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Mycolyltransferase from *Mycobacterium leprae* Excludes Mycolate-containing Glycolipid Substrates

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Trehalose dimycolate (TDM) is a major surface-exposed mycolyl glycolipid that contributes to the hydrophobic cell wall architecture of mycobacteria. Nevertheless, because of its potent adjuvant functions, pathogenic mycobacteria appear to have evolved an evasive maneuver to down-regulate TDM expression within the host. We have shown previously that *Mycobacterium tuberculosis* (M.tb) and *Mycobacterium avium* (M.av), replace TDM with glucose monomycolate (GMM) by borrowing host-derived glucose as an alternative substrate for the FbpA mycolyltransferase. *Mycobacterium leprae* (M.le), the causative microorganism of human leprosy, is also known to down-regulate TDM expression in infected tissues, but the function of its mycolyltransferases has been poorly analysed. We found that, unlike M.tb and M.av FbpA enzymes, M.av FbpA was unexpectedly inefficient in transferring α-branched mycolates, resulting in impaired production of both TDM and GMM. Molecular modelling and mutational analysis indicated that a bulky side chain of leucine at position 130 of M.le FbpA obstructed the intramolecular tunnel that was proposed to accommodate the α-branch portion of the substrates. Notably, even after a highly reductive evolution, M.le FbpA remained functional in terms of transferring unbranched acyl chains, suggesting a role that is distinct from that as a mycolyltransferase.

Key words: glycolipid, glucose monomycolate, *Mycobacterium leprae*, mycolyltransferase, trehalose dimycolate.

Abbreviations: Fbp, fibronectin-binding protein; GMM, glucose monomycolate; M.av, *Mycobacterium avium*; M.le, *Mycobacterium leprae*; M.tb, *Mycobacterium tuberculosis*; TDM, trehalose dimycolate; TMM, trehalose monomycolate.

Mycobacteria are unique in their highly lipid-rich cell wall that is critical not simply for their acid-fast properties but also for their survival and replication. The cell wall contains mycolic acids, a family of α-branched, β-hydroxy long-chain fatty acids, which are densely aligned in covalent association with the underlying arabinogalactan sugar layer or exist as free molecules complexed to either trehalose or glucose. The arabinogalactan-bound mycolic acids are proposed to extend outwards and interact closely with carbon chains of the surface-exposed glycolipids, thereby forming the hydrophobic cell wall architecture that is essential for long-term survival of pathogenic mycobacteria within host cells (*1*).

Trehalose-6,6′-dimycolate (TDM) comprises a major surface-exposed mycolyl glycolipid that can be readily synthesized when mycobacteria are cultured in artificial media, and therefore, its biological activities as well as its relevance to pathogenesis have been studied extensively over the past two decades. A single dose of TDM can induce granuloma formation *in vivo* in animal tissues, and its outstanding ability to stimulate host innate immune cells, such as macrophages and dendritic cells, has been fully documented (*2*). Further, we recently reported TDM-elicited eosinophilic responses in mycobacteria-infected guinea pigs (*3*). All of these immunostimulatory or adjuvant functions mediated by TDM could potentially jeopardize the microbes by allowing the host to efficiently monitor and control infection. The generally accepted picture of the cell wall structure of mycobacteria, highlighting abundant TDM expression at the outermost layer, has been drawn, based primarily on biochemical analysis of the microbes cultivated in artificial media, but adaptive changes that minimize TDM functions may occur after pathogenic mycobacteria infect into the host.

Indeed, we have recently shown that a switch of glycolipid biosynthesis from TDM to another surface-exposed mycolyl glycolipid, glucose-6-monomycolate (GMM), occurs in *Mycobacterium tuberculosis* (M.tb) and *Mycobacterium avium* (M.av) upon exposure to host-derived glucose (*4*). Previous studies have

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established that the final step of TDM biosynthesis is catalysed by the mycolyltransferase activity of the fibronectin-binding protein (Fbp), using its biosynthetic precursor, trehalose-6-monomycolate (TMM) as a substrate (*5*). However, at the physiological concentration of glucose present in mammalian hosts, TDM down-regulation and concomitant GMM up-regulation occur within hours as a result of competitive acceptor substrate selection of TMM and glucose by the Fbp enzyme (*4*). Given that GMM is much less potent than TDM in stimulating innate immune cells, the swift switch from TDM to GMM biosynthesis by utilizing the pre-existing Fbp enzyme and the host-derived glucose could function as an efficient evasive maneuver for pathogenic mycobacteria (*4*).

Leprosy is an ancient disease caused by *Mycobacterium leprae* (M.le) infection, but remains an important health problem worldwide. Unlike M.tb and M.av, M.le is not cultivable in artificial media, and therefore, its lipid chemistry and biology have not been studied so extensively as for the other pathogenic mycobacteria species. Previous studies have suggested that only a trace amount of TDM is produced by M.le grown in infected tissues while production of its biosynthetic precursor, TMM, is readily detectable (*6*). Nevertheless, the M.tb and the M.le genomes share all the four Fbp genes (*fbpA*, *fbpB*, *fbpC* and *fbpD*), and the deduced amino acid sequences indicate that products of M.le *fbpA*, *fbpB* and *fbpC*, designated FbpA, FbpB and FbpC, respectively, contain a catalytic triad that is essential for the mycolyltransferase activity, as found in other serine esterases (*7*). Therefore, it was initially hypothesized that M.le should have evolved an efficient strategy for TDM down-regulation that might be similar to maneuvers employed by M.tb and M.av. Surprisingly, however, we provide evidence that the M.le-derived FbpA protein excludes mycolate-containing glycolipid substrates, resulting in profound down-regulation of both TDM and GMM. This 'intrinsic' mechanism of TDM down-regulation has not been observed for other pathogenic mycobacteria species tested so far, and may uniquely support survival of M.le within the host.

MATERIALS AND METHODS

Chemical Reagents and Bacteria—Chemical reagents were purchased from Nacalai Tesque (Kyoto, Japan) unless otherwise indicated. *Mycobacterium avium* ATCC 35767 (serovar 4) was obtained from American Type Culture Collection (Manassas, VA, USA). The bacteria were grown in Middlebrook 7H9 media (BD, Franklin Lakes, NJ, USA) supplemented with the albumin-dextrose-catalase enrichment (BD) and 0.05% Tween 80. *Corynebacterium matruchotii* JCM 9386 was obtained from Japan Collection of Microorganisms (Tsukuba, Japan), and maintained on a plate of brain-heart-infusion agar media (BD). For isolation of TMM from *C. matruchotii*, the bacteria were grown in brain-heart-infusion liquid media containing 5% trehalose.

Preparation of Recombinant Enzymes—The recombinant M.av FbpA was prepared as described previously (*4*). The gene that encoded the mature M.le FbpA protein lacking the signal sequence was amplified by PCR, using genomic DNA of the M.le Thai-53 strain (*8*) as a template and a specific primer set as follows: 5′-ggaattcca tatgttctcccggccgggattgcc-3′ (sense primer) and 5′-cccaagct tagcacccggggtagccccta-3′ (antisense primer). The amplified PCR product was digested with NdeI and HindIII, and ligated to a NdeI–HindIII-digested pET-21c plasmid vector (Merck Japan, Tokyo, Japan). The nucleotide sequence of the cloned M.le *fbpA* was identical to that reported elsewhere (http://genolist.pasteur.fr/Leproma/). To optimize the codon usage for efficient expression in *Eschericia coli*, PCR was carried out with the cloned M.le *fbpA* gene as a template and a primer set as follows: 5′-cgcatatgttctctcgtccgggtctgccggttgagtacctgcaa-3′ and 5′-gccccggggtagcacccaggtactgctgcaggtccggtttcatg-3′ (sense and antisense primers, respectively, in which mutated nucleotides are underlined). The PCR product was digested with NdeI and SmaI, and exchanged for the corresponding fragment of the cloned M.le *fbpA* gene in pET-21c.

Site-directed mutagenesis was performed by PCR with the PrimeStar HS DNA polymerase (Takara Co. Ltd, Tokyo, Japan), using the codon-modified plasmids as a template. The primer sets used was as follows: 5′-cgg ttcttcggccctgacgctggcgatctaccaccc-3′ and 5′-ggccgaagaaccg gccatcgaaagaccgaccgccgc-3′ (for M.le *fbpA*); 5′-cggcctgtcgg cgctgatcctggccgcctaccac-3′ and 5′-ccgacaggccggccatcgac aggccgacgacacc-3′ (for M.av *fbpA*), in which mutated nucleotides are underlined. The cycling conditions for PCR amplification were as follows: 95°C, 1 min, followed by 10 cycles of 95°C, 15 s and 72°C, 10 min, and a final extension step of 72°C, 10 min. The amplified PCR products were digested with DpnI, and used for transformation of *E. coli*. Introduction of the mutation was confirmed by DNA sequencing.

Escherichia coli BL21 (DE3) was transformed with each of the plasmids, and the His-tagged recombinant FbpA proteins were obtained as described (*4*) with slight modifications. The protein expression was induced with 0.1 mM IPTG at 25°C for 20 h. The cells were then harvested and disrupted by sonication in ice-cold 20 mM Tris–HCl (pH 7.9) buffer containing 0.5 M NaCl and 60 mM imidazole (sonication buffer). The sonicates were centrifuged at 6,000*g* for 30 min at 4°C to remove insoluble materials, and the supernatants were applied onto a Ni-resin column equilibrated with the sonication buffer. After washing the column with the sonication buffer, the recombinant proteins were eluted with the 20 mM Tris–HCl (pH 7.9) containing 0.5 M NaCl and 0.5 M imidazole. The eluates were concentrated and dialyzed overnight at 4°C against 50 mM sodium phosphate buffer (pH 7.5) containing 10% glycerol at 4°C. Purity and quantity of the enzymes were assessed by SDS–PAGE and Coomassie staining.

Preparation of Substrates—The M.av serovar 4-derived long-chain TMM was purified as described (*4*). For purification of the *C. matruchotii*-derived short-chain TMM, the cultured cells were harvested, and lipids were extracted with chloroform/methanol (C/M, 1 : 1, v/v), followed by fractionation by TLC with a solvent system of C/M/water (65 : 25 : 4, v/v/v). The TMM fraction was then extracted with C/M (1:1, v/v) from the silica gels.

The identity of the purified TMM preparations was confirmed by mass spectrometry.

Trehalose-6-monolaurate (TML) was synthesized by modification of a method of Raku *et al*. (*9*). The reaction mixture (dimethylformamide/water, 97 : 3, v/v) containing 160 mM α-trehalose (Wako Pure Chemicals Co. Ltd., Osaka, Japan), 450 mM vinyl laurate (Tokyo Chemical Industry Co., Ltd, Tokyo, Japan) and 31 mg/ml Bioprase OP (Nagase Chemtex Co. Ltd, Osaka, Japan) was incubated at 35°C for 7 days, and then extracted with *n*-hexane to remove unreacted vinyl laurate. The dimethylformamide phase was applied onto a C18 reverse-phase column (Presep C18 ODS, Wako Pure Chemicals) that was equilibrated with water. Subsequently, the column was washed with water, and the TML-containing fraction was eluted with acetonitrile/water (50 : 50, v/v). After evaporation, the fraction was dissolved in methanol and applied onto a silica gel G TLC plate (Analtech, Newark, NJ, USA). The plate was developed with a solvent system of C/M/acetone/acetic acid (50 : 30 : 20 : 1, v/v/v/v), and TML was extracted from the gel. Its identity was confirmed by mass spectrometry and ^{1}H NMR.

Enzyme Assays—FbpA enzyme assays were performed as described (*4*) with slight modifications. Each of the lipidic substrates (63 μM long-chain TMM, 130 μM short-chain TMM and 1.9 mM TML) was prepared in 20 mM sodium phosphate buffer (pH 7.5) (reaction buffer) unless otherwise indicated. In experiments monitoring GMM production, the reaction mixtures also contained 4% D-glucose (w/v). The reaction was started by the addition of either M.le-derived or M.av-derived FbpA enzymes (1.5 nmol for long-chain TMM, 160 pmol for short-chain TMM, and 7.8 pmol for TML) with a total volume of 200 ml per tube, and after 20 min of incubation at 37°C, the reaction was stopped by the addition of 3 ml of C/M (2 : 1) and 0.3 ml of distilled water as well as *n*-tetradecanol (20 μg per sample) that served as an indicator for extraction efficiency. The lipids were extracted by the method of Kremer *et al*. (*10*) and analysed by silica gel TLC with a solvent of either C/M/acetone/acetic acid (90 : 10 : 10 : 1) (for detection of long-chain mycolyl glycolipids) or C/M/acetone/acetic acid (80 : 15 : 10 : 1) (for detection of lauryl trehalose and short-chain mycolyl glycolipids). The lipids on the TLC plates were visualized by spraying 50% sulphuric acid and baking. The amounts of each compound were calculated, based on the intensity of spots of serially diluted *n*-tetradecanol. The molecular identity of the products was confirmed by mass spectrometry, using an electrospray-ion trap-time of flight mass spectrometer (Shimadzu LCMS-IT-TOF, Shimadzu Co. Ltd, Kyoto, Japan) as described (*11*).

Kinetic Analysis of Enzyme Reactions—Enzyme assays were carried out with wild-type and mutant FbpA enzymes, using four different concentrations of the *C. matruchotii*-derived short-chain TMM as a substrate. The lipids were extracted and separated on TLC plates, followed by densitometrical determination of the amount of the products, using serially diluted *n*-tetradecanol as a reference. Data were collected from three independent experiments, and kinetic analysis was performed by Hanes–Woolf plotting.

Molecular Modelling of the FbpA S130L Mutant—Molecular modelling of the M.tb-derived FbpA S130L mutant protein was performed, using the homology modelling software PDFAMS (Protein Discovery Full Automatic Modeling System; In-Silico Sciences, Inc., Tokyo, Japan) as described (*12*, *13*). Briefly, the primary sequence and the molecular model of the M.tb FbpA protein were obtained from the Protein Data Bank (1SFR). The serine residue at position 130 was mutated into leucine, and the obtained 3D structure was optimized by the simulated annealing method. Subsequently, the molecular model was subjected to energy minimization, using the SYBYL software (version 7.3; Tripos Inc., St Louis, MO, USA). The surface of the channel that was proposed to accommodate the α-branch portion of the substrates was depicted by utilizing the MOLCAD module of SYBYL.

RESULTS

M.le FbpA Exhibits Reduced Mycolyltransferase Activity—Recombinant FbpA enzymes derived from M.le and M.av were obtained and tested for their mycolyltransferase activity in an *in vitro* enzyme assay, using mycobacteria-derived natural TMM as a substrate. As we have shown previously (*4*), an efficient transfer of the mycolyl acyl group from one TMM substrate (donor) to the other TMM substrate (acceptor) occurred in the presence of the M.av FbpA protein, evidenced by generation of the reaction product, TDM (Fig. 1, lane 1).

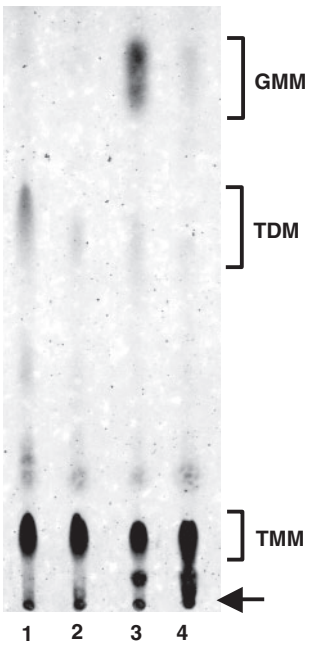

Fig. 1. **Reduced mycolyltransferase activity of M.le FbpA.** Enzymatic reactions with the long-chain TMM as a substrate were performed at 37°C for 20 min at conditions indicated below, and the lipids were extracted from the reaction mixtures, followed by analysis on a TLC plate. Lane 1, M.av FbpA and TMM; lane 2, M.le FbpA and TMM; lane 3, M.av FbpA, TMM and glucose; lane 4, M.le FbpA, TMM and glucose. The TLC origin is indicated with an arrow.

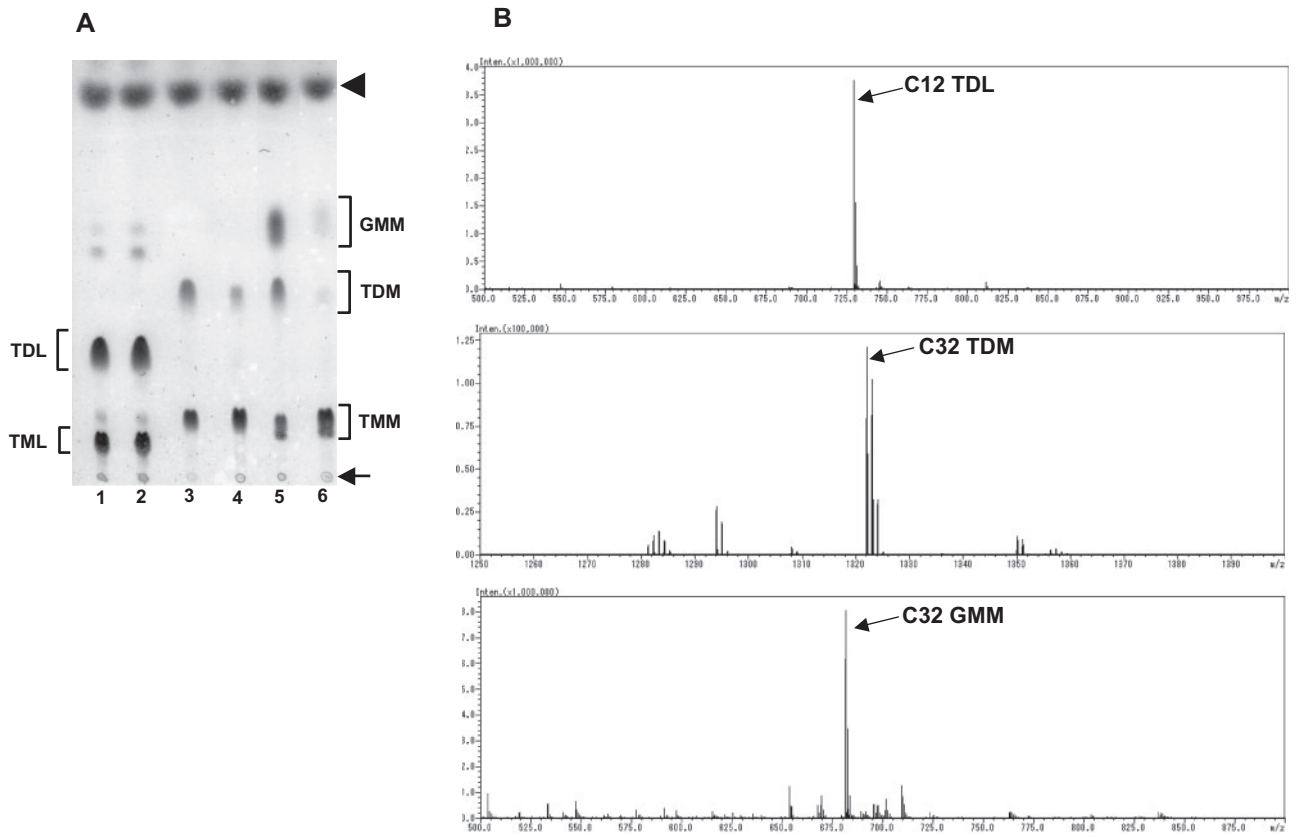

Fig. 2. **Efficient utilization of unbranched, but not α-branched, substrates by M.le FbpA.** (A) Enzymatic reactions were performed as in Fig. 1, using either TML or the short-chain TMM as a substrate. Lane 1, M.av FbpA and TML; lane 2, M.le FbpA and TML; lane 3, M.av FbpA and TMM; lane 4, M.le FbpA and TMM; lane 5, M.av FbpA, TMM and glucose; lane 6, M.le FbpA, TMM and glucose. The position of the extraction efficiency indicator, *n*-tetradecanol, is indicated with an arrowhead. Note that equivalent amounts of *n*-tetradecanol were visualized throughout the lanes. (B) The reaction products, TDL (top panel), *C. matruchotii* TDM (middle panel) and GMM (bottom panel), were extracted from the TLC plates and analysed by mass spectrometry. The major ions of m/z 729.4 (top panel), m/z 1322.0 (middle panel) and m/z 681.5 (bottom panel) were indicated with arrows that represent sodium adducts of C_{12} TDM, TDM with two molecules of C_{32} mycolate, and C_{32} mycolate-containing GMM, respectively.

In sharp contrast, only a tiny amount of TDM was detected when an equivalent amount of the M.le FbpA protein was used (lane 2). The mycolyltransferase activity of the M.av and M.le FbpA proteins was also assessed in parallel, using glucose as an alternative acceptor substrate. Similar to TDM, much more efficient generation of the reaction product, GMM, was observed for M.av FbpA (lane 3), and only a trace of GMM was produced by M.le FbpA (lane 4). Therefore, these results detected apparently reduced mycolyltransferase activity for M.le FbpA.

M.le FbpA Retains the Ability to Transfer Unbranched, but not α-branched, Fatty Acids—The mycobacteria-derived TMM molecules used as a substrate for the enzyme reactions above were those containing α-branched, long-chain (mainly C_{85}) fatty acids. To gain insight into the molecular basis for the decreased mycolyltransferase activity exhibited by M.le FbpA, similar *in vitro* enzyme reaction experiments were performed, using two monoacyl trehalose compounds as model substrates; namely, synthesized TML with a C_{12} unbranched acyl chain, and *C. matruchotii*-derived TMM with α-branched, short-chain (mainly C_{32}) fatty acids. We found that both M.av and M.le FbpA proteins were capable of transferring the C_{12} unbranched acyl chain from the donor TML molecule to the acceptor TML molecule efficiently, resulting in generation of a comparable amount of trehalose-6,6′-dilaurate (TDL) by both enzymes (Fig. 2A, lanes 1 and 2). The molecular identity of the product as TDL was confirmed by mass spectrometry, in which the mass numbers of given ions were matched with those for sodium adducts of C_{12} TDL (Fig. 2B, top panel). On the other hand, the *C. matruchotii*-derived α-branched, short-chain TMM was not utilized efficiently as a donor substrate for M.le FbpA, evidenced by reduced TDM and GMM production as compared with M.av FbpA (Fig. 2A, lane 3 versus lane 4 for TDM production, and lane 5 versus lane 6 for GMM production). The molecular identity of the products as TDM and GMM was confirmed by mass spectrometry (Fig. 2B, middle and bottom panels, respectively). From these observations, we hypothesized that, unlike monoacyl trehalose compounds with an unbranched fatty acid, those containing the fatty acyl branching of mycolic acids could

not gain easy access to the donor substrate-binding site of the M.le FbpA protein.

Leucine at Position 130 of M.le FbpA Limits Access of Mycolate-containing Glycolipid Substrates to their Binding Site—Crystallographic studies of M.tb-derived mycolyltransferases detected a tunnel extending through the core of the protein that could accommodate the α-branch portion of mycolates while their meromycolate chain fitted in a long cleft formed on the surface of the enzyme (*14–16*). The fact that even the α-branch of the short-chain TMM could not be efficiently utilized by the M.le FbpA enzyme might indicate a failure of the α-branch to penetrate deeply into the tunnel due to blockade at or near its opening. We therefore looked closely at the α4 helix (Fig. 3A, boxed area) that contributed to the formation of the tunnel in the proximity of the position 126 serine residue (indicated with an asterisk) of the catalytic triad. Alignment of the amino acid sequences of M.le-, M.av- and M.tb-derived FbpA proteins shows that the α4 helix sequence is highly conserved, but the position 130 serine residue found in both M.av and M.tb is replaced by leucine in M. le (Fig. 3, indicated with an arrowhead).

In the crystal structure of the M.tb FbpA protein reported previously, the side chain of the position 130 serine residue (Ser130) protrudes into the lumen of the tunnel (demarked with dotted lines), generating a kinky channel, but there is still a plenty of open space that allows smooth accommodation of the α-branch portion of the substrates (Fig. 3B, left panel). On the other hand, molecular modeling after serine-to-leucine mutation was introduced at position 130 revealed that the bulky side chain of the leucine residue protruded further, resulting in total obstruction of the tunnel (Fig. 3B, right panel). Thus, we suspected that the impaired utilization of α-branched fatty acid-containing substrates by M.le FbpA might be accounted for by the presence of the leucine residue at position 130.

To test this possibility directly, recombinant M.le- and M.av-derived FbpA proteins with a single amino acid mutation at position 130 were generated and assessed for their acyl-chain transfer activity. The mutant M.av FbpA protein in which the serine residue was replaced with leucine (M.av FbpA S130L) exhibited a reduced mycolyltransferase activity (Fig. 4A, lane 4) as compared with the wild-type M.av FbpA protein (lane 3) while maintaining the ability to transfer the C_{12} unbranched acyl chain (lanes 1 and 2). In sharp contrast, the mutant M.le FbpA protein in which the leucine residue was replaced with serine (M.le FbpA L130S) exhibited an enhanced mycolyltransferase activity (Fig. 4B, lane 4) as compared with the wild-type M.le FbpA

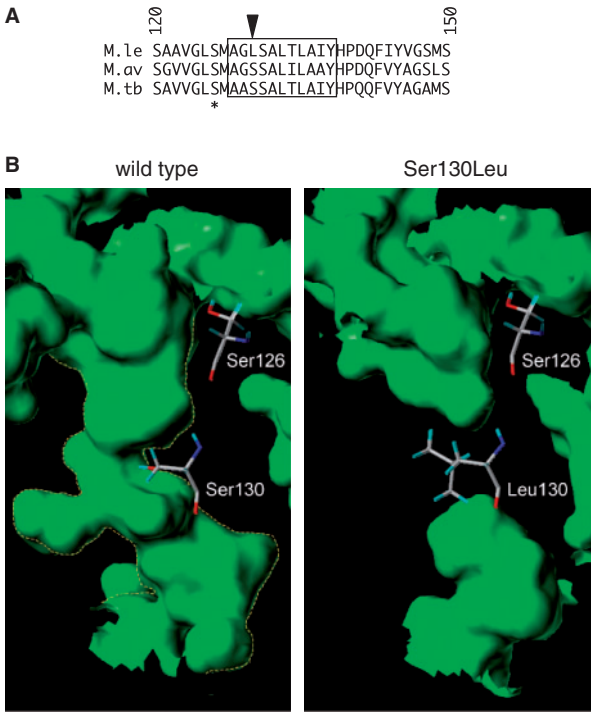

Fig. 3. **A critical amino acid residue at position 130 of FbpA proteins that regulates interaction with α-branched substrates.** (A) Partial amino acid sequences of M.le-, M.av- and M.tb-derived FbpA proteins are aligned. Residues from position 120 to position 150 are shown, and the position 130 is indicated with an arrowhead. The boxed area indicates amino acid sequences of the α4 helix. The serine residue of the catalytic triad located at position 126 is indicated with an asterisk. (B) Structure of the M.tb wild type FbpA (left panel) and molecular modeling of its Ser130Leu mutant (right panel) are shown. The surface of open space is depicted, and the intramolecular channel that accommodates the α-branch portion of the substrates is demarcated with dotted lines. Note that the tunnel totally collapsed after the introduction of the mutation.

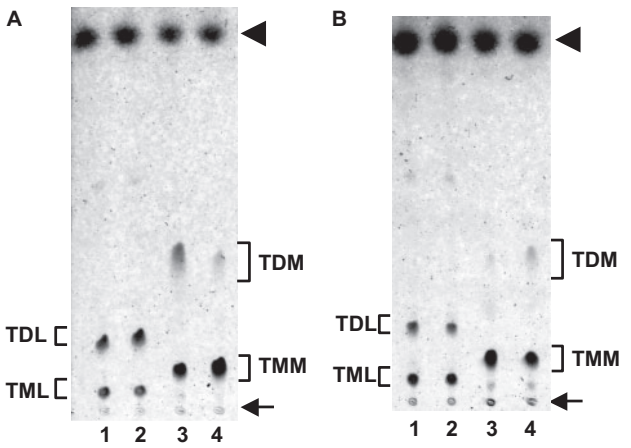

Fig. 4. **Effects of amino acid substitution at position 130 of FbpA proteins on mycolyltransferase activity.** (A) Enzymatic reactions were performed with either the wild-type M.av FbpA or its mutant (M.av FbpA S130L) as in Fig. 1 except for the use of TML at 0.3 mM. Lane 1, wild-type M.av FbpA and TML; lane 2, M.av FbpA S130L and TML; lane 3, wild-type M.av FbpA and short-chain TMM; lane 4, M.av FbpA S130L and short-chain TMM. (B) Similar enzymatic reactions were performed with either the wild-type M.le FbpA or its mutant (M.le FbpA L130S). Lane 1, wild-type M.le FbpA and TML; lane 2, M.le FbpA L130S and TML; lane 3, wild-type M.le FbpA and short-chain TMM; lane 4, M.le FbpA L130S and short-chain TMM. The TLC origin and the position of *n*-tetradecanol are indicated with arrows and arrowheads, respectively.

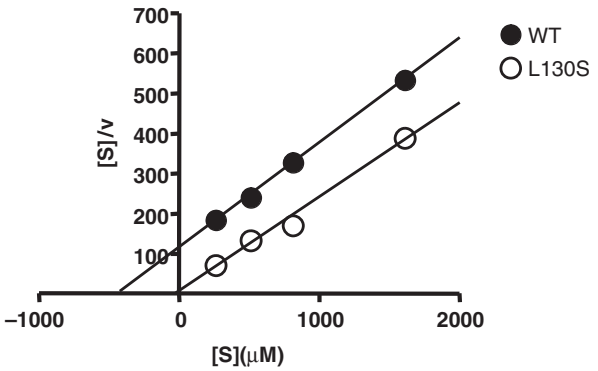

Fig. 5. **Distinct enzyme kinetics for M.le wild-type and mutant FbpA proteins.** Enzyme assays were performed with either wild-type or mutant FbpA proteins, using different concentrations of the short-chain TMM as a substrate. The lipids were extracted, and the amount of the product was densitometrically determined on TLC plates. Hanes–Woolf plots are shown for the wild-type (closed circles) and the mutant (open circles) FbpA enzymes.

protein (lane 3), while the ability to transfer the unbranched acyl chain was unchanged (lanes 1 and 2).

To quantitatively assess the enzyme activity of the M.le FbpA wild-type and mutant proteins, enzyme assays were carried out in the presence of varying amounts of the short-chain TMM substrate, and the amount of the product, TDM, generated at each concentration was determined, followed by kinetic analysis of the reactions using Hanes-Woolf plotting. As shown in Fig. 5, separate, but almost parallel, linearity was obtained for the wild-type and mutant enzymes, indicating a sharp decrease in the apparent K_m value after the amino acid substitution while the V_{max} values remained almost unaffected. The calculated K_m values were 459 μM for the wild-type and 43 μM for the mutant, and the V_{max} values were 3.83 nmol/min/nmol enzyme for the wild-type and 4.27 nmol/min/nmol enzyme for the mutant, underscoring an increased affinity of the TMM substrate to the mutant enzyme. Therefore, the amino acid substitution experiments detected a critical role for the leucine residue at position 130 in excluding α-branched mycolates, which could account for the reduced mycolyltransferase activity of the M.le FbpA enzyme.

DISCUSSION

TDM is produced abundantly by virtually all cultivable mycobacterial pathogens, and its relevance to pathogenesis has been proposed (*1*, *2*). Nevertheless, given its potent adjuvant effects on a wide variety of host immune cells, it would be reasonable to speculate that, upon infection into the host, pathogenic mycobacteria may down-regulate the TDM expression to allow for their escape from the host immune system. In this respect, the mycolyltransferase-mediated switch of glycolipid biosynthesis from TDM to GMM by borrowing host-derived glucose could function as an exquisite maneuver that pathogenic mycobacteria, such as M.tb and M.av, are able to employ (*4*). The cell wall lipid components of M.le remain to be fully characterized due to the highly limited sources of the microbe, but it has been suggested that the expression of TDM could be far below the level that would be expected for other mycobacteria (*6*), implicating a highly efficient mechanism for TDM down-regulation. Therefore, it is important to determine how TDM biosynthesis is regulated in M.le in order to understand the unique immunopathological features of leprosy.

The function of M.le-derived mycolyltransferases has not been specifically addressed previously. By utilizing recombinant proteins and an array of substrates in *in vitro* enzyme assays, the present study has disclosed for the first time unexpected features of the M.le-derived FbpA enzyme that are unique to M.le, but not to other pathogenic mycobcteria. Unlike the M.av FbpA protein capable of catalysing TDM synthesis from its precursor, TMM, the M.le enzyme was inefficient as a mycolyltransferase to generate TDM while maintaining its catalytic activity to transfer an unbranched acyl chain (Figs 1 and 2). Further, the site-directed mutagenesis studies, identifying the amino acid residue at position 130 as a key determinant for the accessibility of the α-branch portion of mycolates, have provided a molecular basis for the reduced mycolyltransferase activity of the M.le FbpA enzyme (Figs 4 and 5). Thus, these observations underscore 'intrinsic' defects of TDM production in M.le, which contrasted sharply with the 'extrinsic' pathway for TDM down-regulation in other pathogenic mycobacteria, borrowing host-derived glucose as an alternative substrate for their functional mycolyltransferases (*4*). Whereas the extrinsic pathway involves concomitant up-regulated expression of GMM, a specific target for host CD1b-restricted cytotoxic T cells that can detect and lyse mycobacteria-infected cells (*17*, *18*), the simultaneous down-regulation of TDM and GMM expression achieved by the intrinsic mechanism would minimize activation of both innate and acquired phases of host immunity, providing better chances for the microbe to survive in the host. Given that M.le is an obligate intracellular parasite that can survive only within the host cells, it might have been critically important for the bacteria to evolve highly efficient maneuvers for adaptation to host environments by even reducing the genuine function of mycolyltransferases. Transcription of *fbpA*, *fbpB* and *fbpC* genes could be detected during M.le growth (*19*). Nevertheless, its TDM expression is highly suppressed (*6*), implicating that the other mycolyltransferases, FbpB and FbpC, also fail to generate TDM. Unlike M.le FbpA that contains a unique amino acid at position 130, the corresponding amino acid residue in FbpB and FbpC is shared between M.le and M.tb. This suggests that distinct, but yet undetermined, mechanisms should have been evolved for M.le FbpB and FbpC that support TDM down-regulation.

As mentioned earlier, detailed lipid biochemical analysis of M.le has been hampered due to the limited sources of the bacteria. Nevertheless, the sequence of the recently decoded M.le genome (*7*) and its comparison with that of its close relative M.tb genome often provide a valuable clue that helps us to understand the yet undefined biology of M.le. The M.le genome is much smaller in size with more pseudogenes, resulting in

significantly reduced numbers of open reading frames that potentially encode functional proteins. It is noteworthy that, even after such a highly reductive evolution, the M.le genome still contains apparently functional *fbp* genes. Surprisingly, while specifically reducing the mycolyltransferase activity by blockade of the tunnel that accommodates the α-branch of mycolates, the M.le FbpA protein still preserves the molecular structure for interaction with the unbranched glycolipid substrates and the catalytic activity to transfer the unbranched acyl chain (Fig. 2). This suggests that the protein integrity of the M.le FbpA has been maintained during the reductive evolution and that it might play a role in the lifecycle of M.le, which is distinct from that as a mycolyltransferase. The present study, detecting differential mycolyltransferase activity in M.le and other pathogenic mycobacteria, provides a clue to the still mysterious biology of the first human pathogenic bacterium to be identified.

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CONFLICT OF INTEREST

None declared.

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