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Mycobacteriophage Lysin B is a novel mycolylarabinogalactan esterase

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Abstract

Mycobacteriophages encounter a unique problem among phages of Gram-positive bacteria, in that lysis must not only degrade the peptidoglycan layer but must also circumvent a mycolic acid-rich outer membrane covalently attached to the arabinogalactan-peptidoglycan complex. Mycobacteriophages accomplish this by producing two lysis enzymes, Lysin A that hydrolyzes peptidoglycan, and Lysin B, a novel mycolylarabinogalactan esterase, that cleaves the mycolylarabinogalactan bond to release free mycolic acids. The D29 LysB structure shows a α/β hydrolase organization with a catalytic triad common to cutinases, but which contains an additional four-helix domain implicated in the binding of lipid substrates. Whereas LysA is essential for mycobacterial lysis, a Giles Δ lysB mutant mycobacteriophage is viable, but defective in the normal timing, progression, and completion of host cell lysis. We propose that LysB facilitates lysis by compromising the integrity of the mycobacterial outer membrane linkage to the arabinogalactan-peptidoglycan layer.

INTRODUCTION

Upon completion of lytic bacteriophage growth the integrity of the host cell wall must be compromised to release progeny phage particles (Wang *et al.*, 2000, Young *et al.*, 2000). Double-stranded DNA (dsDNA) tailed phages typically encode an endolysin that degrades the peptidoglycan mesh, together with a means of allowing this enzyme access to its substrate, either *via* a membrane-localized holin, or through the action of holin-independent SAR-endolysins (Xu *et al.*, 2005, Wang *et al.*, 2000, Young *et al.*, 2000). For phages infecting Gram-positive hosts, not only are these two requirements sufficient for cell lysis (Loessner *et al.*, 1998), but exogenously added endolysin can efficiently kill host cells, with considerable therapeutic potential (Fischetti, 2008, Loeffler *et al.*, 2001, Schuch *et al.*, 2002). In Gram-negative hosts, the outer membrane presents a further barrier to lysis, and phages typically encode an additional set of lysis proteins (Rz/Rz1 proteins or spanin equivalents) that are proposed to complete lysis by fusing the inner and outer membranes (Berry *et al.*, 2008, Summer *et al.*, 2007).

Although the mycobacteria are members of the Gram-positive *Actinomycetales*, they are unusual in possessing a mycolic acid rich outer membrane that is covalently attached to the arabinogalactan-peptidoglycan complex (Hoffmann *et al.*, 2008, Zuber *et al.*, 2008). Mycolic acids are α -alkyl, β -hydroxy C₆₀₋₉₀ fatty acids with a relatively short (C₂₀₋₂₅) saturated α and a longer (C₆₀) meromycolate chain (the β -hydroxy branch) containing

Accession code: The atomic coordinate and structure factor have been deposited in the PDB with accession code 3HC7 for D29 LysB.

double bonds, cyclopropane rings, and oxygenated groups, depending on the *Mycobacterium* species (Hamid *et al.*, 1993, Watanabe *et al.*, 2001). The outer membrane mycolic acids are linked to the cell wall *via* an ester linkage to the terminal pentaarabinofuranosyl components of arabinogalactan (Hoffmann *et al.*, 2008, Brennan & Nikaido, 1995, Niederweis, 2008, McNeil *et al.*, 1991), which in turn is covalently anchored to peptidoglycan (Brennan & Nikaido, 1995, McNeil *et al.*, 1990). In addition to being part of the mycobacterial outer membrane, mycolic acids are constituents of trehalose dimycolate (TDM, also known as cord factor), a secreted molecule important for mycobacterial pathogenesis (Brennan, 2003). Mycolic acid synthesis is essential for viability and is the target of the major anti-tuberculosis drug isoniazid (Vilcheze & Jacobs, 2007). The mycobacterial outer membrane also plays roles in nutrient acquisition (Niederweis, 2008) and as an immune target (Karakousis *et al.*, 2004). Furthermore, it presents a potential barrier to phage-mediated lysis.

A total of 60 mycobacteriophage genomes have been sequenced (Pedulla *et al.*, 2003, Hatfull *et al.*, 2006, Morris *et al.*, 2008, Pham *et al.*, 2007) (our unpublished observations), all of which encode a putative endolysin, Lysin A (LysA) (Garcia *et al.*, 2002, Hatfull *et al.*, 2006). The LysA proteins are highly diverse in sequence and modular in nature (Hatfull *et al.*, 2006), containing combinations of amidase, glycosidase and peptidase motifs. The LysA of phage Ms6 has been shown to have mureinolytic activity in *E. coli* (Garcia *et al.*, 2002) and the LysA of mycobacteriophage Giles is essential for lytic growth (Marinelli *et al.*, 2008). Many of these genomes have a putative holin gene, which is closely linked to *lysA*, whereas in others there is no closely linked and easily recognizable holin gene. Holin function may be encoded from elsewhere as in phage T7 (Hausmann, 1988) or from within another open reading frame as proposed for phage 187 (Loessner *et al.*, 1999). Mycobacteriophage endolysins could contain SAR (signal-anchor-release) domains that allow secretion of the endolysin into the periplasmic space thus rendering them holin-independent (Xu *et al.*, 2005), but none have yet been described. Some mycobacteriophages also encode a second proposed lysis protein, Lysin B (LysB) (Garcia *et al.*, 2002), which does not resemble endolysins and has no identifiable homologs in bacteriophages other than those that infect mycobacterial hosts. The LysB of Ms6 has been shown to be a lipolytic enzyme (Gil *et al.*, 2008) and contains a G-X-S-X-G motif characteristic of serine esterases (Carvalho *et al.*, 1999, Gil *et al.*, 2008, Gupta *et al.*, 2004, Longhi & Cambillau, 1999, Ollis *et al.*, 1992) although its specific substrate is unknown.

We show here that LysB of mycobacteriophage D29 is a novel mycolylarabinogalactan esterase that cleaves the ester linkage joining the mycolic acid-rich outer membrane to arabinogalactan, releasing free mycolic acids. We propose that LysB acts at a late stage in lysis, severing the connection of the mycobacterial outer membrane to the cell wall and completing lysis of the host.

RESULTS

Organization, conservation, and location of mycobacteriophage lysis cassettes

Comparative analysis of mycobacteriophage genomes reveals them to be highly diverse with mosaic architectures (Hatfull *et al.*, 2006, Pedulla *et al.*, 2003). The 6,858 predicted protein products can be assorted into 1,523 phamilies (Phams) of related sequences and their distributions among the mycobacteriophages analyzed (Hatfull *et al.*, 2006) (unpublished observations). While there are few Phams represented in all 60 completely sequenced genomes, one of these is the *lysA* phamily (Pham 66), and the LysA proteins are predicted from sequence comparisons to have peptidoglycan hydrolyzing activity (Hatfull *et al.*, 2006, Garcia *et al.*, 2002, Marinelli *et al.*, 2008). We have confirmed this for three LysA proteins (Corndog gp69, Bxz1 gp236, Che8 gp32) all of which catalyze peptidoglycan hydrolysis in

zymograms (Fig. 1A). The *lysB* gene which located downstream of *lysA*, is also implicated in lysis, primarily because of its linkage to *lysA* (Fig. 1B) and the demonstration of lipolytic activity by Ms6 LysB (Gil et al., 2008). LysB homologs are present in 56 of the 60 completely sequenced mycobacteriophage genomes and are located downstream of *lysA* and separated from it by no more than 4 intervening genes (Fig. 1B). Some of the intervening genes encode putative holins and exhibit holin-like function (e.g. D29 gene 11, data not shown), whereas others (e.g. Omega gene 51) code for putative homing endonuclease HNH motifs (Fig. 1B), or have homologs elsewhere in other mycobacteriophage genomes (e.g. Troll4 gene 37).

Although *lysA* and *lysB* are closely linked, this linkage is not a simple consequence of synteny in the context of the broader genome organizations, because these presumptive lysis cassettes are situated in different chromosomal locations (Fig. 1C). Of the 53 phages with a siphoviral morphotype, the thirteen genomes constituting Cluster A [including L5, D29, Bxb1 (Hatfull & Sarkis, 1993, Ford et al., 1998, Mediavilla et al., 2000) and Che12 (Hatfull et al., 2006)] have the lysis cassette situated to the left of the virion structural and assembly genes, whereas all of the other genomes have the lysis cassette positioned to the right of the structural genes (Fig. 1C). The invariable linkage of *lysA* and *lysB* regardless of genome location further supports a role for LysB in lysis.

Sequence alignment of the phamily of LysB proteins shows that they are highly diverse, and only three residues are completely conserved (Fig. S1). Although the proteins vary in length [from 254 (D29 gp12) to 451 (PG1 gp50) residues] and there are many gaps throughout the alignment, these proteins do not have modular constructions as seen in the LysA proteins (Hatfull et al., 2006). However, the sequence divergence is sufficiently high that some members of the phamily have little identifiable sequence similarity (<20% identity) to each other (Figs. S1, S2). In a search for conserved domains, 14 LysB proteins (including D29 gp12) are predicted to contain a cutinase domain (pfam01083), and Wildcat gp52 has an esterase/lipase domain (pfam c109107). Eight proteins have a predicted peptidoglycan-binding domain (pfam01471) at their N-terminus, although this region is not present in D29 LysB (Fig. S1).

The three absolutely conserved residues include the putative active site serine (position 82 in D29 LysB) and a GXP motif located ~40 residues downstream (residues 117-119 in D29 LysB; Fig. S1). The cutinase- and lipase-like conserved domains suggests that these enzymes are serine esterases, which typically have a Ser-Asp-His catalytic triad. Of three aspartic acids and a histidine residue previously implicated in catalysis through their conservation (Gil et al., 2008), only the aspartic acid corresponding to position 166 in D29 LysB is highly conserved among the larger group of proteins, with the single departure being the LysB protein encoded by a putative *Mycobacterium avium* prophage that has a glutamic acid residue at that position (Fig. S1). The alignment does not reveal a well-conserved candidate for the histidine component of the catalytic triad (Fig. S1). The GXP motif is not absolutely conserved in all serine esterases and its role in LysB functions is not clear.

D29 LysB has lipolytic activity

To further characterize the structure and function of mycobacteriophage LysB proteins, several Pham73 members were cloned and expressed. Although expression and solubility varies among these, we found that the 254-residue D29 gp12 (LysB) expressed well and was readily purified to near homogeneity and high solubility (>10mg/ml) (Fig. 2A). D29 LysB shares only 40% amino acid sequence identity with the previously characterized Ms6 LysB protein (Gil et al., 2008), and lacks 90 N-terminal residues present in Ms6 LysB (Fig. S1).

We also constructed, expressed and purified a mutant version of the protein with a substitution of alanine for the putative catalytic serine at position 82 (S82A).

To test for lipolytic activity of D29 LysB we measured hydrolysis of *p*-nitrophenyl butyrate (*p*NPB) to release *p*-nitrophenol (Gilham & Lehner, 2005) (Fig. 2B). We observed a specific activity of 0.72 U/mg (Fig. 2B), somewhat higher than the 0.12U/mg reported for Ms6 LysB protein (Gil *et al.*, 2008), or for any of the seven cutinase-like proteins found in *M. tuberculosis* (West *et al.*, 2009). The S82A mutant is inactive, consistent with this residue being part of the catalytic triad (Fig. 2B). We also tested D29 LysB, as well as the purified lipase from *Pseudomonas fluorescens* for activity on *p*-nitrophenyl substrates with different carbon chain lengths. While D29 LysB was more active than the lipase on *p*NPB, we also observed decreasing activity of D29 LysB with longer substrates (Fig. 2B), as was reported for Ms6 LysB (Gil *et al.*, 2008). D29 LysB thus shares lipolytic activity with Ms6 LysB (Gil *et al.*, 2008), with serine-82 providing critical catalytic functions.

Crystal structure of D29 LysB

The structure of D29 LysB was determined at 2.0-Å resolution in crystals of the P 43 21 2 space group containing a single molecule in the asymmetric unit (Table 1). Structural alignment with known protein structures using Dali showed that D29 LysB is similar to members of the α/β hydrolase family, which includes cutinases, acetyl xylan esterases and lipases (Holm *et al.*, 2008, Masaki *et al.*, 2005). The five closest structural relatives of D29 LysB are all members of the cutinase family (Table 2), although they share no more than 21% amino acid sequence identity with D29 LysB.

Of the 253 determined residues in D29 LysB, 170 residues (Ser2-Tyr161, Arg244-Gln253) are distributed in a doubly wound α/β sandwich, having a central parallel β -sheet flanked by two parallel α -helices on each side (Fig. 3A). This fold is typical of the α/β hydrolase family and the structure of Lysin B is remarkably similar to that of a *Cryptococcus* cutinase-like protein (Fig. 3A). Despite their low sequence similarity, the rms differences in α -helix

positions are 2.45 Å and a search using the Dali server gave a high Z-score (20.3) when LysB was submitted as the query. The remainder of the protein (Ala162-Asn243) forms an 81-residue linker domain composed of four α -helices connecting the C-terminal α -helix (Arg244-Gln253) back to the side of the central β -sheet. It occupies the general location where the acyl chain is positioned in co-crystals of the *Fusarium solani* cutinase with a covalently-linked n-undecanoylphosphonate methyl ester inhibitor (Longhi *et al.*, 1996). Given the high B-factor and poor electron-density of the Arg231-Lys237 loop connecting the helical-linker and the C-terminal helix, it is reasonable to propose that movement of the helical-linker helps to modulate the active site to accommodate the bulky and hydrophobic acyl chains from mycolic acid. We note that the unusual glycine-rich segment corresponding to a 55-amino acid segment of PG1 LysB (and related proteins) inserted between D29 LysB residues 199 and 200, of which 19 are glycine residues, is located within this linker between the second and third helices (Fig. 3A; S1).

The catalytic triad Ser82-Asp166-His240 is located at the edge of the central β -sheet between the α/β sandwich and the linker domain. The position of the D29 LysB catalytic triad is very similar to those in other members of the α/β hydrolase family as can be seen in the superimposition of D29 LysB on the *Cryptococcus* cutinase-like protein (Fig. 3B). Although Ser-82 is invariant and Asp-166 is very strongly conserved in the alignment of LysB proteins (Fig. S1), the sequence alignment around His-240 varies among the LysB members, reflecting poor conservation of the flanking residues and positioning of the catalytic histidine within a loop region (Fig. S1). In contrast, the GXP motif — of which the glycine and proline residues are invariant — lies immediately adjacent to the catalytic triad.

In D29 LysB, the Gly117-Asn118-Pro119 segment defines the end of β 4 strand and makes a 180 degree turn just beneath Asp166. Besides the turn-makers Gly117 and Pro119, Asn118 forms hydrogen bonds with Met120, Arg121 and Asp160, which may be energetically favorable to the scaffold.

D29 LysB is a mycolylarabinogalactan esterase

The D29 LysB structure confirms its enzymatic function as a serine esterase, and while it clearly has lipolytic activity, the unusual linker positioned adjacent to the active site — together with the inclusion of *lysB* genes within lysis cassettes — suggests that its substrate could be a cell wall component containing ester-linked lipids. We reasoned that an excellent candidate substrate is mycolyl-arabinogalactan—peptidoglycan (mAGP), such that LysB hydrolysis would release free mycolic acids and separate the outer membrane from the arabinogalactan-peptidoglycan layer. To test this we isolated a cell wall fraction from *M. smegmatis* that is highly enriched in mAGP (Besra, 1998) and determined whether it is a substrate for D29 LysB hydrolysis (Fig. 4A, B). We observed both time- and enzyme concentration-dependent release of lipids that migrate similarly to mycolic acids released from the same mAGP preparation by alkaline hydrolysis of mAGP with tetrabutylammonium (TBAH) (Hamid et al., 1993, Watanabe et al., 2001, Besra, 1998) (Fig. 4A, B). To further characterize the lipid products we methyl-esterified them with iodomethane, which yielded methyl esters of α , α' and epoxy mycolates similar to those from TBAH treatment and specific to *M. smegmatis* (Fig. 4C). Preliminary analysis of the released lipids by mass spectrometry and NMR was consistent with them being free mycolic acids (data not shown). To our knowledge, this is the first mycolylarabinogalactan esterase to be described. As expected, hydrolysis of mAGP by D29 LysB is dependent on the catalytic serine residue at position 82 and the S82A mutant enzyme exhibits no observable activity (Fig. 4D). Moreover, mAGP was not a substrate for a *Pseudomonas*-derived lipase (Arpigny & Jaeger, 1999, Gupta et al., 2004) as no release of free mycolic acids was observed (Fig. 4D).

Giles *lysB* facilitates completion of host cell lysis

To explore the role of LysB in mycobacteriophage lytic growth, we first asked whether it is an essential function for plaque formation, using a recently described recombineering strategy to delete *lysB* (gene 32) from the mycobacteriophage Giles genome (Marinelli et al., 2008). We targeted Giles because it is a demonstrated substrate for recombineering (Marinelli et al., 2008), we know that Giles *lysA* is essential (Marinelli et al., 2008), and we anticipated needing to complement with a *lysB* gene that is sufficiently different genetically as to avoid recombination (i.e. D29 gene 12). It should be noted however that Giles LysB—cloned and purified in the same manner as D29 LysB—was also able to hydrolyze mAGP (data not shown). A 200 bp substrate containing 100 bp sequences flanking *lysB* was designed to introduce a 1,146 bp internal deletion in Giles *lysB*, fusing 15 codons at the 5' and 3' ends of the gene to minimize effects on expression of adjacent genes as well as avoiding genetic polarity (Fig. 5A). Following co-electroporation of the 200 bp deletion substrate and Giles genomic DNA into an *M. smegmatis* recombineering strain, plaques were recovered on a lawn of *M. smegmatis* mc²155pMKC2 cells expressing D29 LysB. Of 22 primary plaques screened by Deletion Amplification Detection Assay (DADA) PCR (Marinelli et al., 2008), two contained mixtures of wild-type and mutant phages (Fig. 5B).

To test for viability of the Giles Δ *lysB* mutant, a mixed plaque was picked, resuspended in buffer, and plated to recover ~600 isolated plaques on both a putative complementing strain (*M. smegmatis* mc²155pMKC2) or a non-complementing control strain. Secondary lysates representing all of the recovered particles were harvested and tested by PCR for the presence of the deletion mutant (data not shown). The mutant genotype was present in approximately

equivalent proportions in both the complementing and non-complementing strains, suggesting that the mutant is viable. Thirty individual plaques from a secondary plating were then screened by PCR and a homogenous mutant plaque identified (Fig. 5C). The Giles *ΔlysB* mutant was plaque purified, and the structure of the deletion confirmed by DNA sequencing (data not shown).

The Giles *ΔlysB* mutant forms plaques at equivalent efficiencies on complementing and non-complementing strains, and titers of wild-type and mutant lysates prepared on a wild-type *M. smegmatis* host under standard conditions are similar; Giles *lysB* thus is not essential for lytic growth (Fig. 6A). However, we observed that the mutant forms somewhat smaller plaques on lawns of wild-type *M. smegmatis* compared to the parental Giles, a phenotype that is exaggerated when higher densities of plating cells are used (conditions that favor fewer bacterial doublings and fewer rounds of phage infection) (Fig. 6A). This difference is directly attributable to the loss of *lysB* since complementation with a plasmid expressing D29 *lysB* restores plaque size to near that of the wild-type parent (Fig. 6A). To eliminate the possibility that plaque size restoration results from acquisition of *lysB* by recombination with the complementing plasmid, several plaques were picked and shown to reproduce the mutant phenotype, and by PCR contain only the mutant genotype (data not shown). Furthermore, at a relatively high cell plating density (2×10^8 cfu/plate) the average number of particles in each plaque is ~100-fold reduced in *ΔlysB* mutant relative to wild-type Giles plaques (5×10^5 and 4×10^7 pfu/ml respectively), consistent with a lysis defect. Therefore, unlike *lysA*, *lysB* is not essential for plaque formation but is required for efficient phage release.

To test whether the small plaque phenotype specifically results from changes in the pattern or timing of lysis, we monitored optical densities (OD) of infected *M. smegmatis* cultures (Fig. 6B). After infection with wild-type Giles the OD increases for approximately 3 hours, after which it steadily declines as the cells lyse, up to a period approximately 5 hours after infection. When infected with the *ΔlysB* mutant, the OD does not begin to decline until 3.5 hours and is incomplete even 5.5 hours after infection (Fig. 6B). In contrast, cells infected with a *ΔlysA* mutant — which does not form visible plaques on a non-complementing *M. smegmatis* strain (Marinelli *et al.*, 2008) — cease to grow after 3-3.5 hours, with only a modest reduction in OD thereafter (Fig. 6B).

We also monitored the progression of phage infections by measuring ATP release (Fig. 6C), which we anticipated to reflect either lysis or permeabilization of the cell wall. In a wild-type Giles infection, little or no ATP release is seen until three hours after infection, followed by a steady increase to 4.5-5 hours after infection (Fig. 6C), closely mirroring the changes in OD shown in Fig. 6B. The *ΔlysB* mutant is delayed in the onset of ATP release by about 30 minutes, and fails to achieve the wild-type level even 5.5 hours after infection, which taken together with the OD changes is consistent with a lysis defect. The phenotype is reminiscent of the conditional lysis defect of phage lambda Rz or Rz1 mutants, where the Rz/Rz1 proteins are implicated in the final lysis step in which the *E. coli* outer membrane is compromised by fusion with the cytoplasmic membrane (Summer *et al.*, 2007, Berry *et al.*, 2008). Interestingly, the *ΔlysA* mutant shows no defect in ATP release at all, and may even release more ATP than cells infected with wild-type Giles (Fig. 6C).

Finally, we tested whether the *lysB* mutant produces the same yield of total phage particles as wild-type Giles in a lytic infection, and determined how the particles distribute between those that are released and those that remain trapped in unlysed cells (Fig 6D, E). As shown in Fig. 6D, both the *ΔlysA* and *ΔlysB* mutants show no major defect in the production of phage particles. However, by 4 hours after infection, although >90% of wild-type particles are present in the culture supernatant, about 45% of the *ΔlysB* particles remain associated

with unlysed cells. In contrast, <10% of Δ lysA particles are released into the supernatant even 5 hours after infection (Fig. 6E). These observations are consistent with a strong defect in cell lysis in the Δ lysA mutant, and a milder defect in the *lysB* mutant.

DISCUSSION

We have shown here that the mycobacteriophage D29 LysB protein is a novel mycolylarabinogalactan esterase required for completion of lysis of host mycobacterial cells, and a model for the role of the Lysin A and Lysin B proteins as well as the reaction catalyzed by LysB is presented in Figure 7. The mycolic acid rich mycobacterial outer membrane presents an unusual problem for phages of Gram-positive bacteria, which typically complete lysis through simple endolysin-mediated degradation of the peptidoglycan layer. The mycolylarabinogalactan linkage is not common among bacteria, since mycolic acids are found primarily in the *Corynebacterineae* suborder of the *Actinomycetales*, which includes *Corynebacteria*, *Gordonia*, *Nocardia*, *Rhodococci* and *Mycobacteria*. Few phages infecting non-mycobacterial members of the *Corynebacterineae* have been characterized, although these would be good candidates for also encoding mycolylarabinogalactan esterases. We note that neither phage P2101 of *Corynebacterium glutamicum* (Chen *et al.*, 2008) nor BFK20 of *Brevibacterium flavum* (Bukovska *et al.*, 2006) encode a LysB relative, although unlike the *Mycobacteria*, mycolic acids are dispensable for growth of *C. glutamicum* (Portevin *et al.*, 2004) and therefore may not pose a barrier to efficient lysis.

While removal of LysB function in mycobacterial Giles results in an apparently mild plaque phenotype that can be rescued by expression of D29 LysB, the problems in lysis are made more apparent by measuring changes in optical density of the culture, ATP release, and phage particle release. The ATP release by the Giles Δ lysA mutant is in contrast to its lysis defect as reflected in optical density measurements. A simple explanation for this observation is that the peptidoglycan layer — which most probably remains intact in the Δ lysA mutant infection — provides no barrier to ATP release at all, whereas both the cytoplasmic membrane and the mycobacterial outer membrane must be compromised for complete ATP release. Presumably, the cytoplasmic membrane is permeabilized through the action of a Giles holin (which has yet to be identified), and the differences observed between the Δ lysA and Δ lysB mutants are consistent with an attached mycobacterial outer membrane providing a significant barrier to ATP release. While it is difficult to eliminate the possibility of secondary effects of the deletion mutation in the Δ lysB mutant on expression of other phage genes (including a putative holin), the complementation studies (Fig. 6A) are consistent with loss of LysB function as the primary cause of the phenotypes observed.

At this time it is unknown how LysB is localized to its substrate, and no signal peptide motifs have been identified (Gil *et al.*, 2008). It would be interesting to see if LysB activity is dependent on a mycobacteriophage holin, similar to endolysins of other bacteriophage and presumably the LysA proteins of mycobacteriophage. However, few mycobacteriophage holins have been predicted bioinformatically, and even fewer functional studies have been conducted; Ms6 gp4, a predicted holin, was lethal when over-expressed in *E. coli* (Garcia *et al.*, 2002), and experiments in our lab indicate a potential holin function for D29 gp11 (L. Marinelli and GFH; unpublished observations).

The question arises as to whether phages Che12, Rosebush, Qyrzula and Myrna that lack *lysB* encode alternative enzymes performing analogous functions. These phages do not form a closely related group (Fig. S2), although comparison of each with their closer phage relatives is informative. For example, in Che12 the surrounding genes are syntenic with those in phages L5, D29, Pukovnik and Bxz2 and it appears as though Che12 has simply lost

lysB (Fig. S3A). The organizations in Rosebush and Qyrzula depart from their close relatives in the inclusion of a gene immediately downstream of *lysA* that is lacking in other Cluster B phages, all of which do encode LysB (Fig. S3B). This predicted protein is small (178aa) and is a distant relative of the D29 putative holin (gp11). In Myrna, neither of the two orfs (244, 245) immediately downstream of *lysA* are related to any other mycobacteriophage proteins (Fig. S3C). Myrna gp244 does have similarity to the N-terminal segment of *Rhodococcus* protein (RHA1_ro08121) that contains both peptidase and muramidase motifs in its C-terminus, and Myrna gp244 may play a yet undefined role in lysis. We also note that although *Propionibacterium acnes* phage PA6 (Farrar *et al.*, 2007) and *Streptomyces* phage R4 (unpublished data) encode proteins with sequence similarity to mycobacteriophage LysA proteins, neither encodes a LysB relative. LysB thus appears to be restricted to those phages infecting hosts with mycolic acid outer membranes.

The release of free mycolic acids from LysB-treated cell walls strongly suggests that mAGP is the substrate for the enzyme. The cell wall preparations may contain additional lipid components, but other predominant mycolic acid-containing constituents — such as trehalose dimycolate (TDM; cord factor) and trehalose monomycolate (TMM) — are not cell wall linked and are not major components of the mAGP preparations (Brennan & Nikaido, 1995, Besra, 1998). Preliminary data suggest that D29 LysB can also hydrolyze TDM (A. Ojha, KP and GFH, unpublished observations), but it seems unlikely that this has physiological relevance for lysis because TDM is not covalently attached to mycobacterial cells. We propose that cleavage of the mycobacterial outer membrane from the peptidoglycan-arabinogalactan layer is the primary role of LysB.

The only known physiological circumstances in which free mycolic acids are released from mycobacterial cells is during maturation of *M. tuberculosis* biofilms (Ojha *et al.*, 2008), although the source of biofilm mycolic acids is more likely to be TDM than mAGP (Anil Ojha and GFH, unpublished observations). While seven cutinase-like proteins (culps) encoded by *M. tuberculosis* have been expressed and characterized (West *et al.*, 2009), they are not evidently related to LysB and have not yet been tested for mAGP hydrolysis. Culp1 and Culp4 have optimal enzyme activity on short (butyrate) *p*-nitrophenyl substrates, and only Culp6 has significant activity on longer carbon chain substrates (West *et al.*, 2009). Also, the Culp4 homolog in *M. smegmatis* has been shown to have phospholipase A activity, which is not found in cutinases (Parker *et al.*, 2007). It remains to be seen whether there are any host-encoded enzymes that share mycolylarabinogalactan esterase activity with LysB.

The D29 LysB structure shows it is a member of the α/β hydrolase family with an α/β sandwich and an active site containing Ser, Asp, and His. The five closest related structures are all cutinases, although there is no greater than 21% amino acid sequence identity with any of them. The LysB catalytic mechanism is expected to be similar to that for other serine esterases, although the strong conservation of the GXP motif and its juxtaposition to the active site may play a critical role in its stabilization. However, the D29 LysB also contains an unusual four-helix domain that projects from close to the active site and is lacking in other cutinase-like protein structures. While the specific role of the large linker is not known, its absence in all other cutinase-like proteins and lipases and its proximity to the active site would suggest a role in binding of the D29 LysB substrate. This linker contributes to the generally larger size of LysB proteins (254-451 residues) compared to other cutinases (~225 residues) (Carvalho *et al.*, 1999, Longhi & Cambillau, 1999).

Acquisition of *lysB* by mycobacteriophages throughout their evolution likely confers a substantial selective advantage over those without it by providing faster and more complete lysis. While considered more similar to Gram-positive bacteria, the existence of the mycobacterial outer membrane composed of mycolic acids and free lipids (Fig. 7A) presents

a second barrier analogous to the outer membrane of Gram-negative bacteria (Hoffmann et al., 2008, Zuber et al., 2008). Phages that infect Gram-negative bacteria have the help of spanin proteins and their Rz/Rz1 counterparts (Berry et al., 2008, Summer et al., 2007, Krupovic et al., 2008) to span the periplasmic space and link the inner and outer membrane, enhancing the completion of lysis. We propose that mycobacteriophage have developed an alternative solution to compromise the mycobacterial outer membrane by severing its linkage to the underlying arabinogalactan-peptidoglycan layer.

Exogenously applied phage-encoded endolysins have been shown to have effective antimicrobial activity against Gram-positive bacterial pathogens including *Streptococcus pneumoniae* and *Bacillus anthracis* (Fischetti, 2008). They are, however, ineffective against Gram-negative bacteria since the outer membrane blocks access to the peptidoglycan targets. The mycobacteria are likely to be similarly intractable to exogenously added endolysins because of their mycolic acid rich outer membrane. While we do not yet know whether mycobacteriophage LysB proteins can reach their mycolylarabinogalactan targets from the outside, this possibility raises the intriguing idea that mycobacterial pathogens such as *M. tuberculosis* may be rendered susceptible to endolysin treatment through co-treatment with LysA and LysB proteins.

METHODS

Bacterial Strains and Growth

M. smegmatis mc²155 (Snapper et al., 1990) and the recombineering strain containing pJV53 (van Kessel & Hatfull, 2007) expressing the Che9c genes 60 and 61 under an acetamide-inducible promoter (Parish et al., 1997) have been described previously. All *M. smegmatis* strains were cultured in Middlebrook 7H9 medium or grown on Middlebrook 7H10 agar supplemented with 10% Albumin Dextrose Complex (ADC), 0.2% succinate, 0.05% Tween-80, 1 mM Ca₂Cl, carbenicillin (50 µg/ml), cyclohexamide (10 µg/ml), and kanamycin (20 µg/ml) as required. *E. coli* was grown in LB supplemented with carbenicillin (50 µg/ml) and kanamycin (20 µg/ml) as needed, with *E. coli* GC5 cells (Stratagene) used for cloning and *E. coli* BL21(DE3) cells (Stratagene) for protein overexpression.

Plasmid Constructions

Plasmids pET21a (Novagen), pLAM12 (van Kessel & Hatfull, 2007), pJV53 (van Kessel & Hatfull, 2007), and pNIT-1 (Pandey et al., 2009) have been previously described. Plasmid pLAM3 was constructed by amplifying the D29 gene 12 (*lysB*) with primers bearing Nde I and Hind III restriction sites and cloning into pET21a to add a C-terminal His tag. To create the inducible D29B active site mutant (pKC20), the pLAM3 plasmid was subjected to site-directed mutagenesis using the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene) to change Ser82 to Alanine. Plasmid pKMC2 was made by amplifying D29 *lysB* from pLAM3 with primers containing Nde I and Nhe I restriction sites and cloning this into pLAM12. Plasmid pKMC9 was constructed by amplifying D29 *lysB* with primers containing Nde I and Hind III restriction sites and cloning this into pNIT-1. Plasmids were purified using QIAprep Spin Miniprep Kit (QIAGEN). Oligonucleotides were supplied by Integrated DNA Technologies, Inc. and are listed in Table S1.

Expression and Purification

E. coli BL21(DE3) carrying plasmids pLAM3 or pKC20 (or vector pET21a) were grown to OD₆₀₀ between 0.4-0.6 in L-Broth (LB) containing carbenicillin (50 µg/ml) and induced with 1 mM IPTG for 4 hours. Cells were resuspended in TWEB (50 mM Tris-HCl pH 8.0, 300 mM NaCl), sonicated, centrifuged, and the clarified lysates passed over TALON Co²⁺ resin (Clontech). The resin was washed sequentially with TWEB containing 10 mM and 20 mM

imidazole, and bound protein eluted with five volumes of 120 mM imidazole. Eluted fractions were concentrated using Vivaspin concentration columns (molecular weight cutoff 10 kDa; Sartorius Inc.) followed by dialysis against storage buffer (50 mM Tris pH 8.0, 50 mM NaCl, 50% glycerol) and stored at -20°C .

D29 LysB was purified for crystallization similarly. *E. coli* BL21(DE3)pLAM3 cells were induced for 18 hours at 25°C , pelleted, resuspended in Buffer A (50mM Tris, 500mM NaCl, 5mM imidazole, pH 8) and disrupted in a French pressure cell three times at 10,000 PSI for 3 passages. The cell lysate was cleared by centrifugation at 15,000 rpm for 30 min and applied to a 5 ml HisTrap FF column (GE Healthcare, Piscataway, NJ), and eluted with a linear gradient to 100% Buffer B (50 mM Tris, 500 mM NaCl, 500 mM imidazole, pH 8). Fractions were pooled and dialyzed against buffer containing 20mM Tris, 50mM NaCl, pH 8. D29 LysB was concentrated to at least 10mg/ml.

Zymogram assays

Zymograms were performed as described previously (Piuri & Hatfull, 2006) by incorporation of 0.2% lyophilized *Micrococcus luteus* cells as a source of peptidoglycan into the gel matrix. Zymograms were developed by renaturation overnight at 37°C in 25 mM Tris (pH 7.5), 1% Triton X-100, and 0.1 mM ZnSO_4 , stained with 0.5% methylene blue with 0.01% KOH before destaining with water.

Lipolytic enzyme assays

Enzymatic assays for lipolytic activity were adapted from those described previously (Gilham & Lehner, 2005). One milliliter of *p*-nitrophenyl substrates (50 μM) (Sigma Inc.) were incubated with 1 μg of D29 LysB, D29 LysB S82A, lipase (*Pseudomonas fluorescens*, Sigma), or 5 μl of a mock purified sample (derived from pET21a containing cells) in buffer (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 0.1% Triton X-100) at room temperature for 30 minutes. Release of *p*-nitrophenol was determined by measuring absorbance at 420 nm (A_{420}).

Assays for hydrolysis of *M. smegmatis* mAGP

Mycolyl-arabinogalactan-peptidoglycan was isolated from *M. smegmatis* as described previously (Besra, 1998). *M. smegmatis* cells were grown, collected, and washed three times in phosphate buffered saline (PBS; pH 7.4), and resuspended in PBS+2% Triton X-100 (PBSX). Cells were disrupted by extensive sonication, centrifuged to collect the insoluble cell wall fraction, resuspended in PBSX and agitated overnight at 4°C . After centrifugation the pellet was resuspended in PBS+2% SDS and incubated at 100°C for 60 minutes; this was done three times. After three rounds of extraction, the pellet was washed once each with H_2O , 80% acetone in H_2O , and acetone. Following evaporation of the acetone, the mAGP-enriched cell wall material (mAGP) was resuspended in PBS+0.1% Triton X-100 (final concentration 10 mg/ml) and frozen aliquots stored at -80°C .

mAGP material was chemically hydrolyzed by addition of an equal volume of 15% tetrabutylammonium (TBAH) to 1 mg mAGP resuspended in PBS+0.1% Triton X-100 and incubated at 100°C overnight. Enzymatic assays were performed by incubation of 1 mg mAGP in 100 μl with varying concentrations of protein at 37°C . TBAH- and enzyme-treated samples were prepared for analysis by thin layer chromatography by addition of an equal volume of dichloromethane and incubation for 15 minutes at room temperature. The lipid-rich lower dichloromethane layer was removed, extracted once with 0.25 M HCl and once with water, and lipids collected by evaporation. Samples were resuspended in dichloromethane, spotted onto silica-aluminum TLC plates, and separated by

chromatography in chloroform/methanol (97:3); lipids were identified by spraying with 5% molybdophosphoric acid (in ethanol) and charring for 15 minutes at 110°C.

Methyl-esterification of lipids was performed by resuspension of enzyme reaction products in 15% TBAH, addition of an equal volume of dichloromethane and 1/10 volume iodomethane. Reactions were incubated with shaking at room temperature for 15 minutes and the lower dichloromethane layer recovered and extracted with HCl and water as described above. Lipids were separated by TLC in 95:5 petroleum ether/ethyl ether and recognized by charring as above.

Bacteriophage Recombineering of Electroporated DNA

A *lysB* deletion mutant of phage Giles was constructed as described previously (Marinelli *et al.*, 2008). The targeting substrate was generated using a 100 bp oligonucleotide with 50 bp of homology upstream and downstream of the deletion site, which was then expanded to a 200bp dsDNA by PCR. The deletion was designed to remove 1,146 bp between Giles genome coordinates 28,384 and 29,529. Phage Giles DNA (350ng) was co-electroporated with 200 ng of the 200bp substrate into induced electrocompetent *M. smegmatis* mc²155 pJV53 cells, recovered at 37°C for 2 hours and plated on top agar lawns with *M. smegmatis* mc²155 pKMC2 cells in the presence of 0.2% acetamide; approximately 100 plaques were recovered. Individual plaques were picked into 100 µl phage buffer (10 mM Tris-HCl, pH 7.5; 10 mM MgSO₄, 68.5 mM NaCl; 1mM CaCl₂) and analyzed by Deletion Amplification Detection Assay (DADA)-PCR (Marinelli *et al.*, 2008) using 1 µl sample and primers GilesB-DiagR3 and GilesB-DADAPCR3 (Table S1). Primary plaques containing both wild-type and mutant alleles were diluted and plated with 300 µl of *M. smegmatis* cells containing pLAM12 or pKMC2, either with or without acetamide inducer. To test for viability of the mutant, lysates from plates containing approximately 600 plaques were harvested and tested by PCR with primers GilesB-DiagR3 and GilesB-DiagF. Individual plaques from the secondary plating were picked and tested using the same two primers. A mutant derivative was plaque purified and the sequence confirmed by DNA sequencing.

To test for complementation of the Giles *ΔlysB* small plaque phenotype, mc²155 cells containing pKMC9 or pNIT-1 vector control were grown to OD₆₀₀ 0.6 and 10µl of dilutions of either wild-type Giles or Giles*ΔlysB* phage containing ~10² pfu were added to 500 µl of cells, adsorbed for 30 minutes at room temperature, and plated as top agar lawns in 0.35% MBTA with 1mM CaCl₂ on 7H10 plates with 0 to 0.4% ε-caprolactam.

Lysis assays

M. smegmatis cells grown in 7H9 supplemented with ADC, carbenicillin, cyclohexamide, and calcium were grown to OD₆₀₀ 0.3-1.0. For the ATP release assay, cells were diluted to OD₆₀₀ 0.03, and infected with phage particles at an m.o.i. of 10. After adsorption for 30 min, cultures were shaken at 37°C, and 100µl samples removed at different times. ATP release was measured by addition of 100 µL of ENLITEN rLuciferase/Luciferin reagent (Promega), and luminescence recorded for a 10 second interval in a Monolight 2010 luminometer. For the OD assay, cells were diluted to an OD₆₀₀ of 0.25 and infected with phage particles at an m.o.i. of 10. After adsorption for 30 min, cultures were shaken at 37°C and 1 ml samples removed at different times. OD was measured at 600 nm using a Beckman Coulter DU 530 Spectrophotometer.

To determine the number of phage released into the supernatant or retained in unlysed cells, *M. smegmatis* cells were grown as above and diluted to an OD₆₀₀ of 0.25. These were infected at an m.o.i. of 0.1 with adsorption for 30 min followed by incubation with shaking at 37°C. One milliliter samples were removed at different times and separated by

centrifugation into supernatant and pellet fractions. The pellet was resuspended in 1 ml phage buffer and sonicated. Both pellet and supernatant were serially diluted and 5 μ l samples were spotted onto top agar lawns containing *M. smegmatis* in 0.35% MBTA with 1mM CaCl_2 on 7H10 plates.

Crystallization and Structure Determination

Purified D29 LysB at 10 mg/ml concentration was used to screen a large number of commercially available crystallization conditions by vapor diffusion method. After optimization upon the hit condition in the initial screen, diffraction quality crystals were obtained with 2mg/ml protein in the condition of 50mM Tris pH8, 20%PEG1000, 100mM $\text{Ca}(\text{OAc})_2$. The best crystals grew in 4 days and were flash frozen in mother liquor with 25% glycerol then stored in liquid nitrogen. Data sets were collected with a Rigaku MM007HF generator with a Bruker Smart 6000 CCD detector, and with synchrotron beamlines at Advanced Photon Source (APS 23-ID), Argonne National Laboratory, Chicago. The data were processed with HKL2000 and the anisotropic scaling was further performed on the diffraction anisotropy server (Strong *et al.*, 2006).

The initial structure was solved by SIRAS combining the native crystal with an iodine derivative obtained by quick soaking of the crystal in the cryo with 0.5M NaI for 2 min. The model was refined against the native dataset iteratively until the R/Rfree reached 0.205/0.252 at 2.0 Å. The Iodine sites were found with Shelxd/e program. The phasing and refinement of the protein structure were carried out with the Phenix package. The model was built in Coot. 20.3 PyMol was used for structure analysis and rendering.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Organization, location, and conservation of mycobacteriophage lysis cassettes

A. Peptidoglycan hydrolysis by three LysA proteins. LysA proteins of Che8 (gp32), Bxz1 (gp236), and Corndog (gp69), were expressed, purified to near homogeneity, separated by SDS-PAGE (top panel) and stained with Coomassie blue. In the lower panel, the proteins were separated through a gel matrix containing *M. luteus*, renatured, and the zymogram developed by staining with methylene blue. Peptidoglycan hydrolysis by renatured proteins within the gel produces clear areas that no longer stain with methylene blue. Lysozyme (Lys) and Ovalbumin (Ova) represent positive and negative controls.

B. Each of the 60 completely sequenced mycobacteriophages together with Ms6 and a putative *M. avium* prophage encode LysA proteins predicted to hydrolyze peptidoglycan, although they have highly modular structures (Hatfull *et al.*, 2006). When present, the second putative lysis gene *lysB* is located downstream and is closely-linked to *lysA*, with no more than four intervening genes. The sixty sequenced genomes can be grouped into clusters and subclusters according to their similarities (manuscript in preparation) and the organization in each cluster (with the number of genomes represented in parentheses) is shown. All of the mycobacteriophage genes have been assorted into phamilies according to their sequence similarities, and the pham number (and number of gene members in that pham in parentheses) shown above each gene.

C. Mycobacteriophage lysis cassettes may be positioned either to the left of the virion structure and assembly genes, as in the 13 cluster A genomes, or to their right as in other genomes, but in either case the *lysA* and *lysB* genes are closely-linked.

Figure 2. D29 LysB is a lipolytic enzyme

A. Phage D29 LysB was expressed from plasmid pLAM3, a derivative of vector pET21a, in *E. coli* BL21(DE3) and purified to near-homogeneity. SDS-PAGE of un-induced cells (U), whole cell lysates of induced strains (W) separated into insoluble (I) and soluble (S) fractions, and a clarified soluble lysate (L) are shown. The 30kDa His-tagged D29 LysB was bound to a cobalt-affinity matrix, and flow-through (F), a 20 mM imidazole wash (W₂₀) sample, and fractions collected at 120mM imidazole elutions (E₁₂₀) are shown.

B. Lipolytic activity of D29 LysB (filled bars; left axis scale) is shown using *p*-nitrophenyl substrates containing different lipid chain lengths (C₄, C₈, C₁₂ and C₁₆); lipase activities on the same substrate are also shown (open bars; right axis scale). Specific activities are shown as Units/mg protein, with 1 Unit corresponding to the release of 1 μ mol *p*-nitrophenol min⁻¹. An active site mutant (S82A), boiled sample, and a mock-purified sample show little or no activity.

Figure 3. Crystal structure of D29 LysB

A. The D29 LysB structure is shown in alignment with the *Cryptococcus sp* cutinase-like protein (pdb: 2czq). D29 LysB is colored according to secondary structure (red: α -helix, yellow: β -strand, green: loops). 2czq is shown in cyan.

B. The catalytic triad of D29 LysB catalytic triad is composed of Ser82, Asp166 and His420 (ball-and-stick representations). Electron density map (2Fo-Fc) around the triad is shown in blue mesh at 1 σ level. The triad of 2czq is in stick.

Figure 4. D29 LysB is a mycolyl-arabinogalactan esterase

A and B. Time- and enzyme-concentration-dependence of D29 LysB-mediated hydrolysis of mycolyl-arabinogalactan-peptidoglycan (mAGP). mAGP was purified from *M. smegmatis*, incubated with D29 LysB, and the lipid products separated by thin layer chromatography. The reaction was monitored as a function of time (panel **A**; incubation times as shown, in minutes) or amount of protein (panel **B**; 0.1, 0.25, 0.5, 1, 5, 10 µg D29 LysB). D29 LysB catalyzes production of a species migrating in the same position as the free mycolic acids released by tetrabutylammonium (TBAH) treatment.

C. Methylesterification of the products generated by D29 LysB cleavage of mAGP generates epoxy, α and α' mycolates.

D. Neither a D29 LysB catalytic mutant (S82A), lipase purified from *Pseudomonas fluorescens*, a mock purified sample, nor buffer release free mycolic acids from mAGP.

Figure 5. Construction of a Giles *lysB* deletion mutant

A. A 200 bp dsDNA substrate was designed that has 100bp homology flanking a 1,146 deletion in Giles gene 32. The locations of primers F1, F2, and D1, used for PCR screening are shown.

B. Following co-electroporation of the 200bp substrate and Giles genomic DNA, primary plaques were recovered and screened by PCR using primers D1 and F2, designed to preferentially amplify the deletion mutant. A mixed plaque containing wild-type and mutant DNA is shown.

C. A mixed primary plaque was diluted and plated, and isolated secondary plaques screened using primers F1 and F2. One homogenous wild-type Giles plaque (left) and one homogenous *lysB* deletion mutant plaque are shown (right).

Figure 6. LysB is required for efficient host cell lysis

A. *M. smegmatis* mc²155 containing either plasmid vector pNIT-1 or plasmid pKMC9 carrying the D29 *lysB* gene was infected with wild-type or Giles Δ lysB phages, as indicated. The Δ lysB mutant forms smaller plaques than its wild-type parent, but restored to larger plaques by complementation with D29 *lysB*. Scale bar indicates one cm.

B. Lysis of phage-infected *M. smegmatis* mc²155 was monitored by measuring OD₆₀₀ of cultures infected with wild-type Giles (red), a Δ lysA mutant (green) or a Δ lysB mutant (yellow) at different times after phage addition; uninfected cells are shown in blue. Thicker lines correspond to trendlines.

C. *M. smegmatis* mc²155 cells were infected with Giles (red), a *ΔlysA* mutant (green) or a *ΔlysB* mutant (yellow) and ATP release measured at different times after infection; uninfected cells are shown in blue. ATP was measured in a luciferase assay and reported as relative light units (RLU).

D. *M. smegmatis* mc²155 cells were infected with wild-type Giles, the *ΔlysA* mutant, or the *ΔlysB* mutant and the total number of phage particles (pfu/ml) determined. Wild-type Giles is shown in red, *ΔlysA* in green, and the *ΔlysB* mutant in yellow.

E. For each of the time points in panel D the distribution (as percentage of total) of phage particles in the supernatant and cell pellet was determined. For wild-type Giles the supernatant is shown in dark grey and the cell pellet in red; the *ΔlysA* mutant supernatant is grey and the pellet is yellow; the *ΔlysB* mutant supernatant is shown in light grey and the pellet in green.

Figure 7. A model for mycobacteriophage lysis of mycobacteria

A. Mycobacterial cell walls are unusual in that the cytoplasmic membrane (CM) is surrounded by a peptidoglycan layer (PG) to which a network of arabinogalactan (AG) is covalently attached. A mycobacterial outer membrane consisting of mycolic acids (MA) and free lipids (FL) is covalently attached *via* an ester linkage of mycolic acids to arabinogalactan. We propose that LysA — assisted by holins encoded by at least some of mycobacteriophages — perform an essential step in lysis involving degradation of the peptidoglycan layer, and the lysis is completed through LysB-mediated cleavage of the outer membrane from arabinogalactan. **B.** Diagram illustrating LysB cleavage of the ester bond linking mycolic acids and arabinogalactan.

Table 1
Crystallography data collection and refinement statistics Accession code

The atomic coordinate and structure factor have been deposited in the PDB with the accession code 3HC7 for D29 LysB.

Data collection ¹	
Space group	P 43 21 2
Cell dimensions a, b, c (Å)	92.5, 9.5, 69.6
Wavelength (Å)	0.9795
Resolution (Å)	2.0 (2.07-2.00)
I/sigI	28.0 (3.2)
Completeness (%)	99.8 (99.5)
Rsym	0.0196 (0.3116)
Redundancy	14.1 (12.8)
Refinement ²	
No. reflections	19777
Rwork/Rfree	0.205/0.250
No. atoms	2094
RMSD bonds	0.004
RMSD angles	0.635

¹ The native D29 LysB dataset was collected at APS 23ID and processed with HKL2000.

² Refinement was done with Phenix.

Table 2

Top 5 results of structural alignment of D29Lys B in Dali database *

pdb	Z-score	rmsd	#aligned position	#residue	%identity	Description
2czq	20.3	2.2	169	205	21	Cutinase-like protein
1qoz	19.2	2.6	170	207	21	Acetyl xylan esterase
1bs9	18.9	2.3	164	207	21	Acetyl xylan esterase
3dd5	16.5	2.7	160	193	19	Cutinase
1ffb	16.4	2.7	157	197	20	Cutinase

* The redundant hits of the same protein have been removed.