THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 42, Issue of October 15, pp. 43540–43546, 2004
Printed in U.S.A.
© 2004 by The American Society for Biochemistry and Molecular Biology, Inc.

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

Received for publication, July 12, 2004, and in revised form, July 29, 2004
Published, JBC Papers in Press, August 4, 2004, DOI 10.1074/jbc.M407782200

Jonathan A. Mills‡§, Kelly Motichka¶, Markus Jucker‡, Henry P. Wu‡, Brian C. Uhlik‡, Richard J. Stern¶, Michael S. Scherman¶, Varalakshmi D. Vissa¶, Fei Pan¶, Manikuntala Kundu‖, Yu Fang Ma¶‡‡, and Michael McNeil¶**

From ‡Chugai Biopharmaceuticals, Inc., San Diego, California 92121, the ¶Department of Microbiology, Immunology, and Pathology, Colorado State University, Fort Collins, Colorado 80523, the ‖Department of Chemistry, Bose Institute, 93/1 APC Road, Calcutta 700009, India, and the ‡‡Department of Molecular Biology and Biochemistry, Dalian Medical University, Dalian 116027, PR China

Temperature-sensitive mutant 2-20/32 of *Mycobacterium smegmatis* mc^{2}155 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 Rv3265c in the *M. tuberculosis* H37Rv genome. Rv3265c shows homology to the *Escherichia coli* gene *wbbL*, which encodes a dTDP-Rha: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 insertionally 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 mc^{2}155 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. 1*A*. The complete structure of the linker is illustrated in Fig. 1*B*, and the reaction catalyzed by the enzyme, dTDP-Rha:α-D-GlcNAc-pyrophosphate polyprenol, α-3-L-rhamnosyltransferase (referred to as rhamnosyltransferase in this study) is shown in Fig. 1*C*. The rhamnosyl residue and much if not all of the arabinogalactan polysaccharide are synthesized on GlcNAc-P-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 microbial approach of isolating conditional lethal mutants was undertaken. Our strategy was to isolate temperature-sensitive (TS)[1] mutants in the genetically amenable and relatively fast growing *Mycobacterium smegmatis* mc^{2}155 (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* mc^{2}155 (7) was inoculated into Middlebrook 7H9 with ADC supplement (Difco) (7H9) and grown at 37 °C to ~10^8 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

* This work was supported by Public Health Service NIAID, National Institutes of Health Grant AI-33706. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
§ Present address: Ilypsa, Inc., Santa Clara, CA 95051.
** To whom correspondence should be addressed. Tel.: 970-491-1784; Fax: 970-491-1815; E-mail: mmcneil@colostate.edu.

[1] The abbreviations used are: TS, temperature-sensitive; ORF, open reading frame; LPS, lipopolysaccharide; MOPS, 4-morpholinepropanesulfonic acid; Rha, rhamnose.

This paper is available on line at http://www.jbc.org

Downloaded from http://www.jbc.org/ by guest on January 10, 2017

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 D-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 D-cycloserine was replaced with ampillicin (Sigma) at a final concentration of 2.3 mg/ml. Ampillicin was used in the second cycle to avoid enriching for D-cycloserine-resistant mutants. The treatment with either D-cycloserine or ampillicin 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^2155 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 Sau3A (9). Gel-purified DNA fragments of about 30 kilobases 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 inserts, they were still used to attempt 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. Fortuitously, one of the plasmids from this library, pCB120, allowed TS mutant 2-20/32 to grow at the non-permissive temperature of 42 °C. Plasmid pCB120 was sequenced and shown to contain a 1764-bp *M. tuberculosis* H37Rv DNA insert. A BLAST search showed that this DNA contained the entire open reading frame (ORF) of Rv3265c (10) as well as incomplete portions of the two flanking genes. Thus, the complementing *M. tuberculosis* DNA fragment contained only one complete ORF. The predicted protein product of this intact ORF showed homology to what was originally described as *rfb* ORF 11 in *E. coli* K-12 (11) but now has been designated as *wbbL* (12).

Construction of Plasmids—Plasmids with their relevant characteristic(s) 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 CCGAGCTCCTGAAGTGACTGACGTCCTGCCGG and GAGGTACCTTATTCAGTGCCGCCCTTCTACC from pCB120. The former primer included a SacI site and a stop codon designed to be in-frame with the β-galactosidase followed by the *wbbL* GTG start. The latter primer included a KpnI site. The PCR products were digested with appropriate enzymes and ligated into SacI/KpnI-digested pBluescript (Stratagene) to generate pCB220. *M. tuberculosis wbbL* was also cloned into the *E. coli* overexpression vector pET 23b (Novagen, Milwaukee WI) to generate pVV1. Primers GGAATTCCATATGACTGACGTCCTGCCGGT containing an NdeI site and CAGCTCGAGTCAGTGCCGCCCTTCTACCAGCT containing a stop codon and XhoI site were used. Amplified products from pCB120 were digested with NdeI and XhoI and ligated into NdeI/XhoI-digested pET 23b.

E. coli wbbL was amplified from *E. coli* strain WG1 (11) genomic DNA using PCR primers CGGAATTCAGAAACATGGGGATTGCCG and GAGGTACCTTATTACGGGTGAAAAACTGAT. EcoRI and KpnI sites in primers facilitated cloning into pBluescript. The resulting plasmid pKM1 produced a fusion protein formed between β-galactosidase and ORF 10 (10). The ATG start site in pKM1 for the *E. coli wbbL* followed two base pairs after the stop codon of ORF 10. Plasmid pCB227 was generated when *E. coli wbbL* was cloned into the mycobacterial expression vector pMX1 (20). Primers AAACTGCAGCCTGAAATGGTATATATAATAATCG, which included a PstI site, and GAGGTACCTTATTACGGGTGAAAAACTGAT (same primer used for pKM1) were used. PCR products amplified from *E. coli* WG1 were blunt end-ligated into SmaI-digested pUC18 (Invitrogen). The insert was then cut out with PstI and KpnI and ligated into PstI/KpnI-cleaved pMX1.

SDS-PAGE and Western Blots of LPS Produced by Various Bacteria—LPS solubilization and SDS-PAGE (13) as well as visualization by silver stain were done as described (14). At the beginning of these studies *E. coli* K12-producing O-antigen was not available. Therefore, antibodies against the O-antigen of *E. coli* O-16, which were known to cross-react (11) against O-antigen of *E. coli* K12 were prepared; the two strains of *E. coli* are known to produce O-antigens of similar structure (Fig. 2A) (11, 15). Rabbit antibodies against O-antigen were raised against whole bacteria suspended in 0.5% formalin, phosphate-buffered saline. The resulting antiserum was cross-absorbed with *E. coli* K12 EMG2, which is devoid of any O-antigen. Western blots were done using the rabbit O-antigen antiserum and goat anti-rabbit secondary antibody conjugated to peroxidase (Roche Applied Science) and detected using a chemiluminescence kit (Roche Applied Science).

Preparation of dTDP-[^{14}C]Rha—dTDP-[^{14}C]Rha was prepared from [U^{14}C]sucrose by conversion of the glucose moiety of the sucrose to glucose 1-phosphate by sucrose phosphorylase followed by further conversion to dTDP-[^{14}C]Glc by α-D-glucose-1-phosphate thymidylyltransferase (RmlA) and further conversion by RmlB-D to dTDP-[^{14}C]Rha. The α-D-glucose-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, 113nmol) of [U-^{14}C]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 mM NADPH, 55 μl of 50 mM HEPES buffer with 10 mM $MgCl_2$ at pH 7.0 were combined 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 lysate (~5 ml/ml protein)) and additional NADPH (35 μl of 10 mM) was added to fully convert the dTDP-[^{14}C]Glc to dTDP-[^{14}C]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-[^{14}C]Rha was purified by high performance liquid chromatography as described (17).

Assay for Rhamnosyltransferase—The assay is essentially that of Reeves and co-workers (11). The acceptor for the rhamnosyl residue, GlcNAc-P-P-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 ~13 mg/ml. To 150 μl of these membrane preparations were added 0.1 μCi of dTDP-[^{14}C]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 Transferases—M. smegmatis mc^2155, *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. 3*A* they were then harvested; for the experiment in Fig. 3*B* they were additionally treated for 6 h at 42 °C and then harvested. Cell-free extract preparation, membrane isolation, and incubation with dTDP-Rha (non-radioactive) and UDP-[^{14}C]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 at 30 °C, and the turbidity was followed at A_{600}. At a culture A_{600} of ~0.2, one of the 2-20/32 (non-transformed) and the 2-20/32 (pCB120) was shifted to 42 °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^2155 TS mutants were isolated in

Downloaded from http://www.jbc.org/ by guest on January 10, 2017

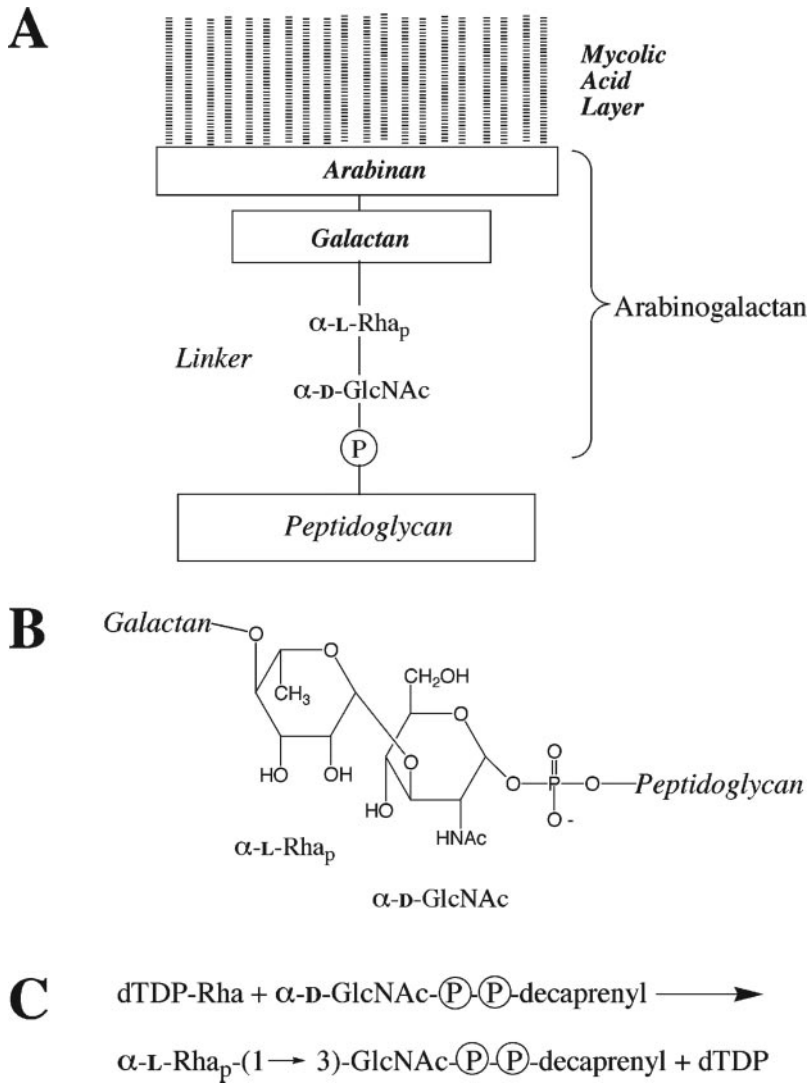

FIG. 1. *A*, the structure of the mycobacterial cell wall. The two layers, mycolic acids and peptidoglycan, are joined by the connecting polysaccharide, arabinogalactan. Arabinogalactan contains about 65 D-arabinofuranosyl, 30 D-galactofuranosyl, 1 L-rhamnopyranosyl, and 1 D-*N*-acetyglucoaminosyl residues (1–5). *B*, the structure of the Rha-GlcNAc linker region attaching the arabinogalactan to peptidoglycan (5). *C*, the reaction is catalyzed by rhamnosyltransferase for both *M. tuberculosis* and *E. coli*.

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-L-Rha-(1→3)-α-D-GlcNAc linker unit (Fig. 1*C*).

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. 2*B*) 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. 2*C*) 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

Downloaded from http://www.jbc.org/ by guest on January 10, 2017

TABLE I
Bacterial strains and plasmids

Stain or plasmid	Relevant characteristic(s)	References or source
Strains		
M. tuberculosis H37Rv	Virulent strain/sequenced by Cole *et al.* (10)	ATTC
M. smegmatis mc^{2}155	Efficient plasmid transformation mutant	7
M. smegmatis 2–20/32	Temperature-sensitive mutant of mc^{2}155	This study
E. coli K12 EMG2	*rfb-50* (*wbbL*)	11
E. coli K12 WG1	*rfb-51*, wbbL+	11
E. coli K12 HMS174 (DE3)	*rfb-50* (*wbbL*), pLysS	Stratagene
E. coli K12 BW 24599	*rfb-50* (*wbbL*) Repaired, O-antigen-competent	17
Plasmids		
p16R1	Mycobacterial *E. coli* shuttle vector; *hyg*	28
pCB7	p16R1 derivative containing a BamHI site and λ *cos* sequence	This study
pCB120	pCB7 containing 1764-bp fragment from H37Rv genome	This study
Pbluescript II SK+	*E. coli* plasmid vector, *amp*	Stratagene
pET 23b	T-7 expression vector	Novagen
pCB220	924-bp fragment (*wbbL*) from pCB120 cloned into SacI-KpnI of pBluescript II SK+	This study
pKM1	*E. coli* K12 WG1 *wbbL* (orf 11) cloned into EcoRI-KpnI of pBluescript II SK+	This study
pMX1	p16R1 derivative containing mycobacterial *HSP*60 promoter	20
pCB227	*E. coli* K12 WG1 *wbbL* (ORF 11) cloned into KpnI-PstI of pMX1	This study
pVV1	*M. tuberculosis wbbL* cloned into the NdeI-XhoI sites in pET 23b	This study

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 Rhamnosyltransferase Activity in E. coli—Direct evidence that the *M. tuberculosis wbbL* encodes for rhamnosyltransferase was obtained using the rhamnosyltransferase assay of Reeves and co-workers (11). Membranes were prepared from *E. coli* strains BW24599, a K12 strain repaired for rhamnosyltransferase (17), and a non-repaired K12 strain, HMS 174 containing the control vector with no insert (pET 23b) and HMS 174 with pVV1, a pET 23b vector with a *M. tuberculosis wbbL* insert. The membranes containing endogenous undecaprenyl phosphate were incubated with UDP-GlcNAc to form *in situ* the acceptor for rhamnosyltransferase, GlcNAc-P-P-decaprenol. dTDP-[^{14}C]Rha donor was added, and the production of product, [^{14}C]Rha-GlcNAc-P-P-undecaprenol was monitored by the production of organic soluble radioactivity. As shown in Table II, the positive control, *E. coli* strain BW24599 and the *E. coli* HMS 174 strain containing pVV1 did show rhamnosyltransferase activity. *E. coli* HMS 174 without plasmid showed no activity.

TS Mutant M. smegmatis 2-20/32 Exhibits Diminished Rhamnosyltransferase Activity—Membranes prepared from *M. smegmatis* strains mc^{2}155, 2-20/32, and 2-20/32 containing pCB120 were tested for rhamnosyltransferase activity using the procedure developed by Mikusova *et al.* (6). This procedure, using radioactive UDP-GlcNAc instead of radioactive dTDP-Rha, allows direct comparison of the amounts of [^{14}C]GlcNAc-P-P-decaprenol and of Rha-[^{14}C]GlcNAc-P-P-decaprenol produced by the *M. smegmatis* membranes. Thus, the membranes were incubated with dTDP-Rha and UDP-[^{14}C]GlcNAc. Endogenous decaprenyl phosphate present in the membrane preparations was utilized by the *M. smegmatis* GlcNAc-1-P transferase to form [^{14}C]GlcNAc-P-P-decaprenyl, which was in turn utilized by the rhamnosyltransferase along with dTDP-Rha to form Rha-[^{14}C]GlcNAc-P-P-decaprenyl. The results, as monitored by TLC, are presented in Fig. 3*A*. Wild-type *M. smegmatis* mc^{2}155 showed a strong Rha-[^{14}C]GlcNAc-P-P-decaprenyl band and a weak [^{14}C]GlcNAc-P-P-decaprenyl band (Fig. 3*A*, *lane 1*, in accordance with previous results (21). In contrast, strain 2-20/32 showed Rha-[^{14}C]GlcNAc-P-P-decaprenyl and [^{14}C]GlcNAc-P-P-decaprenyl in about equal amounts (Fig. 3*A*, *lane 3*), suggesting that even with cell growth and enzyme incubation at 30 °C, the rhamnosyltransferase activity was lower than in wild type. Complementation of *M. smegmatis* 2-20/32 with pCB120 restored the rhamnosyltransferase activity to wild-type levels (Fig. 3*A*, *lane 2*). When enzyme incubations were shifted from 30 to 42 °C a similar TLC profile with no decrease of the rhamnosylated lipid was produced. Therefore, the effect of varying the growth temperatures of the cells was ascertained. Cells of all three strains were again grown at 30 °C and then incubated at the non-permissive temperature of 42 °C for another 6 h. It was expected that in strain 2-20/32 no new active rhamnosyltransferase would be formed at 42 °C and the previously folded rhamnosyltransferases would be depleted by normal turnover events. Membranes were prepared and assayed for rhamnosyltransferase activity at 30 °C (Fig. 3*B*). The results showed that under these conditions strain 2-20/32 produced very little Rha-[^{14}C]GlcNAc-P-P-decaprenyl in comparison to its precursor, [^{14}C]GlcNAc-P-P-decaprenyl (Fig. 3*B*, *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. 3*B*, *lanes 1* and *2*). The fact that the rhamnosyltransferase 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* mc^{2}155 wild-type (GenBank^{TM} accession number AF187550) and 2-20/32 (GenBank^{TM} accession number AF187551) were sequenced, and a single base changed at position 364 from a C to T was found. This translates into a change from proline to serine at amino acid 122 in the WbbL polypeptide. The wild-type *wbbL* sequence agreed with the sequence for the incomplete *M. smegmatis* genome on Tigr (www.tigr.org). The nucleotide sequence of the *M. tuberculosis wbbL* from pCB120 was in complete agreement with the published Rv3265c sequence (10). The deduced amino acid sequences of WbbL from *M. tuberculosis*, *Mycobacterium leprae* (88%), *Mycobacterium avium paratuberculosis* (85%), and *M. smegmatis* (72%) are similar, whereas the *E. coli* WbbL shows only 24% identity to the *M. tuberculosis*. Proline at position 122 in WbbL is conserved in all four wild-type mycobacterial sequences but not in *E. coli* (in Rv3265c this proline resides at position 114).

M. smegmatis TS Mutant 2-20/32 Is Non-viable after Incubations at the Non-permissive Temperature—Two cultures of *M. smegmatis* 2-20/32 and one culture of *M. smegmatis* 2-20/32

Downloaded from http://www.jbc.org/ by guest on January 10, 2017

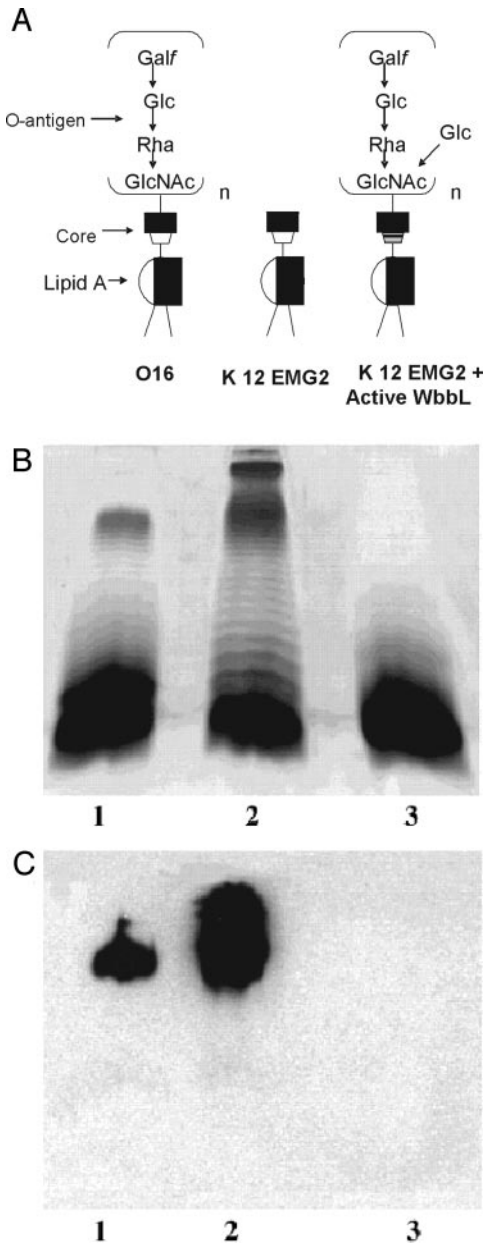

FIG. 2. *A*, depiction of the O-antigen in *E. coli* O16 (strain used to raised antibodies cross-reactive against *E. coli* K12), *E. coli* K12 EMG2 (O-antigen negative), and *E. coli* K12 EMG2 complemented with a functional *wbbL* gene. SDS-PAGE of O-antigen from *E. coli* K12 EMG2 constructs as visualized by silver stain (*B*) and Western blot (*C*) using antiserum raised against O-antigen. *Lane 1*, *E. coli* K12 EMG2 with pCB120 (*M. tuberculosis wbbL*); *lane 2*, *E. coli* K12 EMG2 with pKM1 (*E. coli wbbL*); *lane 3*, *E. coli* K12 EMG2 with no plasmid.

TABLE II
Rhamnosyltransferase activity in membranes prepared from various E. coli strains containing different plasmids

E. coli strain (plasmid)	Incubation time	Total cpm in organic phase[a]
	min	
E. coli BW24599	0	20
E. coli BW24599	60	1080
E. coli HMS 174 (pET 23b)	0	180
E. coli HMS 174 (pET 23b)	60	220
E. coli HMS 174 (pVV1)	0	200
E. coli HMS 174 (pVV1)	60	5000

[a] Substrate dTDP-[^{14}C]Rha was converted from aqueous to organic counts by the presumed attachment of the [^{14}C]rhamnosyl residue to *in situ* GlcNAc-P-P-decaprenol. In total, 250,000 cpm of dTDP-[^{14}C]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-[^{14}C]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-[^{14}C]Rha into the organic phase.

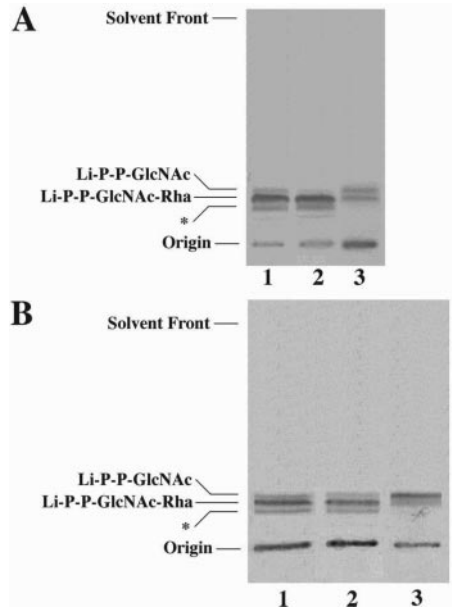

FIG. 3. **TLC analysis of radioactive glycolipid extracts from membranes of *M. smegmatis* after incubation with UDP-[^{14}C]-GlcNAc and dTDP-Rha.** The cells used were grown at 30 °C (*A*) or at 30 °C followed by a 6 h shift to 42 °C (*B*). *Lane 1*, *M. smegmatis* mc^{2}155; *lane 2*, *M. smegmatis* 2-20/32 containing pCB120; *lane 3*, *M. smegmatis* 2-20/32 with no plasmid. The identity of the indicated radioactive glycolipids for *M. smegmatis* mc^{2}155 was determined previously (6), and the glycolipids produced by this strain were used as a standard for these experiments. The identity of the glycolipid indicated by an asterisk is unknown; it might be the C-35 polyprenyl version of Rha-GlcNAc-P-P-polyprenyl. The identity of the radioactivity at the origin is also unknown. *Li-PP*, polyprenyldiphosphoryl.

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 A_{600} was monitored. After a brief growth spurt, 2-20/32 with no plasmid stopped growing (Fig. 4*A*). 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. 4*B*) 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. 1*A*) (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 *M. smegma-*

Downloaded from http://www.jbc.org/ by guest on January 10, 2017

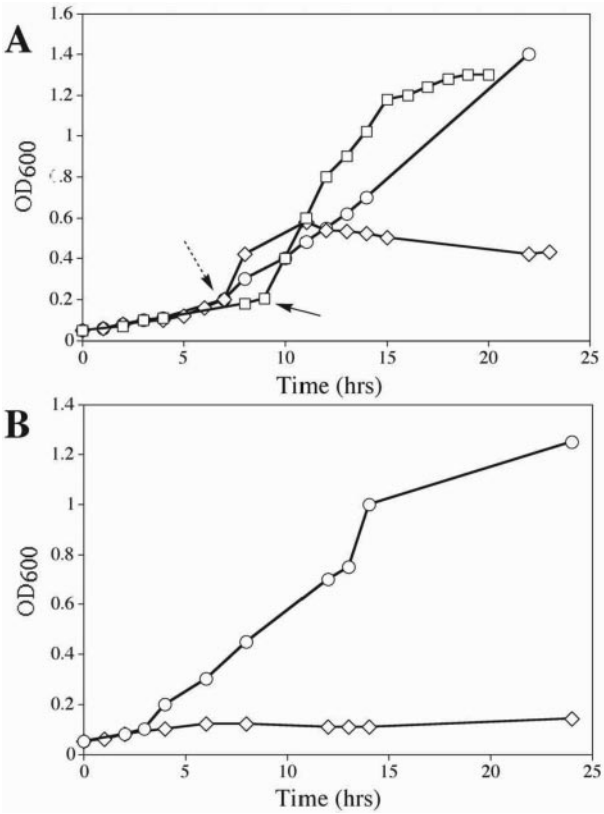

FIG. 4. *A*, growth curves of *M. smegmatis* 2-20/32 (no plasmid) (◇ and ○) and *M. smegmatis* 2-20/32 (□) with pCB120. After the culture A_{600} reached 0.2, the temperature was shifted for one (◇, see the *dashed arrow*) of the *M. smegmatis* 2-20/32 cultures and for the *M. smegmatis* 2-20/32 containing pCB120 (□-see the *solid arrow*). *B*, the non-plasmid containing *M. smegmatis* 2-20/32 cultures from *A* at the 22-h time point were inoculated into fresh media and incubated at 30 °C. The same symbols used in *B* are as in *A*.

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 rhamnosyltransferase in *E. coli* K12 O-antigen biosynthesis utilizes the same substrates, dTDP-Rha and GlcNAc-diphosphoryl polyprenol, to form the exact same product, α-L-Rha-(1→3)-α-D-GlcNAc-diphosphoryl polyprenol, as mycobacteria. This product is then utilized in very different cell wall structures by *E. coli* and by mycobacteria. In *E. coli* it becomes part of the moiety that forms the O-antigen that is attached to LPS core (Fig. 2*A*), but in mycobacteria it is metabolized to generate the linker region that attaches the arabinogalactan to the peptidoglycan (Fig. 1*A*). Moreover, O-antigen is nonessential to the host, whereas the linker is essential.

Given the conservation of cell wall core structure in mycobacteria, it was presumed that *M. tuberculosis* cell wall-associated homologues would be able to complement TS mutations in *M. smegmatis*. Expecting *E. coli* and *M. tuberculosis wbbL* genes to function interchangeably to help confirm enzymatic roles was more uncertain. Fortunately, the *E. coli wbbL* did permit the growth of the TS mutant at the non-permissive temperature of 37 °C, but not at 42 °C, where *M. tuberculosis wbbL* still functioned. Similarly, the *M. tuberculosis wbbL* allowed O-antigen units to be added to the LPS core but not to same degree as the *E. coli wbbL (*Fig. 2). Although complementation was observed in both experiments, the foreign *wbbL* was not expressed as well and/or the WbbL protein did not interact as well as the endogenous WbbL with the native host environment to allow the same level of production. Nevertheless, these complementation results and biochemical evidence of linker-associated metabolites in *E. coli* (Table I) and *M. smegmatis* (Fig. 3) confirmed the putative WbbL to be the rhamnosyltransferase involved in synthesis of the linker region in mycobacteria.

In addition to *wbbL*, the *M. tuberculosis* genome has annotated sequence Rv1525 as *wbbL2* (10). This gene shows no homology to the *wbbL* genes discussed herein, and there is no reason to suspect that it encodes a protein that catalyzes the reaction shown in Fig. 1*C*. Nor does *M. leprae*, *M. avium paratuberculosis*, or *M. smegmatis* carry a known homologue to *wbbL2*. At this time the role of *wbbL2* is unknown. Other rhamnosyltransferase genes have been identified in mycobacteria that function to generate other cell wall-associated structures. The gene *rtfA* encodes a rhamnosyltransferase required for the synthesis of serovar 2-specific oligosaccharides in *M. avium* (25). 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.

Acknowledgments—We gratefully acknowledge the gift of *E. coli* strain BW24599 from Dr. Barry Wanner and the technical assistance of Wenxin Yan and Sara Kimbrel.

REFERENCES

1. McNeil, M., Besra, G. S., and Brennan, P. J. (1996) in *Tuberculosis* (Rom, W. N., and Garay, S. M., eds) pp. 171–186, Little, Brown, and Co., Boston
2. Besra, G. S., Khoo, K.-H., McNeil, M., Dell, A., Morris, H. R., and Brennan, P. J. (1995) *Biochemistry* **34,** 4257–4266
3. McNeil, M., Daffe, M., and Brennan, P. J. (1991) *J. Biol. Chem.* **266,** 13217–13223
4. Daffe, M., Brennan, P. J., and McNeil, M. (1990) *J. Biol. Chem.* **265,** 6734–6743
5. McNeil, M., Daffe, M., and Brennan, P. J. (1990) *J. Biol. Chem.* **265,** 18200–18206
6. Mikusova, K., Mikus, M., Besra, G., Hancock, I., and Brennan, P. J. (1996) *J. Biol. Chem.* **271,** 7820–7828
7. Snapper, S. B., Melton, R. E., Mustafa, S., Kieser, T., and Jacobs, W. R. (1990) *Mol. Microbiol.* **4,** 1911–1919
8. Hooke, A. M. (1994) *Methods Enzymol.* **235,** 448–457
9. Pascopella, L., Collins, F. M., Martin, J. M., Lee, M. H., Hatfull, G. F., Stover, C. K., Bloom, B. R., and Jacobs, W. R., Jr. (1994) *Infect. Immun.* **62,** 1313–1319
10. Cole, S. T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S. V., Eiglmeier, K., Gas, S., Barry, C. E., Tekaia, F., Badcock, K., Basham, D., Brown, D., Chillingworth, T., Connor, R., Davies, R., Devlin, K., Feltwell, T., Gentles, S., Hamlin, N., Holroyd, S., Hornby, T., Jagels, K., Krogh, A., McLean, J., Moule, S., Murphy, L., Oliver, K., Osborne, J., Quail, M. A., Rajandream, M. A., Rogers, J., Rutter, S., Seeger, K., Skelton, J., Squares, R., Squares, S., Sulston, J. E., Taylor, K., Whitehead, S., and Barrell, B. G. (1998) *Nature* **393,** 537–544
11. Stevenson, G., Neal, B., Liu, D., Hobbs, M., Packer, N. H., Batley, M., Redmond, J. W., Lindquist, L., and Reeves, P. R. (1994) *J. Bacteriol.* **176,** 4144–4156
12. Reeves, P. R., Hobbs, M., Valvano, M. A., Skurnik, M., Whitfield, C., Coplin, D., Kido, N., Klena, J., Maskell, D., Raetz, C. R., and Rick, P. D. (1996) *Trends. Microbiol.* **4,** 495–503

Downloaded from http://www.jbc.org/ by guest on January 10, 2017

13. Hitchcock, P. J., and Brown, T. M. (1983) *J. Bacteriol.* **154,** 269–277
14. Tsai, C. M., and Frasch, C. E. (1982) *Anal. Biochem.* **119,** 115–119
15. Jann, B., Shashkov, A. S., Kochanowski, H., and Jann, K. (1994) *Carbohydr. Res.* **265,** 305–311
16. Ma, Y., Mills, J. A., Belisle, J. T., Vissa, V., Howell, M., Bowlin, K., Scherman, M. S., and McNeil, M. (1997) *Microbiology* **143,** 937–945
17. Stern, R. J., Lee, T. Y., Lee, T. J., Yan, W., Scherman, M. S., Vissa, V. D., Kim, S. K., Wanner, B. L., and McNeil, M. R. (1999) *Microbiology* **145,** 663–671
18. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) *J. Mol. Biol.* **215,** 403–410
19. Liu, D., and Reeves, P. R. (1994) *Microbiology* **140,** 49–57
20. Jackson, M., Raynaud, C., Laneelle, M. A., Gullhot, C., Laurent-Winter, C., Ensergueix, D., Gicquel, B., and Daffe, M. (1999) *Mol. Microbiol.* **31,** 1573–1587
21. Khoo, K. H., Suzuki, R., Dell, A., Morris, H. R., McNeil, M. R., Brennan, P. J., and Besra, G. S. (1996) *Biochemistry* **35,** 11812–11819
22. Danner, M., and Seckler, R. (1993) *Protein Sci.* **2,** 1869–1881
23. McNeil, M. (1996) in *Mycobacterium Avium Complex Infection* (Korvick, J. A., and Benson, C. A., eds) pp. 263–283, Marcel Dekker, Inc., New York
24. Ma, Y., Pan, F., and McNeil, M. R. (2002) *J. Bacteriol.* **184,** 3392–3395
25. Eckstein, T. M., Silbaq, F. S., Chatterjee, D., Kelly, N. J., Brennan, P. J., and Belisle, J. T. (1998) *J. Bacteriol.* **180,** 5567–5573
26. Philipp, W. J., Poulet, S., Eiglmeier, K., Pascopella, L., Balasubramanian, V., Heym, B., Bergh, S., Bloom, B. R., Jacobs, W. R., and Cole, S. T. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 3132–3137
27. Sassetti, C. M., Boyd, D. H., and Rubin, E. J. (2003) *Mol. Microbiol.* **48,** 77–84
28. Garbe, T. R., Barathi, J., Barnini, S., Zhang, Y., Abou-Zeid, C., Tang, D., Mukherjee, R., and Young, D. B. (1994) *Microbiology* **140,** 133–138

Downloaded from http://www.jbc.org/ by guest on January 10, 2017

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

Jonathan A. Mills, Kelly Motichka, Markus Jucker, Henry P. Wu, Brian C. Uhlik, Richard J. Stern, Michael S. Scherman, Varalakshmi D. Vissa, Fei Pan, Manikuntala Kundu, Yu Fang Ma and Michael McNeil

J. Biol. Chem. 2004, 279:43540-43546.
doi: 10.1074/jbc.M407782200 originally published online August 4, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M407782200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 26 references, 10 of which can be accessed free at
http://www.jbc.org/content/279/42/43540.full.html#ref-list-1

Downloaded from http://www.jbc.org/ by guest on January 10, 2017