Inactivation of the Mycobacterial Rhamnosyltransferase, Which Is Needed for the Formation of the Arabinogalactan-Peptidoglycan Linker, Leads to Irreversible Loss of Viability*

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Temperature-sensitive mutant 2-20/32 of Mycobacterium smegmatis mc²155 was isolated and genetically complemented with a Mycobacterium tuberculosis H37Rv DNA fragment that contained a single open reading frame. This open reading frame is designated wbbL, which encodes a dTDP-Rha:(–1→3)-L-Rha-(1→3)-α-D-GlcNAc-pyrophosphate polyprenol, α-3-L-rhamnosyltransferase. In E. coli this enzyme is involved in O-antigen synthesis, but in mycobacteria it is required for the rhamnosyl-containing linker unit responsible for the attachment of the cell wall polymer mycolyl-arabinogalactan to the peptidoglycan. The M. tuberculosis wbbL homologue, encoded by Rv3265c, was shown to be capable of restoring an E. coli K12 strain containing an insertional inactivated wbbL to O-antigen positive. Likewise, the E. coli wbbL gene allowed 2-20/32 to grow at higher non-permissive temperatures. The rhamnosyltransferase activity of M. tuberculosis WbbL was demonstrated in 2-20/32 as was the loss of this transferase activity in 2-20/32 at elevated temperatures. The wbbL of the temperature-sensitive mutant contained a single-base change that converted what was a proline in mc²155 to a serine residue. Exposure of 2-20/32 to higher non-permissive temperatures resulted in bacteria that could not be recovered at the lower permissive temperatures.

The acid-fastness of all mycobacteria is based upon a shared universal cell wall core structure. The mycobacterial cell wall consists of an outer lipid layer and an inner peptidoglycan layer. The outer layer is highly impermeable and is composed of unique 70–90 carbon-containing lipids, known as mycolic acids. The mycolic acids are esterified to the non-reducing terminal arabinosyl residues of the polysaccharide arabinogalactan (1–5). The reducing end of arabinogalactan is connected to the peptidoglycan via the disaccharide linker, α-L-Rha-(1→3)-α-D-GlcNAc-(1→phosphate). Structural analyses showed that the integrity of the whole two-layer mycolic acid peptidoglycan assembly hinges on the presence of the rhamnosyl moiety as depicted in Fig. 1A. The complete structure of the linker is illustrated in Fig. 1B, and the reaction catalyzed by the enzyme, dTDP-Rha:a-D-GlcNAc-pyrophosphate polyprenol, α-3-L-rhamnosyltransferase (referred to as rhamnosyltransferase in this study) is shown in Fig. 1C. The rhamnosyl residue and much if not all of the arabinogalactan polysaccharide are synthesized on GlcNAc-P-decaprenyl carrier lipid (6). The eventual transfer of the arabinogalactan-Rha-GlcNAc-phosphate unit to the O-6 of a muramic acid places the polysaccharide in mass onto the peptidoglycan. Finally, at some still to be defined point, the mycolic acids are attached to arabinofuranosyl residues at the non-reducing end of arabinogalactan.

To further define and characterize the essential steps involved in the synthesis of the mycobacterial cell wall core, the classic microbrial approach of isolating conditional lethal mutants was undertaken. Our strategy was to isolate temperature-sensitive (TS) mutants in the genetically amenable and relatively fast growing Mycobacterium smegmatis mc²155 (7). A preferred large temperature range that would support growth precluded Mycobacterium tuberculosis from serving as the host for the generation of TS mutants. TS mutants would be genetically complemented with M. tuberculosis genomic DNA in hopes of identifying essential genes encoding cell wall biosynthetic enzymes. Herein, we describe the isolation of a TS cell wall mutant and the independent genetic complementation of that mutant with a M. tuberculosis gene and an E. coli gene. We report biochemical characterization of the TS mutant, the deduced amino acid change due to the mutation, the genetic complementation of an E. coli mutant to confirm the function of a M. tuberculosis gene, and the effect of the mutation on mycobacterial viability after exposure to non-permissive temperatures.

EXPERIMENTAL PROCEDURES

Isolation of TS Mutants—The strategy for the isolation and enrichment of bacterial TS mutants in a culture as outlined by A. Morris Hooke (8) was adapted for use in this study. M. smegmatis mc²155 (7) was inoculated into Middlebrook 7H9 with ADC supplement (Difco) (7H9) and grown at 37 °C to ~10⁸ colony-forming units/ml. Nitrosoguanidine (Sigma) was added to a final concentration of 0.1 mg/ml, and cultures were incubated at 37 °C without aeration for 40 min. The cells were recovered by centrifugation and washed three times in fresh 7H9 medium to remove the mutagen. Samples of cells were suspended in at least 20 volumes of fresh 7H9 broth distributed into several separate
flasks to help minimize the isolation of siblings and incubated with shaking at the permissive temperature of 30 °C. When cell density reached ~5 × 10^7 colony-forming units/ml cultures were transferred to a shaking water bath at the non-permissive temperature of 42 °C for 1 h. To enrich the population of TS mutants in the cultures r-cycloserine (Sigma) was added to a final concentration of 0.4 mg/ml. Cells were incubated at 42 °C for another 6 h, recovered by centrifugation, and washed 3 times in fresh medium to remove the antibiotic. Surviving cells were cultured in fresh broth at 30 °C until density again reached ~5 × 10^7 colony-forming units/ml. The cultures were subjected to another cycle of enrichment at the non-permissive temperature as described, except the r-cycloserine was replaced with ampicillin (Sigma) at a final concentration of 2.3 mg/ml. Ampicillin was used in the second enrichment for r-cycloserine-resistant mutants. The treatment with either r-cycloserine or ampicillin resulted in a 99–99.9% reduction in colony-forming units. After the second enrichment the surviving cells were washed free of the antibiotic, diluted, plated on Luria Bertani agar supplement with 1% Tween 80 (Sigma), and incubated at 30 °C. *M. smegmatis* mc^2^155 grown in the presence of Tween 80 produce mucoid colonies on Luria-Bertani agar, which make them readily transferable to other plates. Colonies that arose after incubation were replica-plated onto Luria Bertani agar supplement with 1% Tween 80 and incubated at 30 °C and 42 °C to identify TS mutants.

**Genetic Complementation of TS Mutant Strain 2-20/32—*M. tuberculosis* H37Rv genomic DNA was isolated and partially digested with SacI. The GTCGTCCTGCCGG and GAGGTACCTTATTCAGTGC- GTATTACGGGTGAAAAACTGAT (same primer used for pKM1) were ligated into BamHI-digested pCB7 (Table I), packaged into λ particles using Gigapack III XL packing extract (Stratagene, La Jolla, CA), and propagated in *E. coli* (XL1-Blue MR, Stratagene) on LB containing 200 μg/ml hygromycin B (Sigma). Although plasmids recovered contained significantly smaller *M. tuberculosis* DNA fragments than the desired 30-kilobase insert, they were still used to complement some of the TS mutants. These smaller plasmids were probably the result of the instability of the larger constructs in the transduced *E. coli* host. The plasmid library was introduced into the TS mutants by electroporation (8). Transformants were selected on Luria Bertani agar supplement with 1% Tween 80 with 50 μg/ml hygromycin B (Sigma) at 42 °C. Furthermore, the plasmids from the plasmid library of the TS mutant 2-20/32 were digested with NdeI and XhoI and ligated into NdeI/XhoI-digested M. smegmatis mc^2^155 into SacI/KpnI-digested pBluescript (Stratagene) to generate pCB220. The PCR products were digested with appropriate enzymes and ligated into the above clonal plasmid.

**Construction of Plasmids—**Plasmids with their relevant characteristics are listed in Table I. AmpliTaq 2 DNA polymerase (Invitrogen) or Vent polymerase (New England Biolabs, Beverly, MA) were used according to the manufacturer’s instructions to amplify fragments for cloning. All constructions were confirmed by sequencing.

- *M. tuberculosis* wbbL was amplified using primers CCGAGCTCCT-GAAGTGACTGACGTCCTGCCGG and GAGGTACCTTATTCAGTGC-GTATTACGGGTGAAAAACTGAT using PCR primers CGGAATTCAGAAACATGGGGATTGCCG GTG start. The latter primer included a KpnI site.

**Isolation and Complementation of TS Mutant 2-20/32**—Forty-seven *M. smegmatis* mc^2^155 TS mutants were isolated in SmaI-digested pUC18 (Invitrogen). The insert was then cut out with PstI and KpnI and ligated into PstI/KpnI-cloned pCB220.

**Preparation of dTDP-[^14C]Rha**—dTDP-[^14C]Rha was prepared from [U-^14C]sucrose by conversion of the glucose moiety of the sucrose to glucose 1-phosphate by sucrose phosphorylase followed by further conversion to dTDP-[^14C]Glc by a-n-glucose-1-phosphate thymidylyltransferase (RmlA) and further conversion by RmlB-D to dTDP-[^14C]Rha. The d-hexose-1-phosphate thymidylyltransferase was prepared from *M. tuberculosis* rmlA (16) expressed in *E. coli*; the remaining Rml enzymes were those found in *E. coli* BL21-DE3 (Stratagene) and *E. coli* B (ATCC, Manassas, VA). Thus, 50 μCi (442/mCi/mmol, 115nmol) of [U-^14C]sucrose (PerkinElmer Life Sciences) were dried in a tube, and 16 μl of 1 m KH_2PO_4, pH 7.0, 80 μl (0.5 units) of sucrose phosphorylase (Sigma), 10 μl of 40 mm TTP, 4 μl (2 units) of inorganic pyrophosphatase (Sigma), 200 μl of crude lysate (~5 mg/ml protein) of *E. coli* BL21 with *M. tuberculosis* rmlA (16) cloned in pET 29, 35 μl of 10 μm NADPH, 55 μl of 50 μm HEFES buffer with 10 μm MgCl_2 at pH 7.0 were then mixed to make a total volume of 400 μl. After 1 h of incubation at 37 °C an additional source of RmlB-D (200 μl of crude *E. coli* B lystate (~5 ml protein)) and additional NADPH (35 μl of 10 mM) was added to fully convert the dTDP-[^14C]Glc to dTDP-[^14C]Rha. Then 700 μl of absolute ethanol was added, and the precipitated protein was removed by centrifugation at 14,000 × g for 5 min. The bulk of the ethanol was removed by evaporation, and the dTDP-[^14C]Rha was purified by high performance liquid chromatography as described (17).

**Isolation of GlcNAc-P-D-decaprenol**—The assay is essentially that of Reeves and co-workers (11). The acceptor for the rhamnosyl residue, GlcNAc-P-D-decaprenol, is formed in situ from exogenously added UDP-GlcNAc and from undecaprenyl phosphate present in the membrane preparations by GlcNAc-1-phosphate transferase also present in the membrane preparations. Thus, bacteria, as indicated in Table II were disrupted by sonication, and cell debris were removed by centrifugation 8,000 × g. The membrane pellets were then prepared by additional centrifugation for 1 h at 100,000 × g and resuspended in 50 mM, pH 8, MOPS containing 5 mM mercaptoethanol and 10 mM MgCl_2 at protein concentrations in all cases of 1 mg/ml. MA1500 membrane preparations were prepared as 0.1 μl of dTDP-[^14C]Rha (221 μCi/μmol) 6 nmol of UDP-GlcNAc, 18 nmol of ATP, and additional MOPS buffer to total 320 μl. After incubation for 1 h at 37 °C, 200 μl of water, 400 μl of methanol, and 800 μl of chloroform were added, and the organic layer removed and counted.

**Combined Assay for Rhamnosyl and GlcNAc-1-phosphate Transf erase—**Two cultures of *M. smegmatis* mc^2^155, *M. smegmatis* 2-20/32, and *M. smegmatis* 2-20/32 complemented with pCB120 were grown at 30 °C to approximately late log. For the experiment reported in Fig. 3A they were then harvested; for the experiment in Fig. 3B they were additionally treated for 6 h at 42 °C and then harvested. Cell-free extract preparation, membrane isolation, and inorganic cations were prepared as described. UDP-[^14C]GlcNAc, extraction with organic solvent, TLC chromatography, and visualization by autoradiography were as described (6).

**Viability of 2-20/32 after Treatment at the Non-permissive Temperature**—Two cultures of *M. smegmatis* 2-20/32 and a single culture *M. smegmatis* 2-20/32 complemented with pCB120 were grown in LB broth and incubated at 30 °C, and the turbidity was followed at 600 nm at 5-min intervals. MA1500 membrane preparations were prepared as 0.1 μl of the non-transformed and the 2-20/32 (pCB120) was shifted to 42 °C; the remaining Rml was kept at 30 °C; the remaining 2-20/32 was kept at 30 °C. The monitoring at A_600 was continued (see Fig. 4). After 24 h at 42 °C an inoculum of 2-20/32 and 2-20/32 (pCB120) was transferred into fresh LB broth and incubated at 30 °C, and the A_600 was monitored.

**RESULTS**

**Isolation and Complementation of TS Mutant 2-20/32—**Forty-seven *M. smegmatis* mc^2^155 TS mutants were isolated in SmaI-digested pUC18 (Invitrogen). The insert was then cut out with PstI and KpnI and ligated into PstI/KpnI-cloned pCB220.
15 independent mutagenic experiments as described under "Experimental Procedures." The mutagenesis and enrichment procedure yielded an average frequency of 1 TS mutant per 420 colonies screened. One TS mutant 2-20/32 was genetically complemented by pCB120, i.e. the plasmid allowed the mutant to grow at the non-permissive temperature of 42 °C. Plasmid pCB120 contained a 1764-bp *M. tuberculosis* H37Rv genomic DNA fragment. Sequence analysis revealed that pCB120 contained only one intact ORF. This ORF was translated and subjected to a BLAST (18) search that revealed it corresponded to *M. tuberculosis* Rv3265c and showed significant homology to the protein product of the *wbbL* gene of *E. coli* K12 (accession L19537; see also Ref. 12). This fragment DNA was known as ORF 264 in the original report (19). It was, therefore, postulated that the ORF in pCB120 was, in fact, the *M. tuberculosis* version of *wbbL* encoding the rhamnosyltransferase responsible for the synthesis of the α-1-Rha-(1→3)-α-d-GlcNAc linker unit (Fig. 1C).

**Complementation of E. coli K12 EMG2 with *M. tuberculosis* DNA Coding for Putative Rhamnosyltransferase**—The work of Reeves and co-workers (11, 19) allowed us to readily conduct complementation experiments that could determine whether the ORF in pCB120 was a rhamnosyltransferase. The ORF in pCB120 was amplified by PCR and inserted in pBluescript to form pCB220 (Table I). In an analogous fashion the *E. coli* *wbbL* was amplified by PCR from *E. coli* WG1 (19) and cloned in pBluescript to form pKM1 (Table I). Plasmids pCB220 and pKM1 were introduced into *E. coli* EMG2. EMG2 contains an insertionally inactivated *wbbL* (11, 19) and, thus, is unable to produce O-antigen. LPS profiles by non-transformed EMG2, EMG2 (pCB220), and EMG2 (pKM1) were examined by SDS-PAGE. Visualization by silver staining (Fig. 2B) showed as expected that LPS complete with O-antigen was produced by EMG2 containing the plasmid encoding the *E. coli* rhamnosyltransferase (19), whereas EMG2 without plasmid or pBluescript was devoid of O-antigen. Remarkably, a plasmid encoding the putative rhamnosyltransferase gene from *M. tuberculosis* also resulted in O-antigen synthesis. Relative to the *E. coli wbbL*, the number of O-antigen units added to the LPS appeared to be diminished with the *M. tuberculosis wbbL*. Blot analysis (Fig. 2C) showed that the O-antigen produced from both plasmids reacted with antibody raised against *E. coli* O-16 O-antigen, but no reaction occurred without plasmid or pBluescript. Known cross-reactivity of antiserum raised against *E. coli* O-16 O-antigen with *E. coli* K12 O-antigen (11) allowed this antiserum to be used.

**Transformation of TS Mutant M. smegmatis 2-20/32 with a Plasmid Containing the *E. coli wbbL* Gene Encoding for Rhamnosyltransferase Allows for Growth at 37 °C**—Further evidence that the ORF on pCB120 encoded for cell wall linker rhamnosyltransferase was obtained by transforming *M. smegmatis* 2-20/32 with pCB227 a plasmid containing *E. coli wbbL* (Table I) under the control of the heat shock promoter found in pMX1 (20). It was found that although this plasmid did not
allow *M. smegmatis* 2-20/32 to grow at 42 °C as did *M. tuberculosis* *wbbL*, pCB227 did support growth at 37 °C, a temperature at which the non-transformed *M. smegmatis* 2-20/32 was unable to grow.

The *M. tuberculosis* *wbbL* Gene Product Exhibits Rhambosyltransferase Activity in *E. coli*—Direct evidence that the *M. tuberculosis* *wbbL* encodes for rhambosyltransferase was obtained using the rhambosyltransferase assay of Reeves and co-workers (11). Membranes were prepared from the *E. coli* strains *BW24599*, a K12 strain repaired for rhambosyltransferase (17), and a non-repaired K12 strain, HMS 174 containing the control vector with no insert (pET 23b) and HMS 174 with the previously folded rhamnosyl transferases would be depleted by normal turnover events. Membranes were prepared and assayed for rhambosyltransferase activity at 30 °C (Fig. 3B). The results showed that under these conditions strain 2-20/32 produced very little Rha-[14C]GlcNAc-P-P-decaprenol in comparison to its precursor, [14C]GlcNAc-P-P-decaprenol (Fig. 3B, lane 3), whereas both the wild type strain and the complemented TS mutant strain continued to produce major amounts of the rhamnosyl containing product (Fig. 3B, lanes 1 and 2). The fact that the rhambosyltransferase activity of 2-20/32 was not dependent on the temperature of enzyme incubation but rather on the temperature of the growth of the bacteria suggests that the mutation affects the conformation of the protein as it is being synthesized (22).

The *wbbL* Gene of *M. smegmatis* TS Mutant 2-20/32 Contains a T Rather than C at Position 364—The *wbbL* genes from *M. smegmatis* mc²155 wild-type (GenBank accession number AF187550) and 2-20/32 (GenBank accession number AF187551) were sequenced, and a single base changed at position 364 from a C to T was found. This translates into a proline at position 122 in WbbL is conserved in all four wild-type mycobacterial sequences but not in *M. tuberculosis* (in Rv3265c this proline resides at position 114).
transformed with pCB120 were grown at 30 °C. When the cultures reached a 600 of 0.2, one 2-20/32 culture and the 2-20/32 (pCB120) culture were shifted to 42 °C, and the A600 was monitored. After a brief growth spurt, 2-20/32 with no plasmid stopped growing (Fig. 4A). In contrast the 2-20/32 with no plasmid but maintained at 30 °C as well as 2-20/32 (pCB120) shifted to 42 °C continued to grow. After 24 h the untransformed 2-20/32 cultures incubated at 30 °C and 42 °C were subcultured to fresh media and incubated at the permissive temperature of 30 °C. As can be seen (Fig. 4B) after the 17-h treatment at the non-permissive temperature, 2-20/32 was no longer viable. Additional experiments (data not presented) showed that an incubation of ~10 h was required before the TS mutant could no longer be recovered; this result is consistent with the mutation being the temperature-sensitive folding type (22). This observation also explains why 2-20/32 could be recovered after a 7-h incubation at 42 °C during the TS enrichment procedure.

**DISCUSSION**

We have previously determined the structure of the linker region of arabinogalactan (Fig. 1A) (5) and predicted because of its key structural location (1, 23) that the synthesis of this linker region would be essential for mycobacterial viability (16). In addition we have shown that RmlD, one of the enzymes required for the formation of linker, is essential in

### Table II

| E. coli strain (plasmid) | Incubation time | Total cpm in organic phasea |
|-------------------------|----------------|----------------------------|
| E. coli BW24599         | 60            | 1080                       |
| E. coli HMS 174 (pET 23b) | 0              | 180                        |
| E. coli HMS 174 (pVV1)  | 60            | 220                        |
| E. coli HMS 174 (pVV1)  | 60            | 5000                       |

*a Substrate dTDP-[14C]Rha was converted from aqueous to organic counts by the presumed attachment of the [14C]rhamnosyl residue to in situ GlcNAc-P-P-decaprenol. In total, 250,000 cpm of dTDP-[14C]Rha were used, and hence, in the case of E. coli HMS 174 (pVV1), 2% of the donor was converted to product. In similar experiments (data not presented) where decaprenyl phosphate and/or detergent was added, the results were similar; the conversion of dTDP-[14C]Rha into product with E. coli HMS 174 (pVV1) remained between 1.5 and 2%. With detergent there was some increase in background activity in the non-producing strains and the zero time points presumably due to the solubilization by the detergent of some dTDP-[14C]Rha into the organic phase.
tis (24). In the present study we took a classical microbial drug target identification approach that could define any type of essential gene and serendipitously isolated a TS mutation in wbbL, another gene involved in linker biosynthesis. These studies were aided enormously by the pioneering work of Peter Reeves and co-workers (11, 19) on E. coli K12 O-antigen biosynthesis. Coincidentally and fortuitously the M. tuberculosis wbbL essential gene and serendipitously isolated a TS mutation in a target identification approach that could define any type of substrate nor do they generate the same products as WbbL. Other putative rhamnosyltransferases in M. tuberculosis and M. leprae have been hypothesized to be involved in the synthesis of rhamnose-containing oligosaccharides on phenolic glycolipids (25, 26). It is important to note that these various rhamnosyltransferases do not have the same substrates nor do they generate the same products as WbbL.

Because wbbL has been shown by a genomic approach (27) and RmlD by a biochemical approach (24) to be essential, it is not surprising that a TS mutation of wbbL could be obtained. However, it was difficult to predict whether the defect would be static or lethal. The behavior of the TS mutant 2-20/32 after treatment at the non-permissive temperature suggests the latter consequence. This phenomenon would predict that inhibitors of the rhamnosyltransferase (and inhibitors of dTDP-rhamnose formation) will also be lethal rather than static. Further studies to purify M. tuberculosis WbbL enzyme and develop a direct assay to identify inhibitors of its activity are ongoing. Obstacles to be overcome include robust expression of the protein, production of required amounts of acceptor, and development of an assay amenable to screening.

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