Lcp1 Is a Phosphotransferase Responsible for Ligating Arabinogalactan to Peptidoglycan in Mycobacterium tuberculosis

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ABSTRACT Mycobacterium tuberculosis, the etiological agent of tuberculosis (TB), has a unique cell envelope which accounts for its unusual low permeability and contributes to resistance against common antibiotics. The main structural elements of the cell wall consist of a cross-linked network of peptidoglycan (PG) in which some of the muramic acid residues are covalently attached to a complex polysaccharide, arabinogalactan (AG), via a unique α-L-rhamnopyranose–(1→3)-α-D-GlcNAc–(1→P) linker unit. While the molecular genetics associated with PG and AG biosynthetic pathways have been largely delineated, the mechanism by which these two major pathways converge has remained elusive. In Gram-positive organisms, the LytR-CpsA-Psr (LCP) family of proteins are responsible for ligating cell wall teichoic acids to peptidoglycan, through a linker unit that bears a striking resemblance to that found in mycobacterial arabinogalactan. In this study, we have identified Rv3267 as a mycobacterial LCP homolog gene that encodes a phosphotransferase which we have named Lcp1. We demonstrate that lcp1 is an essential gene required for cell viability and show that recombinant Lcp1 is capable of ligating AG to PG in a cell-free radiolabeling assay.

IMPORTANCE Tuberculosis is an infectious disease caused by the bacterial organism Mycobacterium tuberculosis. Survival of M. tuberculosis rests critically on the integrity of its unique cell wall; therefore, a better understanding of how the genes and enzymes involved in cell wall assembly work is fundamental for us to develop new drugs to treat this disease. In this study, we have identified Lcp1 as an essential phosphotransferase that ligates together arabinogalactan and peptidoglycan, two crucial cell wall macromolecules found within the mycobacterial cell wall. The discovery of Lcp1 sheds new light on the final stages of mycobacterial cell wall assembly and represents a key biosynthetic step that could be exploited for new anti-TB drug discovery.

Tuberculosis (TB) remains the single most important bacterial cause of global mortality and morbidity, causing around 9.6 million new cases and 1.5 million deaths each year (1). This prevalence is largely due to the increased susceptibility of HIV-infected individuals, and the rise of multidrug-resistant (MDR), extensively drug-resistant (XDR), and, more recently, totally drug-resistant (TDR) TB strains (2). It is of paramount importance that we extend our understanding of the pathogenicity and physiology of the tuberculosis bacillus in the hope of priming novel therapeutic approaches against this ancient human adversary. Mycobacterial peptidoglycan (PG) forms the basal layer of the mycolyl-arabinogalactan–peptidoglycan (mAGP) complex and is composed of alternating N-acetylg glucosamine (GlcNAc) and modified muramic acid (Mur) residues, linked in a β(1→4) configuration (3). Unlike archetypal PG commonly found in bacteria such as Escherichia coli, the mycobacterial acid residues in Mycobacterium tuberculosis contains a mixture of N-acetyl and N-glycolyl derivatives, whereby the N-acetyl function has been oxidized to an N-glycolyl function to form MurN Gly (4–6). In addition, approximately 10 to 12% of the muramic acid residues of PG are covalently tethered to arabinogalactan (AG) via a phosphodiester bond (7). The 6′-OH groups of these muramic acid residues serve as attachment sites forming a phosphodiester bond via a unique α-L-rhamnopyranose–(1→3)-α-D-GlcNAc–(1→P) linker unit (LU) (8). Collectively, PG and AG form a huge macromolecular positioned between the cytoplasmic membrane and the outer mycolic acid layer of the TB bacillus. Our understanding of both the structure and biosynthesis of mycobacterial AG has developed steadily over the last 2 decades, with much emphasis being placed on investigating the molecular genetics of how this complex structure is assembled (extensively reviewed in reference 9). While the cytoplasmic and extracytoplasmic intermediate biosynthetic steps of cell wall assembly have been almost fully delineated, our understanding of the latter stages of AG biosynthesis, specifically the attachment of AG to PG, remains fragmented at best. Previous studies have demonstrated that attachment of AG to the mycobacterial cell wall occurs only when the PG has been fully matured via the cross-linking action of endogenous transpeptidases (10). However, the enzyme responsible for this action remains elusive. In Gram-positive bacteria, the structure of the linker unit (1,−
GlcNAc-D-ManNAc) that connects wall teichoic acids (WTA) to PG via a phosphodiester bond is remarkably similar to the linker unit found within the \textit{M. tuberculosis} cell wall (D-GlcNAc-L-Rhap) (11). Other commonalities also exist between mycobacterial and Gram-positive cell wall glycan biosynthetic pathways. For instance, in organisms such as \textit{Staphylococcus aureus} polyribitolphosphate (Rbo-P) is assembled upon a polyprenyl pyrophosphoryl lipid carrier which, after biosynthesis is completed, is ultimately attached to the C6-hydroxyl of MurNAc within the PG glycan strands (8, 11). Mycobacterial AG is also assembled upon a polyprenyl pyrophosphoryl lipid carrier before its final deposition onto specific muramic acid residues of PG (12). We postulated that WTA-containing Gram-positive organisms and members of the \textit{Actinomycetales} might utilize similar ligases from the same protein superfamily. Kawai et al. conducted a structural and genetic study and proposed that a family of genes encoding the LytR-CpsA-Psr (LCP) proteins catalyze the ligation of the murine linkage unit of WTA to the MurNAc units of PG in \textit{Bacillus subtilis} (13). More recent studies have revealed further corroborating evidence that the LCP family of proteins are responsible for attaching capsular polysaccharides to the murine sacculus of \textit{S. aureus} (14) and \textit{Streptococcus pneumoniae} (15). Here, we demonstrate that the open reading frame contained in \textit{Rv3267}, one of three LCP ortholog genes in \textit{M. tuberculosis}, encodes a peptidoglycan–arabinogalactan ligase, which we have termed Lcp1.

RESULTS

Genome comparison of the Lcp1 locus. Using \textit{S. aureus}, \textit{S. pneumoniae}, and \textit{B. subtilis} LCP proteins as search sequences, we performed a bioinformatic analysis of mycobacterial and corynebacterial genomes to identify putative genes that might encode the mycobacterial LCP homologs (see Fig. S1 in the supplemental material). We identified three putative LCP homologs in \textit{M. tuberculosis}, with \textit{Rv3267} sharing 20% amino acid sequence identity with Cps2A from \textit{S. pneumoniae}, which is in accordance with a previous phylogenetic study of bacterial LCP proteins (16). The remaining two putative LCP orthologs present in \textit{M. tuberculosis}, encoded by \textit{Rv3484} and \textit{Rv0822c}, share 15% and 12% amino acid identity with Cps2A from \textit{S. pneumoniae}. \textit{Rv3267} shares 36% and 26% amino acid sequence identity with \textit{Rv3484} and \textit{Rv0822c}, respectively. Both \textit{Rv3484} and \textit{Rv0822c} are predicted to contain a single N-terminal transmembrane (TM)/H9251-helix, similar to that of \textit{Rv3267}, and are predicted to contain domains belonging to both LCP and LytR_C superfamilies. Interestingly, the genome of \textit{Mycobacterium leprae}, which shows massive gene decay and is thus considered to represent a “minimal” mycobacterial genome, has retained only two LCP orthologs in the form of ML0756 and NCgl0708, respectively. Both \textit{Rv3484} and \textit{Rv0822c} are predicted to contain a single N-terminal transmembrane (TM) α-helix, similar to that of \textit{Rv3267}, and are predicted to contain domains belonging to both LCP and LytR_C superfamilies. Interestingly, the genome of \textit{Mycobacterium leprae}, which shows massive gene decay and is thus considered to represent a “minimal” mycobacterial genome, has retained only two LCP orthologs in the form of ML0750 and ML2247, sharing 79.1% and 32.7% amino acid sequence identity with \textit{Rv3267}, respectively. Inspection of the \textit{Mycobacterium smegmatis} genome reveals four putative orthologs, MSMEG_1824, MSMEG_6421, MSMEG_0107, and MSMEG_5775, each sharing amino acid sequence identities of 72%, 35%, 31%, and 24% with \textit{Rv3267}, respectively. Furthermore, an alignment of the genes surrounding \textit{Rv3267} reveals genetic synteny with orthologous regions from \textit{M. smegmatis}, \textit{M. leprae}, and \textit{Corynebacterium glutamicum} (Fig. 1A). This close arrangement of genes is highly indicative of conserved functionality. Interestingly, \textit{Rv3267} is positioned immediately upstream of \textit{rmlD} and \textit{wbbL1}, both of which...
encode enzymes responsible for the formation of the linker unit during early-stage AG biosynthesis (Fig. 1A) (17, 18). Based on these analyses, we hypothesized that Rp3267 was the primary LCP homolog responsible for ligating PG to AG in mycobacteria, which we have named Lcp1.

**In silico analysis of **$^{\text{Mtb}}$**Lcp1 and identification of copurifying polyisoprenoid phosphate.** Analysis of the *M. tuberculosis* Lcp1 ($^{\text{Mtb}}$Lcp1) amino acid sequence suggests that a single transmembrane-spanning a-helix occurs at the N terminus of the protein (Fig. 1B). Similarly, a single N-terminal transmembrane a-helix has also been observed in other LCP proteins, such as Cps2A from *S. pneumoniae* (13, 16). An alignment of the amino acid sequences of $^{\text{Mtb}}$Lcp1 and Cps2A from *S. pneumoniae* indicates that these two proteins share 20% sequence identity (see Fig. S1 in the supplemental material). The crystal structure of $\Delta$/H9252T-MtsLcp1 (residues 98 to 481) has been solved to a resolution of 1.69 Å with one molecule of decaprenol-1-monophosphate bound in a central hydrophobic cavity (2XXP) (13). Although we were able to express large amounts of pure, soluble His6-tagged $^{\text{Mtb}}$Lcp1 (see below), our attempts to obtain an experimental crystal structure of $^{\text{Mtb}}$Lcp1 have so far proven unsuccessful. Therefore, we generated a $^{\text{Mtb}}$Lcp1 homology model using the I-TASSER server (utilizing the *S. pneumoniae* Cps2A structure as the template), resulting in a C-score of $-2.64$ (19, 20). Unlike the *S. pneumoniae* $\Delta$/TMS2A structure, which also harbors an N-terminal accessory domain, the $^{\text{Mtb}}$Lcp1 amino acid sequence and homology model suggests that only a single “LCP-like” domain is present (Fig. 1C) (13). Our $^{\text{Mtb}}$Lcp1 homology model indicates that a 5-stranded b-sheet forms the core of the protein, with a-helices surrounding a central b-sheet. Our homology model also retains the polyisoprenoid-binding cavity of Cps2A (Fig. 1C) (13). Overexpression of His6-tagged $^{\text{Mtb}}$Lcp1 in *E. coli* BL21(DE3) cells and subsequent purification by immobilized metal affinity chromatography (IMAC) resulted in a preparation of His6-tagged $^{\text{Mtb}}$Lcp1 that was stable in solution at a concentration of 50 mg/ml. Since Cps2A was shown to copurify with a polyisoprenoid phosphate, we also investigated whether His6-tagged $^{\text{Mtb}}$Lcp1 copurified with a similar lipid (13). We performed an organic solvent extraction on His6-tagged $^{\text{Mtb}}$Lcp1 followed by thin-layer chromatography (TLC) analysis, which revealed a spot that migrated with an R$_f$ identical to that of decaprenyl-1-monophosphate (Fig. 2A). Mass spectrometric analysis of this copurifying lipid revealed an m/z of 777, which correlates with the expected mass of decaprenyl-1-monophosphate (Fig. 2D). This evidence was further corroborated by comparison to known standards of decaprenyl-1-monophosphate and undecaprenyl-1-monophosphate, which reveal m/z values of 777 and 845, respectively (Fig. 2B and C). We also performed an identical solvent extraction on a highly purified preparation of EmbC$^{\text{CT}}$ (the C-terminal globular domain of the arabinofuranosyltransferase EmbC from *M. tuberculosis* [21]) (Fig. 2A). This negative control confirms that the product extracted from $^{\text{Mtb}}$Lcp1 is not a contaminating impurity from the extraction process.

**$^{\text{Mtb}}$Lcp1 is essential for viability of **$^{\text{M. smegmatis}}$. Because of its putative role in covalently attaching AG to PG in mycobacteria, we hypothesized that $^{\text{Mtb}}$Lcp1 would likely be an essential gene. In order to test this hypothesis, we used *M. smegmatis* as a model system to investigate the essentiality of Lcp1 in mycobacteria using the conditional expression specialized transduction essentiality test (CESTET) (22–25). We constructed a knockout phage, $\phi^{\text{Mtb}}$Lcp1, designed to replace MSMEG1824 (*M. smegmatis* lcp1 [M$\text{ts}$lcp1]) with a hygromycin resistance cassette (22), but as predicted, we were unable to directly generate a null mutant due to the failure to yield any transductants. However, we were able to generate an $^{\text{Mts}}$lcp1 null mutant (*M. smegmatis* $^{\Delta}$/H9262T-McLcp1 pMV306-ACET-M$\text{ts}$lcp1) by transducing a merodiploid strain containing a second, inducible copy of $^{\text{Mtb}}$lcp1. Expression of this recombinant copy of $^{\text{Mtb}}$lcp1 was induced by the addition of acetamide to the growth medium, and only when this inducer was present on agar plates were we able to generate conditional knockout mutants. In this regard, the conditional knockout mutant (*M. smegmatis* $^{\Delta}$/H9251T-McLcp1 pMV306-ACET-M$\text{ts}$lcp1) exhibited an altered colony phenotype compared to the wild type, which is suggestive of a defect or lesion in the cell wall, which might not be fully recovered by complementation via the acetamide-inducible pMV306-ACET-M$\text{ts}$lcp1 plasmid (Fig. 3A). To further investigate the essentiality of $^{\text{Mtb}}$lcp1, we cultured *M. smegmatis* $^{\Delta}$/H9251T-McLcp1 in liquid medium and withdrew the inducer acetamide from the growth medium, with subsequent measurement of CFU. We observed a rapid loss of cell viability immediately after acetamide was removed from growth medium, indicating that expression of the pMV306-ACET-driven copy of $^{\text{Mtb}}$lcp1 was required for cell growth (Fig. 3B). This confirms that $^{\text{Mtb}}$lcp1 is indeed essential in *M. smegmatis*. The rapid loss in cell viability of the $^{\Delta}$/H9251T-lcp1 mutant made any attempt to characterize the resultant phenotype (specifically cell wall lesions) technically challenging. Therefore, we turned our attention toward the biochemical characterization of recombinant $^{\text{Mtb}}$Lcp1.

**Carbohydrate binding of linker unit mimetics to **$^{\text{Mtb}}$**Lcp1.** Our laboratory has previously synthesized neoglycolipid acceptors modeled on motifs found within mycobacterial AG, which have been successfully employed to probe both enzymatic function and ligand binding relationships of cell wall processing enzymes (21, 26, 27). In this regard, we synthesized a panel of analogues of the mycobacterial linker unit (compounds 1 to 4) (Fig. 4A; also see Text S1 in the supplemental material) in order to probe the interaction of $^{\text{Mtb}}$Lcp1 with decaprenyl-D-phosphoryl-3-deoxy-D-mannooctulosonic acid-Rha-Galactose, its natural physiological substrate. Each of the ligands used (compounds 1 to 4) was synthesized to include an octyl (C$_8$) group attached to the anomic carbon. Historically, these alkyl groups are useful for performing solvent extractions from glycosyltransferase assay mixtures when used as neoglycolipid acceptors (26). In this study, their sole use was to mimic the hydrophobicity of the lipid moiety of the natural substrate, which would beaccommodated by decaprenyl-D-phosphoryl. Due to the chemical complexity and labile nature of the natural substrate (12, 28), using synthetic analogues offered us an alternative approach to investigate the biochemical interaction of this key enzymatic step. By way of intrinsic tryptophan fluorescence (ITF), we probed the binding of four potential ligands (compounds 1 to 4) to $^{\text{Mtb}}$Lcp1, each of which is a variant of the mycobacterial linker unit (Fig. 4A). ITF is an extremely useful approach to study protein-ligand interactions (29) and has been successfully used by our laboratory to biochemically characterize several mycobacterial proteins (21, 27, 30, 31). Fitting the binding data to a single-site saturation model yielded an equilibrium dissociation constant, K$_D$, of 57.68 mM for compound 1, the ligand which resembles the mycobacterial linker unit disaccharide L-Rha-3-deoxy-D-manno-octulosonic acid (Fig. 4B). ITF experiments repeated
with a tetrasaccharide ligand that resembles an extension of the linker unit by two Gal\textsubscript{f} residues afforded an ~10-fold increase in affinity, with a $K_D$ of 5.13 $\mu$M. Further elongation with a third Gal\textsubscript{f} unit resulted in a calculated $K_D$ of 20.39 $\mu$M. The cytoplasmic steps of mycobacterial galactan formation proceed via a linear biosynthetic pathway; therefore, compounds 2 and 3 represent chemical mimetics of glycolipid-3 (GL-3) and glycolipid-4 (GL-4) intermediates of this pathway, respectively (12, 28), without the pyrophosphoryl-decaprenyl moiety linked to the anomeric position of the GlcNAc unit. In addition, we investigated the binding of a fourth ligand against $\text{MtbLcp1}$. Compound 4, a disaccharide of D-Gal\textsubscript{f}-(1→4)-L-Rhap (27), mimics the terminal component of the linker unit with a single Gal\textsubscript{f} residue and displays an apparent $K_D$ of 97.61 $\mu$M. However, compound 4 achieves a $B_{\text{max}}$ of only 177, which is significantly lower than those of compounds 1 to 3 and reflects the changes in fluorescence observed upon addition of equivalent volumes of water. Therefore, compound 4 displays no significant binding affinity (above background) toward $\text{Mt-Lcp1}$ in ITF assays (Fig. 4B).

$\text{MtbLcp1}$ ligates newly synthesized galactan to peptidoglycan in a cell-free assay. We sought to investigate whether $\text{MtbLcp1}$ is capable of ligating AG to PG (from decaprenyl-pyrophosphoryl-GlcNAc-Rha-galactose) by developing a cell-free biochemical functional assay. Since $\text{MtbLcp1}$ contains a single N-terminal TM-\alpha-helix, and attachment of AG to PG is likely to occur at or around the cytoplasmic membrane, we postulated that membranes prepared from mycobacteria should contain endogenous PG-AG ligation activity and, as such, could be investigated using an appropriate $[^{14}\text{C}]$carbohydrate radiolabeling assay. We have previously developed a radiolabel assay for monitoring the synthesis of decaprenyl-pyrophosphoryl-linked intermediates of mycobacterial cell wall biosynthesis (28). In this study, we modified this assay system so that we could directly investigate the in vitro covalent attachment of AG intermediates to PG. Briefly, membranes and the P60 fraction from $\text{M. smegmatis}$ (both rich in endogenous cell wall biosynthetic activity) were combined with substrates in the form of purified peptidoglycan (PPG) and UDP-$[^{14}\text{C}]$Galp. The endogenous mutase activity within the P60 fraction of this assay mix (used to convert UDP-$[^{14}\text{C}]$Galp to UDP-$[^{14}\text{C}]$Galp) was sufficient to allow direct incorporation of $[^{14}\text{C}]$Galp into cell wall intermediates, as previously demonstrated (12, 28). PPG is a highly purified preparation of nascent $\text{M. smegmatis}$ PG free from AG covalently attached to the 6'-OH position of MurNAc via a phosphodiester bond (32). The purpose of PPG is both to provide a substrate for attachment of $^{14}\text{C}$-labeled linker unit-galactan intermediates and to serve as a "vehicle" for extraction from the assay mixture for subsequent radiochemical quantification. Fi-

**Figure Legend Continued**

monophosphate and undecaprenyl-1-monophosphate are in lanes 1 and 2, respectively. Lane 3 contains material extracted from $\text{MtbLcp1}$, while lane 4 contains a negative control of material extracted from $\text{EmbC}$ (21). Lipids were analyzed by TLC using silica gel plates (5735 silica gel 60F254; Merck) and developed in CHCl\textsubscript{3}-CH\textsubscript{3}OH-NH\textsubscript{4}OH-H\textsubscript{2}O (65:25:0.5:3.6, vol/vol/vol/vol), and plates were sprayed with 5% ethanolic molybdophosphoric acid and charred to visualize the lipids. (B) ES-MS analysis of decaprenyl-1-monophosphate standard (TLC, lane 1). (C) ES-MS analysis of undecaprenyl-1-monophosphate standard (TLC, lane 2). (D) ES-MS organic extracted material from $\text{MtbLcp1}$ (TLC, lane 3).
nally, the assay mixture was initiated by addition of highly purified recombinant \emph{Mtb}Lcp1. The analytical strategy for the products formed from this assay is illustrated as a flow chart in Fig. S4 in the supplemental material. Analysis of the fraction containing the CHCl\(_3\)-CH\(_3\)OH organic extraction by TLC reveals three major bands migrating toward the bottom of the TLC plate (Fig. 5A, lane 2). These bands correspond to \(^{14}\text{C}\)-labeled decaprenyl-P-P-GlcNAc-Rha-[\(^{14}\text{C}\)]Galf (GL-3) and decaprenyl-P-P-GlcNAc-Rha-[\(^{14}\text{C}\)]Galf\(_2\) (GL-4), with decaprenyl-P-P-GlcNAc-Rha-[\(^{14}\text{C}\)]galactan remaining at the origin of the TLC plate (12, 28). Interestingly, the intensity of each of these bands diminished in a titration-dependent response as the amount of \(^{14}\text{Mtb}\)Lcp1 increased in the assay mix (Fig. 5A, lanes 3 and 4), which was also quantified by densitometric analysis. When we repeated the assay at the highest \(^{14}\text{Mtb}\)Lcp1 concentration in the absence of purified PG (PPG), we observed a product profile on the TLC with band intensities almost identical to that of the control reaction (Fig. 5A, lane 5). These data clearly demonstrate that upon addition of \(^{14}\text{Mtb}\)Lcp1 to the assay mixture, the relative amount of \[^{14}\text{C}\]\text{Galf}-labeled material is reduced in the extracted organic fraction. We continued our analysis of the assay products by turning our attention to the insoluble fraction that contains PG and PG-linked \[^{14}\text{C}\]\text{Galf}-labeled material. Complete acid hydrolysis for the purpose of \[^{14}\text{C}\]sugar identification was conducted on the insoluble material (fraction 2) that contains nascent PG with newly synthesized AG material. Hydrolysates were then reduced and per-O-acetylated before analysis via silica TLC. Analysis of per-O-acetylated alditol acetates from this insoluble fraction revealed a major band that migrated to the position of per-O-acetylated galactitol (Fig. 5B, lane 2). Other bands that correspond to per-O-acetylated glucitol and per-O-acetylated N-acetyl glucitol were also identified (Fig. 5B, lane 2). However, we observed no statistically significant variation in the intensity of these bands across all samples and repeats and attribute this phenomenon to metabolism of UDP-[\(^{14}\text{C}\)]Gal into other sugars under these reaction conditions (Fig. 5B, lane 2), and this phenomenon has also been previously reported (12). Overall, this background activity \[^{14}\text{C}\]\text{Gal} incorporation can be attributed to endogenous \(^{14}\text{Mtb}\)Lcp1 present in the \emph{M. smegmatis} membrane preparation used in the assay mixture (Fig. 5B, lane 2). Due to the essentiality of \(^{14}\text{Mtb}\)Lcp1, we were unable to culture \emph{M. smegmatis} depleted of \(^{14}\text{Mtb}\)Lcp1 in order to derive membranes free from \(^{14}\text{Mtb}\)Lcp1 activity. Therefore, we adopted a strategy whereby we assayed for Lcp1 ligase activity over and above endogenous background activity (Fig. 5B, lane 2). The addition of recombinant \(^{14}\text{Mtb}\)Lcp1 to reaction mixtures resulted in a concomitant increase in band density that corresponds to per-O-acetylated galactitol (Fig. 5B, lanes 3 and 4), which is also quantified by densitometric analysis. However, in control reaction mixtures where PPG is removed from the assay mixture, we observed no significant incorporation of \[^{14}\text{C}\]\text{Galf}-labeled material, as manifested by the disappearance of a band corresponding to per-O-acetylated galactitol (Fig. 5B, lane 3). Taken together, these data clearly demonstrate two major findings. First, we are able to directly monitor the ligation of \[^{14}\text{C}\]\text{Galf} cell wall material to PG. Second, recombinant \(^{14}\text{Mtb}\)Lcp1 is capable of using decaprenyl-P-P-[\(^{14}\text{C}\)]Gal\(_2\) as a substrate for subsequent attachment to PG.

**DISCUSSION**

After conducting a bioinformatics analysis of organisms belonging to the \emph{Actinomycetales}, we identified \(^{14}\text{Mtb}\)Lcp1 (Rv3267) as the primary putative LCP homolog in \emph{M. tuberculosis}, sharing 20% identity with Cps2A from \emph{S. pneumoniae} (see Fig. S1 in the supplemental material). Since \emph{lcp1} is positioned immediately upstream of two genes involved in linker unit biosynthesis, in the form of \emph{wbbL1} and \emph{rmd0} (Fig. 1A), we decided to investigate the \emph{M. smegmatis} lcp1 ortholog (MSMEG_1824) as a model system to study the molecular genetics of this gene in order to assess its essentiality and to confirm its role as a primary LCP protein involved in mycobacterial cell wall assembly.

Mycobacterial AG is a highly branched heteropolysaccharide that serves to attach PG to the outer “mycomembrane,” and the biosynthesis of this essential cell wall component has been extensively studied (reviewed in reference 9). Since we have previously used \emph{M. smegmatis} to successfully study the molecular genetics of mycobacterial cell wall biosynthesis (22, 24), we attempted to generate a clean deletion of \(^{14}\text{Mtb}\)lcp1 from \emph{M. smegmatis}. However, due to the essential nature of \(^{14}\text{Mtb}\)lcp1 in attaching AG to PG in the cell envelope, we anticipated that a direct deletion of \(^{14}\text{Mtb}\)lcp1 would result in a nonviable, lethal strain, and our hypothesis was justified by our inability to generate a null mutant. The essentiality of \(^{14}\text{Mtb}\)lcp1 was confirmed when we performed a CESTET essentiality experiment (Fig. 3) (22). Generating null mutants of genes in mycobacteria that are involved in essential aspects of cell envelope biosynthesis is technically challenging and can be attributed to corresponding defects in the cell wall that produce nonviable phe-
(A) Chemical structure of the mycobacterial linker unit and novel chemical scaffolds used as ligands to probe the interaction of the linker unit with \textsuperscript{Mtb}Lcp1. (B) Saturation binding experiments using intrinsic tryptophan fluorescence of \textsuperscript{Mtb}Lcp1 to study binding of ligands 1 to 4. Data plotted represent the mean ± standard deviation from three independent biological replicate experiments.
notypes. The *M. smegmatis* ΔaffC mutant represents a strain of mycobacteria for which we have succeeded in generating a significant cell wall defect via genetic manipulation (24). Due to the complete loss of (1→3)-arabinosyltransferase activity, the *M. smegmatis* ΔaffC mutant produces a significantly altered AG but, crucially, still connects PG to the outer (albeit reduced) mycolate layer (24). Unsuccessful attempts by our laboratory to generate a direct knockout (KO) of any other gene(s) involved in cell wall biosynthesis upstream of this event are due to the formation of nonviable mutants which are completely unculturable. Given the essential nature of the mycobacterial cell wall toward cell viability, the generation of conditional mutants followed by phenotypic characterization via the CESTET procedure is an extremely useful tool to confirm gene essentiality. Furthermore, *M. smegmatis* encodes three additional Lcp orthologs in the forms of the products of MSMEG_0107, MSMEG_6421, and MSMEG_5775, none of which can complement the function of MSMEG_1824 KO. In *M. tuberculosis*, two additional Lcp orthologs are encoded by Rv3484 and Rv0822c. In this regard, the function of these remaining lcp genes requires further investigation.

The presence of a hydrophobic channel within the *Mtb*Lcp1 homology model is consistent with similar cavities found within LCP proteins from *B. subtilis* and *S. pneumoniae* (13, 15) (Fig. 1C). Indeed, biochemical analysis of recombinant *Mtb*Lcp1 clearly demonstrates that decaprenyl-1-monophosphate copurifies and is likely to be bound within the hydrophobic cavity of the protein (Fig. 2). In order to investigate the biochemical function of *Mtb*Lcp1, we chemically synthesized a panel of four ligands that represent chemical structures found within the mycobacterial AG proximal to the linker unit and used intrinsic ITF to study the ligand binding properties of these ligands against *Mtb*Lcp1. We observed a ligand binding relationship that reached saturation with a defined *B*max for each of the compounds 1 to 3, all of which display a binding affinity in the low-micromolar range. Compound 1 was synthesized to mimic the L-Rha-(1→3)-D-GlcNAc disaccharide moiety of the linker unit, and we recorded a *K*<sub>D</sub> of 58.71 μM. However, our binding data clearly demonstrate that the ligand with highest affinity for *Mtb*Lcp1 is the tetrasaccharide Galf₂-Rha-GlcNAc-O-C₈ (compound 2) with a *K*<sub>D</sub> of 5.13 μM. Interestingly, extension of this molecule with an additional Galf
residue (compound 3) caused a subtle 4-fold decrease in binding affinity (Fig. 4B). For technical reasons, we were unable to synthesize Gal\textsubscript{f}-Rha-GlcNAc-O\textsubscript{C8} and therefore were unable to study the binding of a ligand that represents the linker unit with only a single Gal\textsubscript{f} extension. In a control experiment, we investigated the ligand binding properties of Gal\textsubscript{f}-Rha-(1\rightarrow4)-L-Rhap (compound 4) toward \textsuperscript{Mtb}Lcp1. This compound displayed no recordable affinity toward the protein (over changes in background fluorescence), which suggests that the presence of D-GlcNAc in the linker unit is vital for biophysical association between protein and ligand. However, since \textsuperscript{Mtb}Lcp1 is able to bind both the linker unit (compound 1) and the linker unit with 2 to 3 Gal\textsubscript{f} residues (compounds 2 and 3, respectively), our data suggest that \textsuperscript{Mtb}Lcp1 might catalyze the phosphotransfer and ligation of AG to PG upon encountering a true physiological substrate that contains a decaprenyl-phosphate-linker unit with at least one (if not two) Gal\textsubscript{f} residue at the nonreducing end of the glycan chain (Fig. 6).

We extended our investigation toward the development of a cell-free functional assay to measure the ligation of AG to PG in the presence of \textsuperscript{Mtb}Lcp1. Chemical analysis of a reaction mixture that contained \textsuperscript{14}C-radiolabeled AG supplemented with nascent PG clearly shows that \textsuperscript{Mtb}Lcp1 is capable of ligating both macromolecules in a titratably dependent manner (Fig. 5). However, because this assay requires the use of mycobacterial membranes that contain endogenous AG-PG ligase activity, an alternative explanation is that recombinant \textsuperscript{Mtb}Lcp1 could indirectly activate this residual membrane activity. It has been previously reported that ligation of AG to PG in mycobacteria requires concomitant synthesis of both cell wall polymers (10, 12); however, until now, the enzyme responsible for this activity has not been reported. This study now provides compelling genetic and biochemical evidence that \textsuperscript{Mtb}Lcp1 is indeed the enzyme that reconciles two major mycobacterial cell wall biosynthetic pathways by forming a covalent phosphodiester between PG and AG (Fig. 6). The discovery of \textsuperscript{Mtb}Lcp1 sheds new light on a key reaction involved during late-stage cell wall assembly which is likely to be critical for all members of the \textit{Actinomycetales} harboring AG attached to PG via a phosphodiester linker unit.

In \textit{S. aureus}, the first two genes of the WTA pathway (\textit{tagO} and \textit{tagA}) are nonessential and can be deleted without retarding growth of both mutant strains (33, 34). However, deletion of any of the subsequent WTA-synthesizing enzymes (TagBDFGH or TarIJL) produces a lethal phenotype, unless each corresponding mutant also contains an inactivation in \textit{tagO} or \textit{tagA} (34). A proposed hypothesis to explain this synthetic viable phenotype relates to the limited availability of the bactoprenol lipid carrier in the cytoplasmic membrane, which is required for not only WTA biosynthesis but also lipid II biosynthesis, which ultimately leads to...
Lcp1 Ligates AG to PG in M. tuberculosis

PG assembly (34). Furthermore, similar effects have been demonstrated in S. aureus by chemical means, whereby tarcogin, an inhibitor of late-stage WTA biosynthesis, causes an accumulation of undecaprenyl-linked intermediates, thus preventing recycling of the undecaprenyl-phosphate lipid carrier, causing synthetic lethality (35). This phenomenon most likely reflects the phenotypic consequence of an \( \Delta^{\text{Ms}} \text{lcp1} \) deletion in M. smegmatis. Upon acetamide withdrawal from growing cultures of M. smegmatis \( \Delta^{\text{Ms}} \text{lcp1} \) pMV306-ACET-\( \Delta^{\text{Ms}} \text{lcp1} \), expression of \( \Delta^{\text{Ms}} \text{lcp1} \) diminishes, which will likely result in two major effects: (i) loss of AG-PG ligation activity and (ii) a concomitant increase in the decaprenyl-pyrophosphoryl-linked galactan intermediate(s). While the former effect induces a devastating cell wall lesion, the latter effect will cause a dramatic reduction in available decaprenyl monophosphate that is also required for PG biosynthesis, via loss of lipid II. Furthermore, Actinomycetales such as M. tuberculosis and C. glutamicum also use decaprenyl phosphate in the production of decaprenyl-phosphoarabinose (DPA) and decaprenyl-phosphomannose (DPM), which is directed toward the formation of \( \delta \)-arabinan and lipomannan (which is the precursor to lipoarabinomannan [LAM]), respectively (36, 37). Benzothiazinones (BTZs) are a class of compounds that are particularly potent inhibitors of mycobacterial growth. BTZs target DprE1, which is an oxireductuase involved in the epimerization of decaprenyl-phosphoribose (DPR) to decaprenyl-phosphoarabinose (DPA), thus arresting \( \delta \)-arabinan biosynthesis (38, 39). However, it has recently been shown that BTZ’s potent mode of action is attributed to the blocking of decaprenyl monophosphate recycling, a process which is required to allow cell wall biosynthesis to function properly (40). In this regard, Lcp1 is a potential drug target, since its inhibition will affect late-stage cell wall maturation and reduce the availability of decaprenyl monophosphate, leading to synthetic lethality.

MATERIALS AND METHODS

Homology modeling and sequence alignment. The amino acid sequence of M. tuberculosis Rv3267 (\( \text{Ms} \)\text{lcp1}, Swiss-Prot ID P96872_MYCTU) was submitted for three-dimensional (3D) structure prediction using the I-TASSER server (19, 20), and the conformation of the secondary structural features for the resultant \( \Delta^{\text{Ms}} \text{lcp1} \) model was predicted using pdbsum (41). The homology model of \( \Delta^{\text{Ms}} \text{lcp1} \) was analyzed in comparison to the Cps2A structures from S. pneumoniae (PDB ID 2XXP) (13).

Chemicals, reagents, and enzymes. All chemicals and solvents were from Sigma-Aldrich (Dorset, United Kingdom), Bio-Rad (CA, USA), and Fisher Chemicals (United Kingdom) unless otherwise stated and were of analytical grade or equivalent. Plasmids were propagated during cloning in E. coli Top10 cells (Invitrogen). All restriction enzymes and Phusion DNA polymerase enzymes were sourced from New England Biolabs. The Bioline quick ligation kit was used to perform ligation reactions. Oligonucleotides were from MWG Biotech Ltd., and PCR fragments were purified using the QIAquick gel extraction kit (Qiagen). Plasmid DNA was purified using the QIAprep purification kit (Qiagen). The preparation of oligosaccharides 1 to 3 using methods previously reported (42) is described in Text S1 in the supplemental material. Disaccharide 4 was obtained by digestion of oligosaccharides 1 to 3 using methods previously reported (42) is described in Text S1 in the supplemental material. Disaccharide 4 was subsequently sequenced to check for construct integrity (Eurofins MWG).

For generating an integrative vector containing MSMEG1824, the open reading frame (ORF) was amplified from M. smegmatis mc\( ^{155} \) genomic DNA using the primers MS1824LF (5’-GATCGATGCTGCTCGACGACTAC-3’) and MS1824RL (5’-GAGCGTGCGAGCGTCGCGCGC-3’). The DNA amplicon was digested with HindIII and EcoRV and subsequently cloned into pMV306 together with the \( \text{Pac}_{\text{etamidase}} \) promoter region from pSD26 as described previously (22), thus yielding pMV306-ACET-MSMEG1824 (pMV306-ACET-\( \Delta^{\text{Ms}} \text{lcp1} \)). The plasmid was subsequently sequenced to check for construct integrity (Eurofins MWG).

For generation of the \( \Delta^{\text{Ms}} \text{lcp1} \) plasmid, approximately 1-kb sequences of the upstream and downstream regions of MSMEG1824 were PCR amplified from M. smegmatis mc\( ^{155} \) genomic DNA with the primer pairs MS1824FL (5’-TTTTTTTTCTACATGACCCCCACCCGGGCGGCATGCCTCA-3’) and MS1824RL (5’-TTTTTTTTCTACATGACCCCCACCCGGGCGGCATGCCTCA-3’) and MS1824RL (5’-TTTTTTTTCTACATGACCCCCACCCGGGCGGCATGCCTCA-3’). The DNA amplicon was digested with HindIII and EcoRV and subsequently cloned into pMV306 together with the \( \text{Pac}_{\text{etamidase}} \) promoter region from pSD26 as described previously (22), thus yielding pMV306-ACET-MSMEG1824 (pMV306-ACET-\( \Delta^{\text{Ms}} \text{lcp1} \)).

Construction of the \( \Delta^{\text{Ms}} \text{lcp1} \) conditional mutant. The\( \Delta^{\text{Ms}} \text{lcp1} \) conditional mutant was generated using the CESTET procedure (22). Briefly, a merodiploid strain was first generated by introducing pMV306-ACET-\( \Delta^{\text{Ms}} \text{lcp1} \) by electroporation into M. smegmatis mc\( ^{155} \) (22). The merodiploid strain M. smegmatis mc\( ^{155} \)=pMV306-ACET-\( \Delta^{\text{Ms}} \text{lcp1} \) was then subjected to specialized transduction as previously described (22) using a temperature-sensitive, recombinant phage, \( \Phi \text{MSMEG1824} \), designed to replace MSMEG1824 with a hygromycin resistance marker. Transductants were selected at the nonpermissive temperature of 37°C on selective plates containing 25 \( \mu \)g/ml kanamycin, 100 \( \mu \)g/ml hygromycin B, and 0.2% (wt/vol) acetamide. Allelic exchange in hygromycin-resistant transductants was confirmed by PCR and Southern blotting.

Conditional deletion of the \( \Delta^{\text{Ms}} \text{lcp1} \) conditional mutant. The \( \Delta^{\text{Ms}} \text{lcp1} \) conditional mutant was grown in tryptic soy broth (TSB; Difco) containing 0.05% Tween 80, 25 \( \mu \)g/ml kanamycin, 100 \( \mu \)g/ml hygromycin B, and 0.2% (wt/vol) acetamide. Cultures were allowed to grow for a further 56 h at 37°C with shaking. Ten-microliter samples were taken at 8-hour intervals and plated on TBS agar plates supplemented with 25 \( \mu \)g/ml kanamycin and 100 \( \mu \)g/ml hygromycin B with or without 0.2% (wt/vol) acetamide to measure CFU.

Expression and purification of \( \Delta^{\text{Ms}} \text{lcp1} \). The plasmid pET28b-\( \Delta^{\text{Ms}} \text{lcp1} \) was transformed into E. coli BL21 (DE3) cells, spread onto LB agar plates containing 50 mg/ml Kan, and incubated at 37°C for 18 h. Single colonies were selected to inoculate 5 ml LB broth containing 50 \( \mu \)g/ml Kan and were incubated at 37°C overnight. The overnight cultures were then used to inoculate 1 liter Terrific Broth containing 50 mg/ml Kan, which was incubated (37°C, 180 rpm) until an optical density at 600 nm
(OD600) of 0.5 was reached. Protein production was then induced by the addition of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside; 12 h at 16°C). Cells were harvested via centrifugation at 6,000 × g for 15 min (4°C). Supernatant was discarded, and cells were washed in 30 ml phosphate-buffered saline (PBS). Pellets were stored at −20°C until further use. A pellet was defrosted and then resuspended in 30 ml lysis buffer (50 mM KH2PO4 [pH 7.9], 300 mM NaCl, 20 mM imidazole), including a proteinase inhibitor tablet (Roche). The mixture was subsequently sonicated using a Soniprep 150 ultrasonic disintegrator (MSE) (30 s on, 30 s off, 10 cycles). The solution was then centrifuged at 27,000 × g for 40 min (4°C), and the supernatant was retained (clarified lysate). Protein was precipitated using a Soniprep 150 ultrasonic disintegrator (MSE) (30 s on, 30 s off, 10 cycles). The solution was then centrifuged at 27,000 × g for 40 min (4°C), and the supernatant was retained (clarified lysate). Protein was loaded onto a HisTrap 5-ml chelating HP column (GE Healthcare), charged with 0.1 M NiCl2, equilibrated in lysis buffer, and eluted via a stepwise 50 to 500 mM imidazole concentration gradient (in lysis buffer). Fractions were analyzed by 12% SDS-PAGE to check for purity, and those fractions containing purified protein were dialyzed into dialysis buffer (25 mM Tris-HCl [pH 7.9], 10 mM NaCl, and 10% glycerol). Protein concentration was measured using a Bradford assay. The inclusion extract was resuspended in CHCl3-CH3OH (100 ml) and stored at −20°C. Material in fraction 1 was subjected to TLC using CHCl3-CH3OH (4:6, vol/vol) on aluminum-backed silica gel plates (5735 silica gel for 20 min and then washed repeatedly with methanol and stored at 20°C.) Functional biochemical assay for MtbLcp1. Membranes (containing membrane-bound enzymes involved in lipid-linked cell wall biosynthetic processes) and P60 (a Percoll-derived cell-free fraction rich in carbohydrates and enzymes associated with cell wall processes) and P60 fractions, respectively.

The basic assay mix consisted of buffer A, 0.5 mg membranes (33 μl), 0.5 mg of P60 (50 μl), 0.2 mM ATP, 0.2 mM NADH, 0.1 μCi of UDP-[14C]galactose (specific activity, 300 mCi/mmol; ARC Radioclemics), and 1 mg PPG; the reaction was initiated with the addition of 0.1 mg/ml MtbLcp1 (final volume of 200 μl); and the reaction mixture was incubated for 17 h at 37°C. Reactions were quenched by the addition of 1,333 μl of CHCl3-CH3OH (1:1, vol/vol). After mixing and centrifugation at 27,000 × g for 15 min at 4°C, the supernatant was removed and dried under nitrogen and the pellet was retained for further processing. The dried supernatant was resuspended into 2 ml CHCl3-H2O (1:1, vol/vol), and the lower organic phase (fraction 1) was removed and dried under nitrogen. Material in fraction 1 was subjected to TLC using CHCl3-CH3OH-H2O-NH4OH (65:25:3:60.5, vol/vol/vol/vol) on aluminum-backed silica gel plates (5735 silica gel 60F254; Merck), and products were visualized by autoradiography, exposing the TLCs to X-ray film (Kodak X-Omat). The pellet from the previous step was resuspended into 1 ml H2O-C6H4OH-(C6H5)2-pyridine-NH2OH (15:15:5:1:0.017, vol/vol/vol/vol/vol) and left at room temperature to be extracted for 2 h. Samples were again centrifuged at 27,000 × g for 15 min, and supernatant was discarded. The remaining pellet (fraction 2) was resuspended in 200 μl H2O. All fractions were quantified by liquid scintillation counting using 10% of the labeled material and 5 ml of EcoScintA (National Diagnostics, Atlanta, GA).

Material in fraction 2 was hydrolyzed in 2 M trifluoroacetic acid (TFA) and reduced with NaBH4, and the resultant alditols were per-O-acetylated as described previously (47). The resulting 14C-labeled per-O-acetylated alditol acetate derivatives were analyzed by TLC using ethyl acetate-hexane (4:6, vol/vol) on aluminum-backed silica gel plates (5735 silica gel 60F254; Merck), and products were visualized by autoradiography, exposing the TLCs to X-ray film (Kodak X-Omat) and comparing them to known standards. All experiments were performed in triplicate as biological replicates.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl?doi=10.1128/mBio.00972-16/-/DSupplemental.

Text S1, PDF file, 0.2 MB.
Figure S1, PDF file, 2.6 MB.
Figure S2, PDF file, 0.2 MB.
Figure S3, PDF file, 0.8 MB.
Figure S4, PDF file, 0.4 MB.

ACKNOWLEDGMENTS

J.H. is a Medical Research Council Quota Student. T.L.L. and M.I. acknowledge support from the Alberta Glycomics Centre and the Natural Sciences and Engineering Research Council of Canada. G.S.B. acknowl-
edges support in the form of a Personal Research Chair from James Bardrick, Royal Society Wolfson Research Merit Award, as a former Lister Institute-Jenner Research Fellow. We acknowledge funding from the MRC (grant G1100127). The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.


**FUNDING INFORMATION**

This work was funded by Medical Research Council (MRC) (G1100127, and MR/K012118/1).


