved in diethyl ether prior to analysis by gas chromatography (GC) and GC-mass spectrometry (GC-MS). GC was performed on a Geldel series 30 apparatus equipped with an OV1 capillary column (0.30 mm × 25 m) using helium gas (0.7 bar) with a flame ionization detector at 310 °C. The temperature separation program involved an increase from 200 to 310 °C at the rate of 5 °C/min, followed by 10 min at 310 °C. GC-MS analyses were performed on a Hewlett-Packard 5890 X mass spectrometer (electron energy, 70 eV) coupled to a Hewlett-Packard 5890 series II gas chromatograph fitted with a capillary column (0.30 mm × 25 m). GC-MS analyses were performed in both electron impact and chemical ionization modes; in the latter mode, NH3 was used as the reagent gas.

Subcellular Distribution of DIM in M. tuberculosis—We determined the distribution of DIMs in the various cell fractions of M. tuberculosis using labeled cultures; 20 μCi of sodium [1-14C]propionate (specific activity, 55 Ci/mmol; ICN) was added to 100 ml of 16-day cultures of the wild type and insertional mutants of M. tuberculosis that were incubated at 37 °C for 16 h with continuous shaking. Cultures were centrifuged for 10 min at 4000 × g, and culture supernatants were filtered twice through membranes with 0.2-μm pores (Millipore) to remove contaminating cells and concentrated to one-tenth of their initial volume. Half of each bacterial pellet was gently shaken with 10 g of glass beads (4-mm diameter) for 30 s, resuspended in 10 ml of H2O, and centrifuged for 10 min at 4000 × g. Supernatants were filtered through membranes (0.2-μm pores) to yield the surface-exposed materials (17). Mini glass beads (500 μl; Polylabo) and 1 ml of H2O were added to the remaining half of each bacterial cell that was disrupted using a mini bead beater for 3 min. Bacterial extracts were centrifuged for 10 min at 5000 × g to eliminate intact cells, and supernatants were centrifuged for 30 min at 16,000 × g. The 15,000 × g supernatants corresponded to the cytoplasmic and cell membrane components, whereas the corresponding pellets contained mainly cell envelope components. These pellets were washed twice with 1 ml of H2O, and the second washing and the other fractions were kept for isocitrate dehydrogenase (ICD) activity assays to check for contamination with cytoplasmic compounds. This enzyme assay was performed as previously described (18) using a 100-μg protein equivalent of each fraction. The fractions were first sterilized by filtration through membranes (0.2-μm pores); protein concentration was then determined using the Coomassie Blue reaction (Bio-Rad protein assay). We checked for contamination with the extracellular fraction by performing Western blot analysis as previously described as described using 30 μg of proteins of the various fractions and antiserum raised against the Erp protein (19). In M. bovis BCG, it was found that in liquid medium without Tween, most of the Erp protein was present in the supernatant.4

All of the extracts were inactivated by incubation for 2 h at 95 °C before extraction with organic solvents for lipid analysis. Lipids were extracted from the various cell fractions by adding 2 volumes of CH3OH and 1 volume of CHCl3, 0.8 volume of Tris buffer (0.2 M, pH 7.4), and 1 volume of petroleum ether (9:1 v/v). The mixtures were incubated for 2 h and then partitioned into two phases by adding 1 volume of H2O/CHCl3 (1:1 v/v). The organic phase was recovered, washed twice with water, and dried to yield the subcellular lipid extracts. 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 (0.5 × 10 cm, E. Merck), which were developed in petroleum ether (9:1 v/v).

The lipid was detected by scanning chromatograms with a Berthold LB 2832 TLC linear analyzer. The total number of counts per min recovered in the region corresponding to DIM on TLC was used to determine the amount of DIM in the portion of each fraction analyzed and, consequently, in the whole bacterial compartment of each mycobacterial strain examined (expressed as relative percentages).

Drug Sensitivity and Permeability Assays—The drug sensitivity of the wild type strain and its isotopic insertional mutants was determined as described previously (15). Permeability assays were performed using labeled cultures; 20 μCi of sodium [1-14C]propionate (specific activity, 55 Ci/mmol; ICN) was added to final concentrations of 1 or 5 mM, and the pH was adjusted to 5.5. The effect of this pH itself on growth of the various M. tuberculosis strains was monitored by incubating the same medium adjusted to pH 5.5 without NaNO2. Cultures were incubated for 10 days, and aliquots were collected after 0, 1, 4, and 10 days of growth. The number of viable bacteria was evaluated by plating serial dilution on 7H11 medium.

For assays of resistance to RNI, SDS was added to a final concentration of 0.01, 0.04, or 0.1%. Cultures were incubated for 9 days, and aliquots were collected after 0, 1, 4, and 9 days of growth. The number of viable bacteria was evaluated by plating serial dilutions on 7H11 medium.

RESULTS

Genetic Organization of the DIM Locus—In silico analysis of the 13 different ORF present in the 50-kb fragment (Fig. 2) showed that ORF fadD26 to papA5 were all transcribed in the same orientation and that, based on their predicted start codons, several of these ORF overlapped (11). The overlapping genes are fadD26/papsA, papsA/papsB, papsB/papsC, papsC/papsD, drrA/drrB, and drrB/drrC, which display a 2-bp overlap. The other three intergenic regions correspond to papsD/papsE (5 bp), papsE/drrA (10 bp), and drrC/papA5 (46 bp). This kind of organization is typical of a polycistronic message with translational coupling. However, it remains possible that other start sites may be used. A similar situation is observed for ORF fadD28 and mmpL7, which may be part of a second operon because they display 5-bp overlaps. We studied the transcriptional organization of this region by using RT-PCR to investigate the transcriptional coupling of all of these ORF using total RNA extracted from the wild type strain Mt103. To show a transcriptional coupling, a primer within a downstream ORF was used to prepare cDNA. This template was then used to amplify a fragment by PCR with one primer specific for the upstream ORF and another specific for the downstream ORF (usually the one used to produce the cDNA). A PCR product was thus expected if the two genes were part of the same operon. This analysis was performed for ORF fadD26 to papA5 and fadD28 and mmpL7 (Fig. 2). PCR amplification products of the expected sizes were observed in the expected genomic regions tested. All of the PCR products had the expected sequences (data not shown). These results strongly suggest that the fadD26 to papA5 genes, as well as fadD28 and mmpL7, are transcriptionally coupled. In the five insertional mutants, transposons had inserted into these two operons.

Biochemical Phenotypes of Mutants with Insertions in the DIM Locus—To correlate data from genetic analysis of the DIM locus with the biochemical phenotypes of the mutants, the lipid contents of the wild type and its isotopic insertional mutants were compared. To facilitate the detection and quantification of DIMs, cells were first labeled with [14C]propionate, a precursor
molecule known to be incorporated into both the multimethyl-branched fatty acid residues and the phthiocerol portion of DIM (2, 20), and then extracted with organic solvents. Extractable lipids recovered from the wild type strain and insertional mutants accounted for about 16\% of the dry bacterial weight. We carried out TLC analysis to compare lipid contents and found that the wild type M. tuberculosis clinical isolate used in this study (Mt103), like most M. tuberculosis strains analyzed to date, did not produce detectable amounts of phenolphthiocerol dimycocerosates (data not shown) but synthesized molecules with mobility similar to DIMA and DIMB (Fig. 1). Two of the five strains examined, MYC2253 (fadD26) and MYC2267 (fadD28), did not produce detectable amounts of these molecules, whereas the remaining three strains, MYC2251 (insertion upstream of fadD26), MYC2260 (mmpL7), and MYC2261 (drrC), did (Fig. 3A). These data are consistent with the genetic organization of the DIM locus because the MYC2253 mutant, which has an insertion within fadD26, was expected to be devoid of DIM because of a polar effect of the mutations on the expression of pps genes. Similarly, an insertion in fadD28, a gene encoding an acyl-CoA synthase thought to be involved in the release and transfer of mycoserosic acid from Mas to diols (9), would likely lead to a lack of production of DIM in MY2267, as recently demonstrated for M. bovis BCG (9) and M. tuberculosis (13). Similar amounts of these substances were present in Mt103 and MYC2260, but smaller amount of these substances accumulated in MYC2251 and MYC2261. This observation was confirmed by quantifying the two purified lipid spots; the radiolabeling of these spots in MYC2251, MYC2260, and MYC2261 corresponded to 5, 20, and 100%, respectively, of that of the wild type strain (Fig. 3B). The phenotype of mutant MYC2251 suggests that transposon insertion upstream from fadD26 has a strong polar effect on the expression of downstream genes.

Structural Analysis of Lipids from the Mutants with Insertions in the DIM Locus—We investigated the nature of the pairs of lipid constituents with mobilities similar to that of DIMs on TLC by fractioning lipids extracted from the various strains on Florisil columns. The native purified substances, which accounted for about 5% of the noncovalently bound lipids extracted from Mt103 and MYC2260 (mmpL7), were further analyzed by NMR and mass spectrometry.

The \(^1\)H NMR spectra obtained for the purified compounds with similar mobility on TLC and originated from the three strains analyzed (Mt103, MYC2260, and MYC2261) could be superimposed, and most of the proton resonances were common to all of these spectra. Proton assignments were validated previously by two-dimensional chemical shift-correlated and J-resolved spectroscopy (21). In addition to the broad signal

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**Fig. 2.** Genetic organization of the DIM locus. Top panel, results of the RT-PCR assays. Each set of three reactions consists of an RT-PCR reaction, a negative control assay without reverse transcriptase (.), and a positive control PCR assay with genomic DNA as the template (+). The cDNA template was generated with the same 3' primer as the one used in the subsequent PCR reaction. The combinations of primers are indicated above each set of three reactions. Bottom panel, schematic diagram and transcriptional organization of the DIM locus. The genes and transcripts are indicated as horizontal arrows. Transposon insertions are indicated as vertical arrows, and the names of the corresponding mutant strains are indicated.

**Fig. 3.** TLC analysis of lipids extracted from the wild type and mutant strains. A, TLC of lipids from M. tuberculosis Mt103 and some of its insertional mutants. Crude lipid extracts were deposited in each lane, separated by TLC, and detected with 10% phosphomolybdic acid in ethanol. Lane 1, Mt103; lane 2, MYC2253; lane 3, MYC2261; lane 4, MYC2260; lane 5, M. tuberculosis Canetti; lane 6, MYC2251; lane 7, MYC2267. The eluent was petroleum ether-diethyl ether (9:1 v/v). B, cells were metabolically labeled with \([1-\text{\textsuperscript{14}C}]\)propionic acid for 16 h, and the crude lipid extracts were run on TLC as described for A and analyzed with a radioscaner.
The chemical structures of DIMs were established by analysis of their degradation products. The two constituents of DIM were obtained by a conventional Hakomori procedure and were analyzed by GC and GC-MS as illustrated in Fig. 6. The same fatty acid trideuteriomethyl ester and per-O-deuteriomethyl phthiocerol profiles were obtained for the degradation products from DIM purified from M. tuberculosis H37Rv strain of M. tuberculosis were similar to those of M. tuberculosis Mt103 and its insertion mutants, whereas a series of DIM (C86–C98) were detected in the mass spectra of M. tuberculosis.

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The structure of the phthiocerol moiety of DIM from M. tuberculosis Mt103 and its insertion mutants produced structurally identical DIM. The structure of the phthiocerol moiety of DIM from M. tuberculosis H37Rv and M. tuberculosis Mt103 and its insertion mutants. In contrast, shorter fatty acid methyl esters typified the DIM from M. tuberculosis, which contained mainly equal amounts of C26 (n,n' = 18; p,p' = 2).
and C29 \((n,n' = 18; p,p' = 3)\), consistent with mass spectrometry data showing that this species produced DIMs with lower molecular masses. The fatty acid composition of DIMs from \(M. tuberculosis\) H37Rv was very similar to that of \(M. tuberculosis\) Mt103.

Subcellular Distribution of DIM in Mt103 and the Various Insertional Mutants—In \(M. tuberculosis\), small amounts of lipids, including DIM, have been found in the outermost layer of the cell envelope (17). This did not account for the totality of these substances, the remaining fractions being found in deeper layers. Because the MYC2260 (\(mmpL7^{-}\)) and MYC2261 (\(drrC^{-}\)) strains had insertions in genes encoding transporters, we investigated the subcellular distribution of these molecules in the wild type and the two insertional mutants, seeking the possible roles of these two transporters in the transfer of DIM in the bacterial cell envelope. \(M. tuberculosis\) cultures were labeled by incubation for 16 h in Middlebrook 7H9 supplemented with ADC using \([14C]\)propionate and four cell fractions (culture filtrate, surface-exposed material, cell wall, and cytosol plus plasma membrane) were prepared and tested for the presence of DIM (Fig. 7B). Cross-contamination of the various fractions was ruled out by assaying the ICD activity (usually restricted to the cytosol) or by looking for the presence of the secreted mycobacterial protein Erp. Both surface and cell envelope fractions displayed very low levels of ICD activity and contained no detectable Erp. As expected, high levels of ICD activity and no Erp were detected in the cytosol plus plasma membrane fraction and low ICD activity, but high levels of Erp were found in the culture medium (data not shown). These controls showed that the level of cross-contamination between the various fractions was very low. DIM were found mostly (more than 65%) associated with the cell wall of the wild type strain, whereas they were primarily located in the cytosol plus plasma membrane fraction of MYC2260 (\(mmpL7^{-}\)) and MYC2261 (\(drrC^{-}\)) (78 and 92%, respectively). Thus both the MmpL7 and DrrABC transporters are required for the correct localization of DIM.

A culture on Middlebrook 7H9 medium supplemented with ADC or Sauton medium resulted in the production of insignificant amounts of DIM in the culture filtrate of \(M. tuberculosis\), consistent with previous data (23) but conflicting with one recent observation (13) in which DIM from the Erdman strain of \(M. tuberculosis\) were found in the culture medium, Middlebrook 7H9 supplemented with OADC, glycerol, and 0.1% Tween 80. We investigated whether this difference in localization was strain-dependent or caused by the presence of the detergent by repeating the localization experiment with cultures on Middlebrook 7H9 supplemented with 0.05% Tween 80 (Fig. 7A). In these growth conditions, 65% of the DIMs were recovered in the culture medium of the wild type strain, whereas no DIM was detected in the culture medium of the two mutants. Thus the presence of DIM in the culture medium was clearly an artifact caused by the addition of detergent to the growth medium.

Complementation of Mutants with Insertions in the DIM Locus—The genetic organization of the DIM locus suggested that...
the biochemical phenotypes of the various strains analyzed may be due to polar effects on genes located downstream from the various insertion sites. These polar effects of transposon insertion do not always lead to complete shut-down of downstream genes expression because transcription may initiate within the transposon or cryptic promotor may be present downstream from the insertion. Therefore some level of complementation may be expected by reintroducing a wild type copy of the mutated gene. To confirm the direct involvement of the disrupted genes in the observed phenotype, the various mutants were complemented with wild type genes, and the production and distribution of DIM were analyzed. Complementation of the mutation in MYC2267 (fadD28) with the fadD28 gene resulted in the production of DIM. However, smaller amounts of these substances accumulated in the complemented MYC2267 mutant than in the wild type strain (~15% of that in the wild type strain). The MYC2267 strain complemented with the fadD28 gene did transport the synthesized DIM into the cell wall, as shown by the presence of DIM in the culture fluid of the strain grown in the presence of Tween (data not shown). This observation shows that mmpL7, the product of which is essential for the transport of DIM into the cell wall, is expressed. The same complementation experiment performed with strain MYC2253 (fadD26) and pLCF26 led to the partial restoration of DIM production (~5% of that in the wild type strain). Thus fadD26 is directly involved in the biosynthesis of DIM. The poor production of DIM in MYC2253: pLCF26 and MYC2267:pLCF28 may be either due to poor expression of the fadD26 and fadD28 genes from pLCF26 and pLCF28 or, in the case of MYC2253, due to a polar effect of transposon insertion on expression of the pps genes, as predicted from the genetic organization of the DIM locus. In the case of the drrC gene, the promotor is expected to be far upstream the start codon because of the operon organization. Therefore we cloned the wild type copy of the drrC gene under the control of an exogenous well characterized mycobacterial promotor, pBluF*, leading to plasmid pLCDC. Introduction of pLCDC into strain MYC2261 (drrC−) led to full restoration of DIM production and translocation. This demonstrates that the DrrABC transporter, like MmpL7, is essential for DIM translocation.

Cell Wall Permeability of M. tuberculosis Mt103 and the Cell Wall-DIM-less Mutant—The permeability of mycobacterial cell wall is unusually low, only one-tenth to one-hundredth that of E. coli for β-lactam antibiotics (24, 25). This feature may be relevant not only to the unusually high resistance of mycobacteria to drugs but also to their pathogenicity by preventing toxic molecules produced by the host from penetrating the mycobacterial cell. This very low permeability has been attributed mostly to the presence of large amounts of long chain molecules, such as the mycolic acids covalently linked to the cell wall arabinogalactan (26). We used attenuated mutants of M. tuberculosis devoid of DIM and strains in which these molecules are not present in the cell walls to investigate the role of DIM in cell wall permeability. We did this by measuring the uptake of chenodeoxycholate by M. tuberculosis Mt103 and its isogenic insertional mutants defective in the production or translocation of DIM (Fig. 8A). This molecule is a negatively charged hydrophobic probe that diffuses through lipid domains and has been used to evaluate the fluidity of mycobacterial cell wall lipids (27, 28). The accumulation of the probe by the three insertional mutants greatly differed from that of the parent strain Mt103 (Fig. 8A). All of the mutants showed significantly higher initial rates of uptake of the compound than did Mt103. To rule out the possibility that this phenotype was due to a smaller amount of mycolates in the insertional mutants, we

![Fig. 8. Role of DIM in the cell envelope permeability barrier and resistance to antimicrobial compounds.](image)
Phthiocerol Dimycolocerosate in M. tuberculosis

19853

(29). We investigated further the consequence of the defect in DIM production by evaluating the sensitivity to SDS of Mt103 and its DIM-less MYC2253 (fadD26) mutant by comparing the survival of these two strains after treatment with various concentrations of the detergent for 1, 4, and 9 days (Fig. 8B). SDS concentrations lower than 0.01% did not affect cell viability in either strain. The DIM-less strain appeared to be much more sensitive than the wild type strain to 0.1% SDS, although the addition of detergent caused a rapid decrease in the number of viable bacteria for both strains. After 1 day of exposure to the detergent, the number of colony-forming units differed between the two strains by a factor of almost 100. This difference decreased over time, being smaller on days 4 and 9. However, even on day 9, the number of viable cells was lower for the DIM-less strain than for the parent strain. This greater susceptibility of the insertional mutant to detergent is consistent with differences in cell envelope structure and a higher level of cell wall permeability.

Changes in the cell wall permeability barrier may also be detected by measuring the sensitivity of bacteria to various antibiotics, which presumably enter the bacterium to exert their effect, provided that the diffusion of the drugs is the limiting step. We therefore determined the minimal inhibitory concentrations for both relatively hydrophilic molecules (ciprofloxacin and ofloxacin) and hydrophobic drugs (ethambutol, isoniazid, and pyrazinamide) of the parent strain and its DIM-less mutant. The minimal inhibitory concentrations of both types of compounds were unaffected by the mutation. This suggests that the diffusion of these antibiotics through the cell wall permeability barrier was not the limiting step for their activity and is consistent with the results obtained for an antigen-85-inactivated mutant of M. tuberculosis, which was affected in cell wall mycolate content and cell wall permeability but for which the minimal inhibitory concentrations of the drugs tested were unaffected (15).

Susceptibility of M. tuberculosis Mt103 and a DIM-less Strain to Reactive Nitrogen Derivatives—Insertional mutants with insertions affecting either the synthesis or transport of DIM in the cell wall are strongly attenuated in mice (12, 13). This growth defect is already visible during the initial stages of the infectious process in which M. tuberculosis is thought to multiply within macrophages and monocytes and to resist the microbicidal responses of these cells. A change in cell wall permeability may modify the susceptibility of M. tuberculosis to toxic metabolites produced by the host cell. Major antimicrobial molecules produced by mouse macrophages include reactive oxygen intermediates such as hydrogen peroxide and RNI such as nitric oxide. RNI has been shown to be essential for the control of M. tuberculosis infection in mice (30). We investigated whether DIM were involved in resistance to RNI by comparing the susceptibility to RNI of Mt103 and its DIM-less mutant MYC2253 (fadD26) (Fig. 8C). Bacterial survival was assessed after exposure to NaNO2 (1 or 5 mM) for 1, 4, or 10 days under acidic conditions (pH 5.5) that did not by themselves affect cell viability (data not shown) and in which NaNO2 generated NO and NO2-. Under these conditions, exposure for less than 1 day did not reduce bacterial growth, whereas exposure to RNI for longer than 1 day reduced cell viability. For example, less than 0.1% survival of the wild type strain was recorded after 10 days of exposure to 5 mM NaNO2 at pH 5.5. A time-dependent inhibition effect was observed with Mt103 for both concentrations of NaNO2 used, but no difference was found between the DIM-less insertional mutant, MYC2253, and its parent strain in terms of sensitivity to NaNO2 (Fig. 8C). We therefore concluded that DIM by itself did not contribute to the resistance of M. tuberculosis to RNI.

DISCUSSION

Following the recent development of genetic tools for mycobacteria and the completion of genome sequencing for M. tuberculosis (11, 31), it has become possible to investigate more thoroughly the molecular bases of the pathogenicity of the tubercle bacillus. Application of the Signature-tagged transposon mutagenesis method to mycobacteria led to the identification of several attenuated mutants with insertions in a 50-kb chromosomal fragment (12, 13). This region has been previously shown to contain 13 genes, some of which are involved in the biosynthesis and transport of DIMs. This study was undertaken to investigate further the genetic organization of this DIM region and the biochemical phenotypes of the insertion mutants. The data we obtained by RT-PCR and the sequence of this region strongly suggested that the DIM locus was divided into three transcriptional units: (i) one covering more than 32 kb and including 10 ORF from fadD26 to papA5, (ii) a second containing only the mas gene, and (iii) a third including the fadD28 and mmpL7 ORF.

In the five mutants studied, transposon insertions were found to have occurred within two different operons. We showed that disruptions of mmpL7 and fadD28, two genes belonging to the same operon, led to different phenotypes in terms of DIM production. A strain with an insertion in mmpL7 produced DIM that were primarily retained in the cytosol or the cytoplasmic membrane. In contrast, a strain with an insertion in the fadD28 gene did not produce any detectable DIM. Therefore it is possible to conclude that the FadD28 protein is directly involved in the biosynthesis of DIM. Support for this conclusion is provided by the results obtained for the complementation of the fadD28-disrupted mutant with the wild type gene, which resulted in the partial restoration of DIM synthesis in the complemented strain. Two different unrelated strains of M. tuberculosis (Mt103 and Erdman) with insertions in this gene did not synthesize DIM, whereas Fitzmaurice and Kolat-tukudy (9) have shown that a BCG strain of M. bovis with an insertion in the fadD28 gene still produced DIM containing shorter chain mycoerosic acid residues. We found that these fatty acids, mainly C26 mycoerosates, were only minor products in the H37Rv strain (less than 5% of mycoerosic acids) and were absent from the Mt103 strain of M. tuberculosis, whereas they accounted for 50% of the mycoerosates isolated from M. bovis BCG. FadD28 therefore seems to be specific for C29–C32 mycoerosic acid residues. This heterogeneity among bacteria of the M. tuberculosis complex has previously been reported for compounds related to DIM. Indeed, unlike DIM, which are produced by all strains of M. tuberculosis examined to date, only a very small proportion of M. tuberculosis, in particular the Canetti and related strains of M. tuberculosis (22), elaborate phenolphthiocerol dimycolates, or their glycosylated derivatives. These differences may be relevant for pathogenicity because DIMs are clearly important virulence factors, whereas the presence of the related mycoside has been associated with a decrease in virulence (4).

The MmpL7 protein is clearly involved in translocation of the synthesized molecules but is not the only protein involved in this phenomenon because an insertion in the drrC gene also resulted in the production of small amounts of DIM present principally in the bacterial cytosol or plasma membrane. The phenotype of this strain with an insertion in the drrC gene may be due to direct involvement of the mutated drrC gene, a polar effect on the expression of papA5 or both. To discriminate between these possibilities, we transformed strain MYC2261 (drrC−) with pLCD, which resulted in the translocation of DIM into the cell wall, showing that the disruption of the drrC gene was responsible for the observed phenotype. The require-
ment of both the MmpL7 and DrrABC transporters for the correct distribution of DIM is worth noting. Because both the drrC and mmpL7 mutants produce DIM with structures identical to those of DIMs synthesized by the wild type strain, it is unlikely that each transporter translocates part of the DIM molecule (the phthiocerol or the mycoscerosate moiety) with the final assembly taking place in the cell envelope. Instead the two proteins may cooperate in the translocation of DIM. MmpL7 is similar to several proteins of the resistance-nodulation-cell division superfamily (10), in terms of both predicted structure and amino acid sequence (data not shown). This superfamily includes ActII-3, which is involved in polyketide export in *Streptomyces coelicolor* (32). It includes also the SecD and SecF proteins, which have been shown to be part of the type II secretion system. In this system SecD and SecF act in association with other proteins including an ATPase to combine proton motive force and ATP hydrolysis for protein translocation (33). A similar situation may apply to the correct localization of DIM with the MmpL7 and the DrrABC transporters interacting for efficient translocation.

For the mutant with an insertion in the *fadD26* gene, the observed phenotype may result from a direct involvement of the disrupted gene, a polar effect on downstream genes, or both. Complementation analysis showed that *fadD26* was directly involved in DIM biosynthesis, possibly by activating substrates for the Pps polyketide synthase. However, the low level of DIM production in MYC2253:pLCF26 suggests that the transposon insertion in *fadD26* exerted a polar effect on the expression of downstream genes.

The low permeability of the mycobacterial envelope has been associated with the existence of an outer pseudobilayer, involving the cell wall-linked mycolates and probably other lipids (34, 35). Clear evidence that covalently bound cell wall mycolates are involved was provided by the analysis of an antigen-85C-35). Clear evidence that covalently bound cell wall mycolates associated with the existence of an outer pseudobilayer, involving expression of downstream genes.

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**Phthiocerol Dimycocerosate in M. tuberculosis**

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