Investigating the Antimicrobial Activity of Two Mycobacteriophage-encoded Lysterases

by

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All double-stranded DNA bacteriophages encode a specialized set of proteins that allow progeny phages to escape the current host cell at the end of the lytic cycle. At a minimum, this lytic cassette consists of a holin and an endolysin to destabilize the host's plasma membrane and peptidoglycan layer, respectively. This two-enzyme system is sufficient to break open most Gram-positive hosts, but the phages of bacteria with more complex cell walls often encode additional lysis proteins. For example, the mycobacteria have thick, waxy, mycolic acid-rich cell walls that aid in their antibiotic resistance. Therefore, their phages—the mycobacteriophages—also encode a lysterase (LysB), in order to efficiently overcome this barrier. *In vitro*, these serine esterase proteins have been shown to hydrolyze mycolic acids from whole mycobacterial cells and purified cell wall components. Our data also show that exogenous addition of purified lysterases has antimicrobial activity against several species within the Order Actinomycetales, including bacteria associated with disease and—surprisingly—bacteria that do not contain mycolic acids, such as *Cutibacterium acnes*. *C. acnes* is the implicated causative bacteria in the skin disease acne vulgaris and is an opportunistic pathogen in eye, blood, and medical device infections. While the identity of the LysB target on *C. acnes* is still unknown, we have tested LysB activity against multiple clinically relevant strains of *C. acnes* and have shown that LysB treatment releases an unidentified lipid from whole *C. acnes* cells.

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PREFACE

This work would not have been possible without the help and support of the people around me. First, I would like to thank Graham for this project, for providing me with a lab home, and for all the support he has given me as an advisor. This project has been super interesting and has given me many opportunities I otherwise wouldn't have had, such as presenting at The Lysin Meeting, meeting the bigwigs in the field of phage-encoded hydrolytic enzymes, and even experience with the patent application process. I wouldn't have been able to do any of these things without your support and guidance. Thank you!

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DISCLOSURE

Graham F. Hatfull (Thesis Director) has significant financial interest in Dermalytica, Inc., which has optioned for development patent-pending technology (No. PCT/US16/44427) based on a subset of this research.

1.0 PHAGE-MEDIATED LYSIS OF BACTERIAL CELLS

The general bacteriophage—a virus that infects bacteria—must be able to accomplish a minimum of four tasks in order to complete its life cycle (Figure 1): 1) the phage must be able to find a host cell and inject its genome; 2) the phage genome must be replicated; 3) progeny phages must be assembled; and 4) progeny phages must be able to escape the host cell so that the life cycle can repeat anew[1–3]. Therefore, phage must be able to exploit host machinery and/or encode their own genes to complete these tasks. For the purposes of this Masters Thesis, I will focus exclusively on the final part of the phage life cycle: escape from the current host cell. All tailed double-stranded (ds) DNA phages encode a specialized set of genes to accomplish this task; these genes are almost always located adjacent to one another and are, therefore, referred to as the lytic cassette[4–6]. These proteins work together in different areas of the bacterial cell wall to efficiently lyse the host cell.

There are three main strategies for phage escape from the current host cell. First are the filamentous bacteriophages, which extrude from the host[7], are not lytic, and will not be further discussed in this document. Other bacteriophages either actively degrade cell wall components—usually with muralytic enzymes targeting the peptidoglycan layer—or encode a single non-muralytic enzyme—such as an inhibitor of murine synthesis—to achieve lysis[5,7,8]. These two strategies are employed by dsDNA phages of a range of hosts and single-stranded (ss) DNA or RNA phages of Gram-negative hosts, respectively[5,7]; though whether these strategies result in complete destruction of the host bacterium or simply cellular emptying depends on the phage in question[5]. Generally, the phage-encoded muralytic enzyme involved in lysis is called an

endolysin and lacks a signal sequence, a length of amino acids which enables the proteins to be translocated through the plasma membrane. Therefore, bacteriophages that utilize endolysins require another protein, a holin, to help them reach the peptidoglycan layer and correctly time lysis[5,6]. In the *Escherichia coli* phage λ—possibly the most-well studied bacteriophage—the holin and endolysin are encoded by the genes *S* and *R*, respectively.

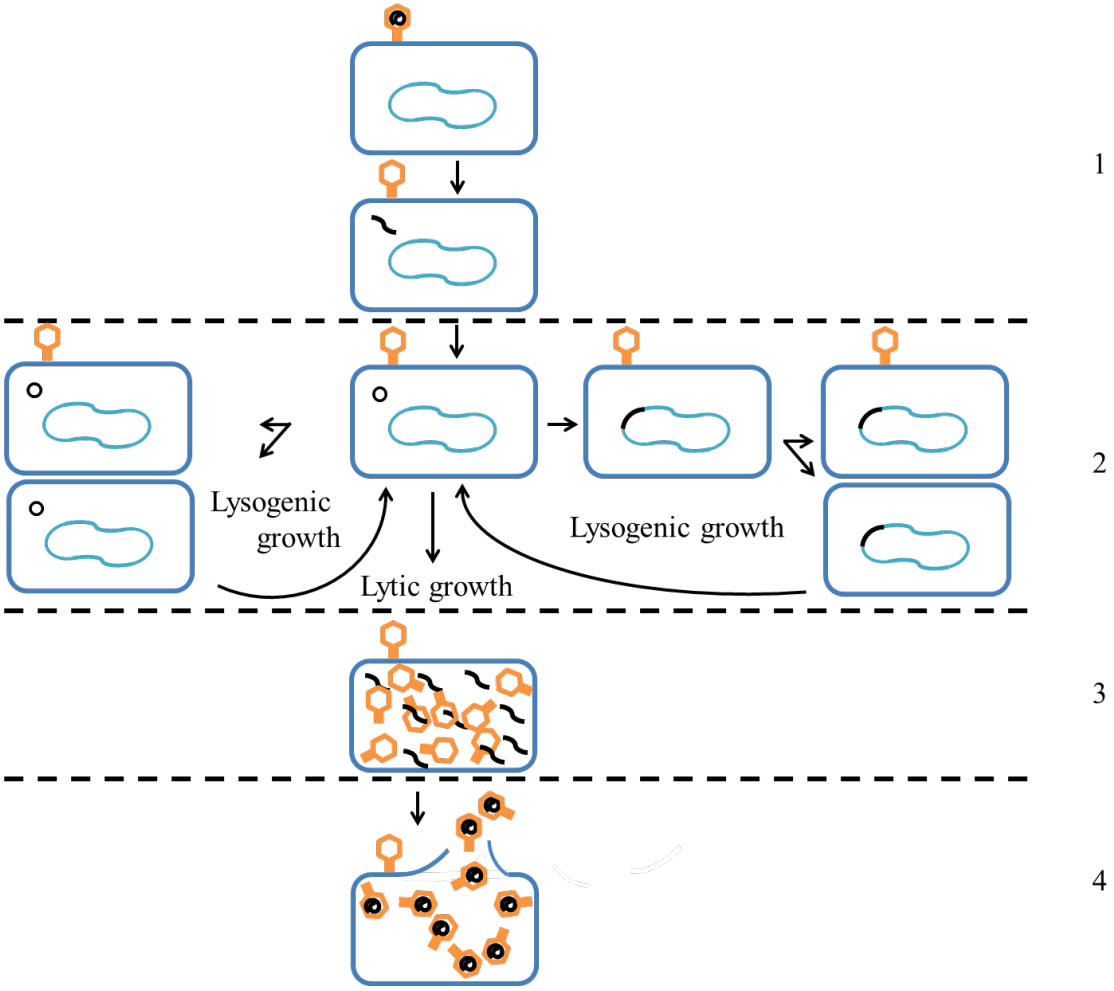


Figure 1. Temperate bacteriophage life cycle

After adsorbing to a bacterium, a bacteriophage (orange) injects its genome (black) into the host. If the phage is temperate, it makes a decision to grow either lytically or lysogenically, in which the phage genome is usually integrated into the genome of the host bacterium (light blue) and as the host replicates, so does the phage. In some cases, the phage genome is not integrated and instead is maintained as a plasmid as the host replicates. Lytic phages and temperate phages that have flipped the genetic switch toward lytic growth, then hijack host transcription and translation machinery to create progeny phages. Finally, progeny phages must escape the current host cell so they may find new hosts. The phage life cycle is numbered to reflect the four necessary tasks a phage must complete, as detailed in the text.

1.1 TWO-ENZYME PHAGE-MEDIATED LYSIS

1.1.1 Holins

While most phages harbor a bioinformatically predicted holin gene, our knowledge about holin structure and function comes almost exclusively from coliphages like λ. Holins are a diverse group of functional homologs that share little sequence similarity and are the first protein involved in bacterial cell lysis. While many details of holin action and timing are still unclear, holins are transmembrane proteins that accumulate in the bacterial plasma membrane and control the timing of lysis[7]. When a sufficient number of canonical holin proteins—exemplified by the protein product of λ gene *S*–have accumulated, they oligomerize and create holes in the plasma membrane[5,8,9]. This action disrupts membrane potential, compromises the structural integrity of the membrane, and creates lesions through which subsequent lysis proteins can access their targets in more distal portions of the cell wall[5]. Translation of *S* can initiate at the first or third codon, making *S* a dual-start gene[7]. If translation starts at the 3rd codon, the resulting 105-amino acid protein is the lethal holin. Translation from the first codon produces a 107-amino acid anti-holin, which opposes holin activity and helps regulate the timing of lysis[5,7]. Many holin systems include an anti-holin, though they are not always encoded by the same gene[10].

1.1.2 Endolysins

The endolysins are a family of modular proteins that participate in host cell lysis through the degradation of bonds in the peptidoglycan layer. Unless the bacteria are grown in iso-osmotic media, high internal osmotic pressure will rapidly empty the cell contents into the environment

once the peptidoglycan layer has been compromised[7]. At a minimum, an endolysin has two domains, a C-terminal cell wall binding domain (CBD) that localizes the enzyme to its specific target and an N-terminal catalytic domain to hydrolyze that bond[11–14]. Endolysins can have different types of catalytic activity in the bacterial cell wall; lysozymes target glucosamine or muramine moieties, amidases target amide bonds linking the glycan strand and peptide moieties, and endopeptidase domains target peptide cross-bridges[14–16].

1.1.3 Pinholins and Signal-Anchor-Release Endolysins

A notable variation to the holin-endolysin system described above is that of pinholins and signal-anchor-release (SAR) endolysins. In this case, pinholin and SAR endolysin proteins are both incorporated into the plasma membrane[5,7]. Unlike canonical holins, pinholins do not cause large lesions in the plasma membrane, although they still disrupt membrane potential and structural integrity[17,18]. SAR endolysins have a hydrophobic N-terminal sequence that keeps the CBD and catalytic domains tethered inside the periplasmic space away from their targets[19,20]. When membrane potential collapses, SAR endolysins are released from the plasma membrane and can reach their targets in the peptidoglycan layer.

1.2 ADDITIONAL LYSIS ENZYMES

These two enzyme holin-endolysin systems are sufficient to lyse most Gram-positive bacterial hosts. However, for the phages of hosts with more complicated cell walls, additional lytic enzymes are sometimes necessary for efficient lysis.

1.2.1 Canonical cell walls

The cell walls of canonical Gram-positive and Gram-negative bacteria are structured dramatically differently, and they impose different challenges for phage lysis. The mycobacterial cell wall poses yet another kind of challenge for phage lysis. In order to highlight why some bacteriophages might need additional lytic enzymes and how and where these proteins participate in lysis, we shall first introduce the basic components of these cell wall types.

While the exact composition of any bacterial cell wall is unknown, certain common features and the properties of those features have allowed essentially all bacteria to be sorted into two major groups. Gram-positive and Gram-negative bacteria are first differentiated by their reaction to Gram stain. Crystal violet is used to stain peptidoglycan purple. The thick peptidoglycan layer of Gram-positive bacteria retains the dye even after the alcohol is added, while the comparatively thinner peptidoglycan layer of Gram-negative bacteria does not. When the counterstain is applied, only the Gram-negative bacteria will appear pink. Moving from the cytoplasm outward, the Gram-positive cell wall (Figure 2A, adapted from Payne[21]) is characterized by a plasma membrane, periplasmic space, and thick peptidoglycan layer[22]. Furthermore, Gram-positive bacteria can have a myriad of lipids and polysaccharides attached to

their cell walls[23], some—like teichoic acid—have been characterized, but most remain unidentified.

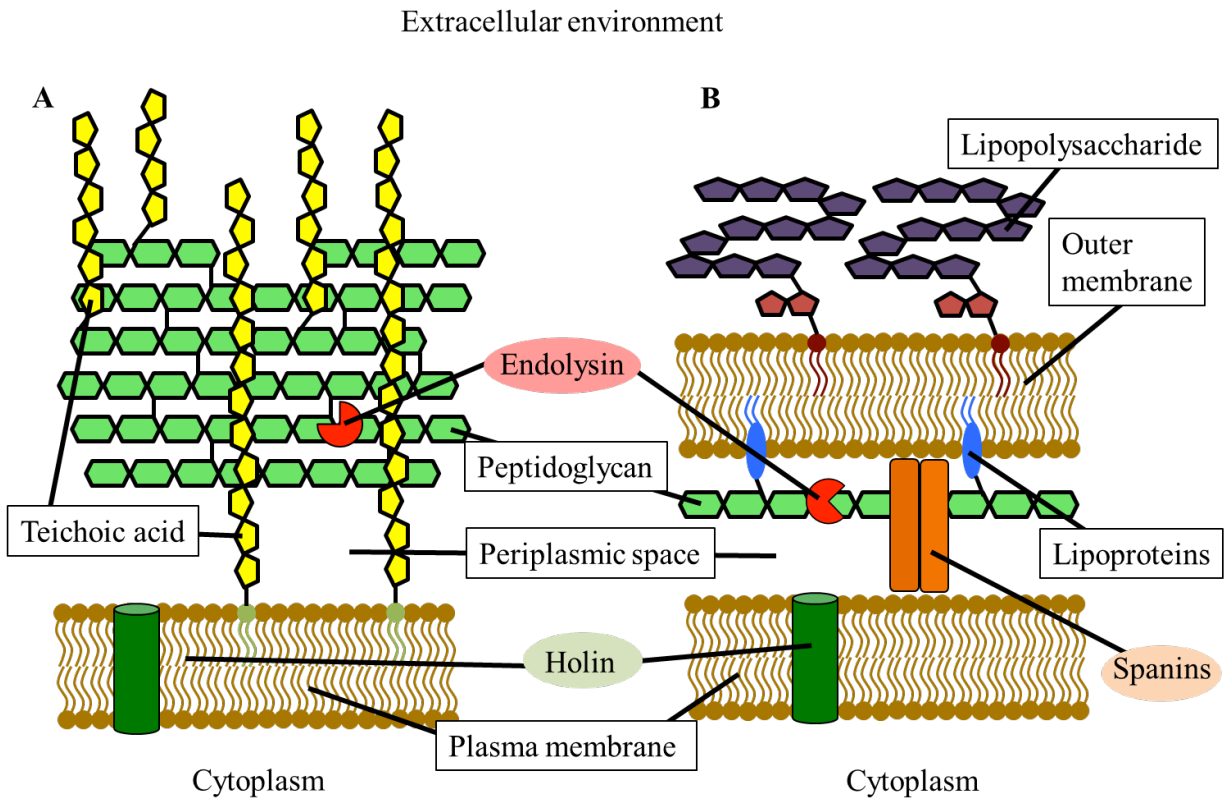


Figure 2. Models of Gram-positive and Gram-negative cell walls and their phages' lysis proteins.

The Gram-positive cell wall consists of a plasma membrane, periplasmic space, and thick peptidoglycan layer with attached teichoic acids. The Gram-negative cell wall also has a plasma membrane and periplasm, but it contains a thinner peptidoglycan layer, an outer membrane, and finally exterior complex carbohydrates. The phages of both types of bacteria encode a holin and endolysin to collapse membrane potential and degrade the peptidoglycan layer, respectively. Some phages of Gram-negative bacteria also encode spanins, which fuse the inner and outer membranes for concurrent destabilization. Bacterial cell wall parts are labeled in white boxes; phage proteins are labeled in colored ovals.

1.2.2 Gram-negative bacteria and spanins

The cell wall of Gram-negative species (Figure 2B, adapted from Payne[21]) is generally comprised of a plasma membrane, periplasmic space, a comparatively thinner peptidoglycan layer, and an outer membrane with exterior complex carbohydrates[24]. While the Gram-positive

cell wall is comprised of 30-70% peptidoglycans[22], peptidoglycan makes up less than 10% of the Gram-negative cell wall[25]. Gram-negative cell walls are also lipid-rich, but, again, little is known about the specific lipids.

In addition to a holin and endolysin, some phages of Gram-negative bacteria, such as phage λ, produce spanins—encoded by genes *Rz* and *Rz1*—which fuse the inner and outer membranes of the *Escherichia coli* host cell, so that both membranes are compromised concurrently[7,26,27]. Under the right conditions, defective Rz and/or Rz1 will terminate lysis in a spherical cell shape[28], presumably because the outer membrane is still intact. However, under standard laboratory conditions, spanins are not essential for lysis[7,28,29].

1.2.3 Mycobacterial cell wall and lysterases

The mycobacteria—exemplified by *Mycobacterium tuberculosis*, the causative pathogen of the lung disease tuberculosis—have historically been categorized as Gram-positive bacteria due to their thick peptidoglycan layer and lack of true outer membrane[30]. However, these acid-fast bacteria are more accurately considered Gram-variable, as they lack a true outer membrane but often do not stain Gram-positive because of their waxy, lipid-rich cell walls. Along with the plasma membrane, the defining characteristics of their cell walls (Figure 3, adapted from Payne[21]) include the mycolylarabinogalactan-peptidoglycan (mAGP) complex, which consists of a peptidoglycan layer covalently attached to an arabinogalactan layer. The arabinogalactan is in turn covalently linked to the mycolic acids that lend their name to the mycobacteria[30–33]. Finally, interdigitated with the mycolic acids is an asymmetric bilayer comprised of a variety of free lipids and glycolipids, such as trehalose dimycolate (TDM)[33]. This outer most region of the cell

wall is sometimes referred to as the "mycobacterial outer membrane" and poses another barrier to lysis.

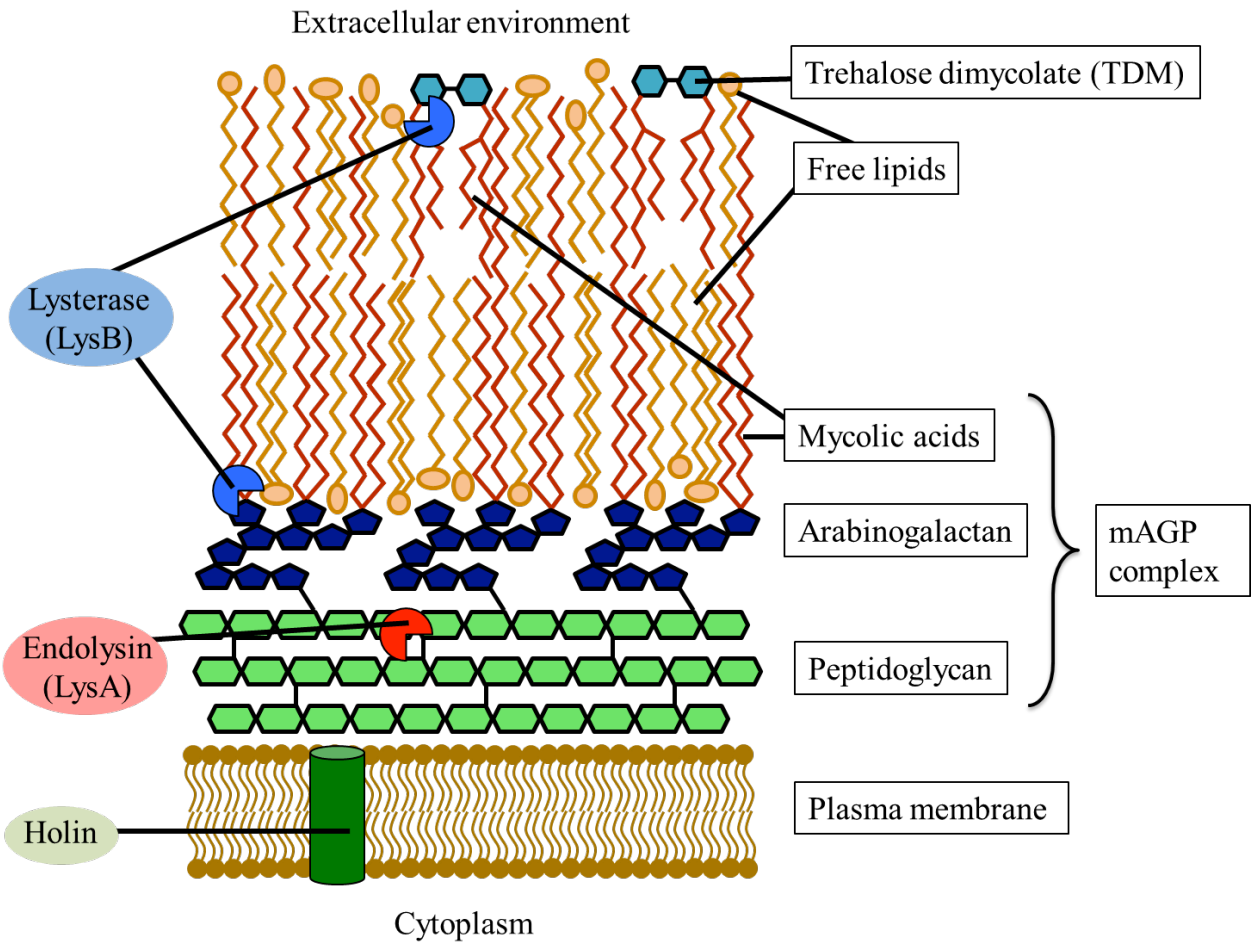


Figure 3. Model of the mycobacterial cell wall and mycobacteriophage lysis proteins.

The mycobacterial cell wall has a plasma membrane, mycolylarabinogalactan-peptidoglycan (mAGP) complex, and an asymmetric bilayer of free lipids and glycolipids such as trehalose dimycolate (TDM). While the mycobacteriophages encode holins and endolysins like the phages of Gram-positive and –negative bacteria, they also encode a lysterase. The lysterase hydrolyzes mycolic acids from the mycobacterial outer membrane to help efficiently lyse the host cell. Bacterial cell wall parts are labeled in white boxes; phage proteins are labeled in colored ovals.

To overcome the barrier of the mycobacterial outer membrane, in addition to a holin and endolysin, mycobacteriophages encode a lysterase (LysB)[6,34,35]. Lysterases have formerly been categorized as a type of endolysin—called Lysin B after the endolysin's Lysin A (LysA) in the mycobacteriophage genome annotation convention[34]. However, we suggest the new

classification of lysterase, as endolysins and lysterases are starkly different in terms of sequence and target molecule. While endolysins target bonds in the peptidoglycan layer, lysterases specifically hydrolyze mycolic acids from their ester-linked substrates in the mycobacterial cell wall. Details of lysterase activity will be discussed further in Chapter 3. Additionally, while all fully sequenced mycobacteriophage genomes on phagesdb.org[36] have annotated LysA genes, only 89% of these genomes contain bioinformatically-identified LysB genes. Payne *et al* 2009[35] used Bacteriophage Recombineering of Electroporated DNA[37] (BRED) to delete the *lysB* gene in mycobacteriophage Giles. The Δ*lysB* mutant was recovered, but resulted in smaller plaques and had a defect in lysis when grown in liquid culture[35]. This suggests that while *lysB* may not be essential, it is necessary for the efficient lysis of mycobacterial hosts, as wildtype Giles phages would have timing and burst size advantages over Δ*lysB* mutants.

2.0 PHAGE-ENCODED ENZYMES AS ANTIMICROBIAL AGENTS

2.1 INTRODUCTION

While understanding how mycobacteriophage achieve lysis of their hosts is inherently interesting to phage biologists, there is obvious translational appeal to studying molecules involved in killing bacteria. Traditional antibiotics have been isolated from fungal and bacterial sources, but with resistance to antibiotics constantly increasing, it is necessary to continue searching for new antimicrobial therapies. Bacteriophage proteins are unsurprisingly an important potential source.

2.2 ENDOLYSINS AS ANTIMICROBIALS

To date, the endolysins are the most studied phage-encoded antimicrobial protein. At the 1st International Symposium on Antimicrobial Hydrolytic Enzymes in November 2016, twenty-two of the thirty speakers presented specifically on endolysins. Because of their inherent ability to lyse bacterial cells during the phage life cycle, it was hypothesized and found that endolysins from phages of Gram-positive bacterial hosts can effectively lyse bacterial suspensions when added exogenously[12–14,38–40]. Furthermore, it has been found that the CBD of various endolysins confer very specific antibacterial activity; that is, in most cases, an endolysin only has antibacterial activity against the natural host species of the phage that encodes the protein[14]. This

is, of course, not without exception, though most endolysins that can kill other bacteria can only kill those very closely related to their original host taxa, often limited to within the same genus[13].

This specificity makes endolysins promising narrow-spectrum antimicrobials, able to discriminate specific pathogens from other microbial flora. For example, in the dairy industry, endolysin are already being used to selectively kill bacteria that contaminate the essential lactobacilli cultures[41,42]. Additionally, the high specificity of endolysin CBDs is being explored as a diagnostic tool. For example, fluorescent proteins fused to CBDs from *Listeria* phages can selectively label *Listeria* species in mixed cultures; some CBDs can even differentiate between *Listeria* species[16,43,44]. Another advantage of endolysins is that, to date, susceptible bacteria have never been seen to acquire resistance[14]. Two companies, Contrafect and Elanco, have optioned patents to develop therapeutic endolysins for human and animal use, respectively. Contrafect has concluded Phase I clinical trials of their CF-301 endolysin[45]. Meanwhile a number of research and commercial laboratories continue to investigate their antimicrobial and diagnostic use on a wide range of bacteria[12,13,16,38–41,43,44,46–49].

2.3 TAIL-ASSOCIATED HYDROLASES

Recently, the ability of phage tail-associated hydrolase proteins is being investigated for antimicrobial potential[50]. *In vivo*, these proteins are located on the tip of a bacteriophage tail, are often structurally essential, and aid in the phage's ability to overcome the barrier of the cell wall during the initial stage of infection[51–53]. Once the cell wall has been compromised, the bacteriophage genome can make its way into the host cell. During instances of high multiplicity of infection (MOI), these tail-associated hydrolases aid in the process of lysis-from-without[54], the

phenomenon of bacterial lysis induced by phage adsorption at high MOI and occurs without progeny phage production[54,55]. Despite their peptidoglycan-degrading activity, these enzymes are distinct from endolysins and have at least been identified in *E. coli*[56–59], *Salmonella*[59], *Lactobacillus lacti*[60], *Bacillus*[61], and *Staphylococcus aureus*[62–64] bacteriophages. One such protein—called a tail-associated muralytic enzyme (TAME)—from *S. aureus* phage K, has been combined with the CBD of lysostaphin and was shown to be effective in killing staphylococci in a rat nasal colonization model[64].

2.4 TOXINS

The term "toxin," in this case, is used to differentiate any bacteriophage-encoded protein with antimicrobial activity that is not used in initial infection or in final lysis of the host bacterium. Many genes in bacteriophage genomes have no predicted known function, and investigating these can lead to the discovery of new antimicrobial gene products. PK34, a synthetic peptide created from gene *63* from mycobacteriophage D29, has been shown to bind trehalose dimycolate in the mycobacterial outer membrane, resulting in *M. tuberculosis* killing and inflammation inhibition in a mouse model[65,66]. The Hatfull laboratory has also been investigating possible mycobacteriophage-derived toxins. For example, the product of mycobacteriophage Fruitloop gene *52* is toxic when overexpressed in *M. smegmatis* and interacts with Wag31, an essential gene involved in cell shape and cell wall integrity (Ching-Chung Ko, unpublished data).

3.0 LYSTERASE ANTIMICROBIAL ACTIVITY

Investigating phage-encoded proteins with potential antimicrobial activity has clear biomedical relevance. The respiratory disease tuberculosis is the world's most deadly disease caused by a single pathogen, killing nearly two million people annually and harbored in an estimated two billion worldwide[67]. The causative pathogen, *M. tuberculosis* (Mtb), is notoriously difficult to diagnose and treat, as it has the thick, waxy mycolic acid-rich cell wall characteristic of its genus, is a slow growing, intracellular pathogen, and is often encased within granulomas in the lung. Diagnosis most commonly relies on microscopic identification and culturing the bacterium, which requires acquiring sufficiently high numbers of the bacilli from patient sputum and several weeks of incubation time, respectively[68]. More recently, a polymerase chain reaction (PCR) diagnosis method has been developed, which is much faster and requires less bacteria[69]. Unfortunately, this method is limited by efficiency of DNA extraction and currently requires a costly combination of harsh chemicals and mechanical sonication to break apart *M. tuberculosis* cells to reach their DNA[70]. Further, the front lines of Mtb infection usually lack access to such expensive diagnostic tools[67].

Treatment is equally difficult and costly, and treatment is often started before a positive diagnosis if the patient has come in contact with a known infected individual[71]. A treatment regime is often several months long, involves multiple antibiotics, and can change as various information is obtained[67,71–73]. For example, a patient may be started on a drug cocktail of

isoniazid, rifampin, pyrazinamide, and either streptomycin or ethambutol. If the Mtb strain in question turns out to be antibiotic susceptible, streptomycin and ethambutol treatment can be withdrawn, but antibiotic resistance rates are always increasing, including multidrug resistant (MDR) and extensively drug resistant (XDR) strains[67,71]. Additionally, these drugs themselves have a number of harmful side effects. Taken together, it is clear that any enzyme with the ability to break down the cell wall and potentially kill mycobacteria is worth investigating.

3.1 LYSTERASE CATALYTIC ACTIVITY

The lysterases were originally considered a second type of endolysin, but early studies showed that the two types of proteins have very different activity. The LysB from mycobacteriophage Ms6 was found to have lipolytic activity, being able to hydrolyze lipase and esterase substrates; the Ms6 LysB did not have hemolytic or proteolytic activity[34]. Lysterases from mycobacteriophages Ms6[34,74], D29[35], and Bxz2[74] are able to hydrolyze *p*-nitrophenyl from carbon chains of varying lengths, representing canonical esterase and lipase activity, though the specific activity (in units per milligram) differed by LysB in these experiments. Treatment with D29 LysB releases free mycolic acids from purified mycobacterial cell wall components such as the mycolylarabinogalactan-peptidoglycan (mAGP) complex and trehalose dimycolate[75]. Additionally, when we treated whole *M. smegmatis* cells with Bxb1 or D29 LysB, we also saw an increase in free mycolic acids (Figure 4A) and a decrease in trehalose dimycolate and corresponding increase in trehalose monomycolate (Figure 4B). Some of the cells were boiled before LysB treatment to control for any stress response the cells might mount (Figure 4 “heat killed cells” vs other lanes). Since the beginning of my investigation into lysterase activity,

exogenous Ms6[74], Bxz2[74], and BTCU-1[76] LysB proteins have demonstrated some antimicrobial activity against *Mycobacterium smegmatis*.

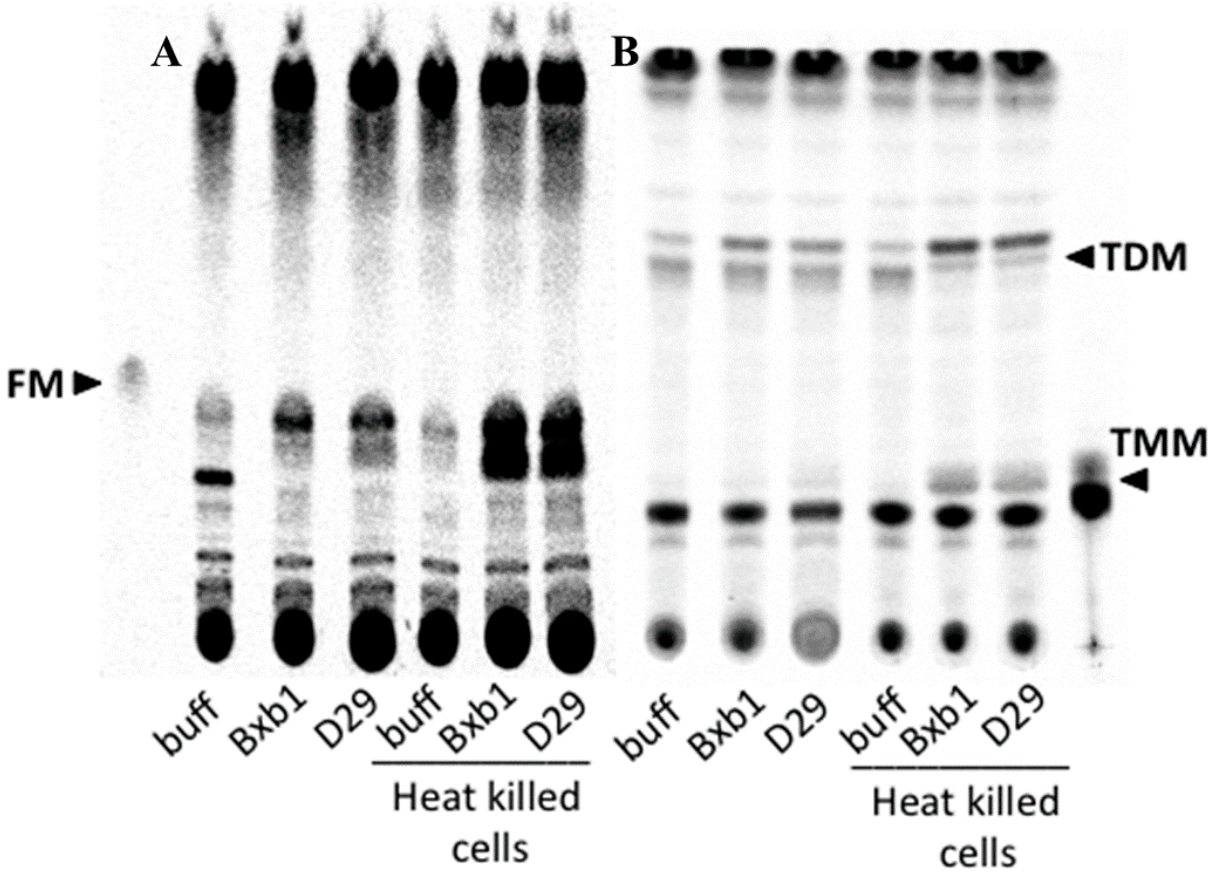


Figure 4. LysB treatment releases lipids from whole *M. smegmatis* cells.

Treatment with either LysB leads to an increase in free mycolic acids (FM), which can be seen as a darkening of the band indicated by the FM arrow in the Bxb1 and D29 lanes in comparison to the buffer control lane (**A**). Similarly, LysB treatment affected cell wall glycolipids, trehalose dimycolate (TDM) and trehalose monomycolate (TMM). Treatment resulted in a decrease in TDM and an increase in TMM, as indicated by the lightening of the TDM band and a darkening of the TMM-denoted band (**B**) in comparison to the control condition. Half of the cells were heat killed (where indicated) prior to treatment in order to control for any stress response by the cells. Optimized running conditions[77] for visualizing (**A**) FM and (**B**) the difference between TDM and TMM as described in the methods section.

In the pages that follow, we tested LysB proteins from two mycobacteriophages: D29 and Bxb1. These proteins share only 21% sequence identity and were selected for multiple reasons. First, the D29-encoded LysB is one of the best-studied lysterases thus far and is the only LysB with a solved crystal structure (PDB: 3HC7)[35] (Figure 5A, grey). Additionally, these two LysBs were easily cloned and overexpressed and represent the two structural classes of lysterase

predicted from sequence data: one defined by the D29 LysB protein and comprised of a single domain, whereas the Bxb1 LysB (Figure 5A, green) has a predicted additional N-terminal domain of unknown function (Figure 5B, green). No studies have investigated the role of the N-terminal domain, but it has HHpred[78,79] predicted structural similarity to the CBD of a *Pseudomonas* phiKZ bacteriophage (PDB:3BKH)[80] (Figure 5B, orange), although the two domains share only 19% sequence identity.

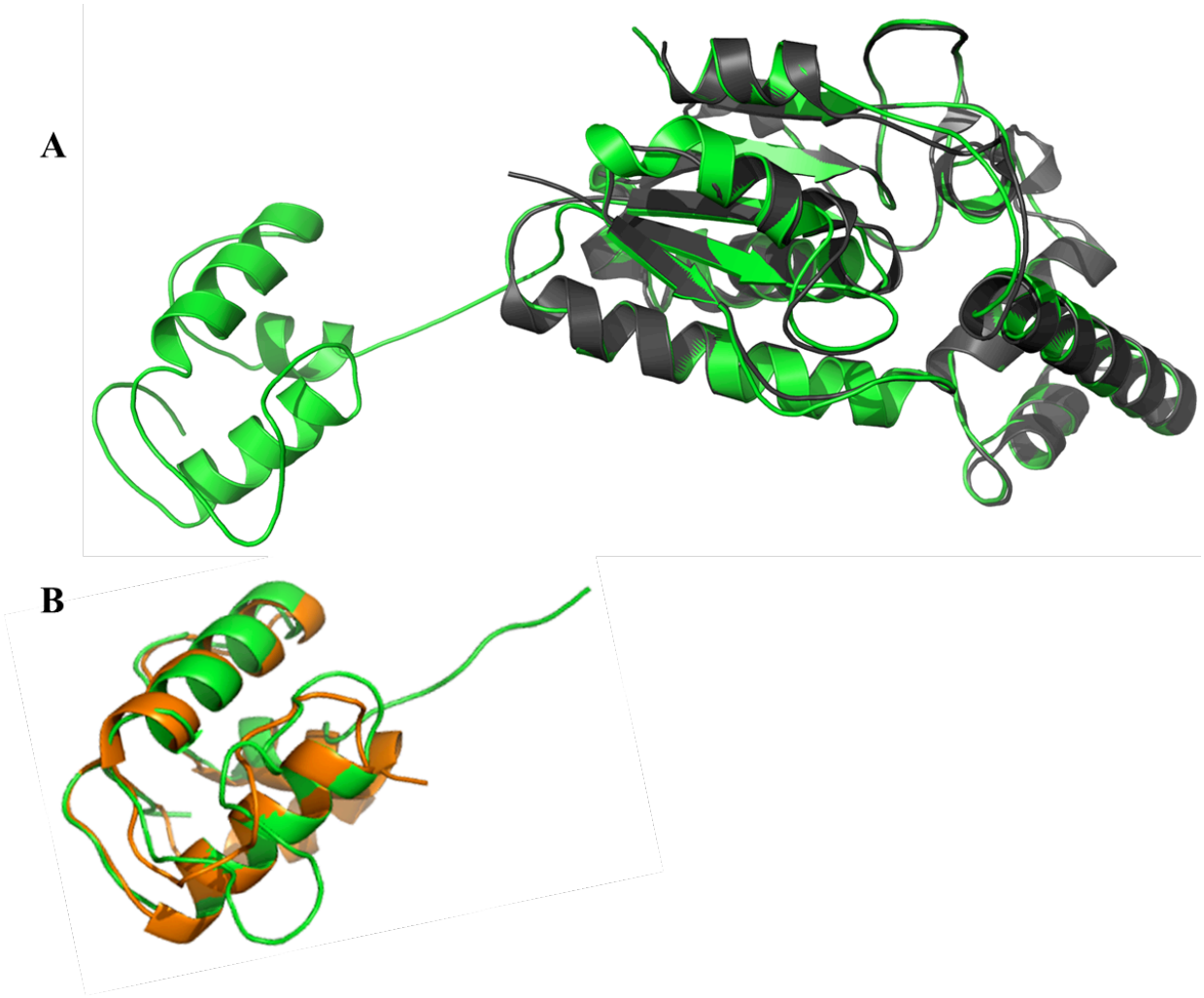


Figure 5. Lysterase structure.

A ribbon diagram alignment of the solved crystal structure of the mycobacteriophage D29 (gray) and a MODELLER[79,81]-predicted Bxb1 (green) lysterase proteins (**A**). The N-terminal domain of the Bxb1 LysB (green) and the cell wall binding domain of the *Pseudomonas* phage phiKZ endolysin (orange) (**B**).

3.2 RESULTS

We first set out to determine whether lysterases had any effect on bacteria when added exogenously. *In vivo*, lysterases are translated within the cell; therefore, they would first encounter the mycobacterial outer membrane at the mAGP complex where ester-linked mycolic acids are abundant. However, unlike in studies with endolysins from Gram-positive bacteriophage, whose targets in the peptidoglycan layer should be accessible to exogenously added enzymes, it was unclear whether lysterases would be able to find their targets from outside of the cell.

3.2.1 Lysterase activity against mycobacteria

M. smegmatis mc^2155 was grown to log-phase and suspended in phosphate buffered saline plus Tween (PBST). The cells were then treated with 2 μM of D29 or Bxb1 LysB or an equivalent volume of protein storage buffer as a negative control and incubated at 37ºC with agitation. At set timepoints, an aliquot of cells was removed, serially diluted, and spotted onto appropriate growth media agar plates. After several days, killing activity was assessed by comparing surviving colony forming units (cfu) from each LysB treatment to those from the negative control-treated cells. Surprisingly, not only were the LysB proteins apparently able to find their target when added exogenously, but their activity led to dramatic reduction of *M. smegmatis* mc^2155 survival within five minutes of incubation (Figure 6A). These experiments were also performed using stationary-phase cells, and similar results were observed.

To be sure this antimicrobial activity was due to the catalytic activity of the protein, as opposed to any artifacts of the experimental methods, site-directed mutagenesis was used to

change the catalytic serine (S158) of the Bxb1 LysB to an alanine. When the killing assay was repeated with this mutant, the catalytically inactive Bxb1 LysB had no effect on *M. smegmatis* survival (Figure 6B). Payne *et al* showed that mutating the catalytic serine (S82) to an alanine in the D29 LysB also abolished catalytic activity[35].

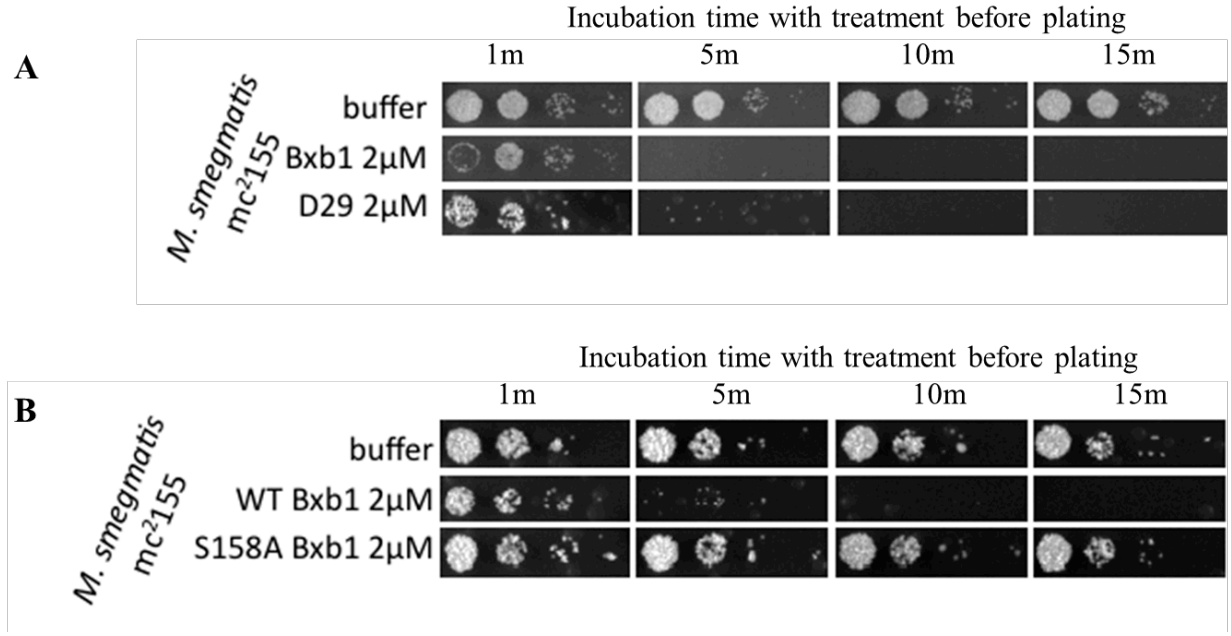


Figure 6. LysB treatment has an antimicrobial effect on *M. smegmatis*

Cells were incubated with LysB or buffer control until the indicated timepoint, at which an aliquot was removed, serially diluted, and spotted onto 7H10 agar plates. After 3-4 days, cell growth was compared across conditions. Incubating *M. smegmatis* cell suspensions with either D29 or Bxb1 LysB results in a dramatic decrease in survival after as little as five minutes (**A**). This killing activity is abolished when the catalytic serine is mutated to an alanine (**B**).

M. smegmatis is the mycobacteria laboratory workhorse, as they are much easier to grow than *M. tuberculosis*. For example, *M. smegmatis* takes only a few days to produce single colonies, whereas *M. tuberculosis* requires three weeks. However, it cannot be assumed that *M. smegmatis* is a perfect surrogate for *M. tuberculosis*. Therefore, we tested LysB activity against *M. tuberculosis* mc^{2}7000, an avirulent mutant strain of *M. tuberculosis*[82]. Similar reduction in surviving cells was observed when *M. tuberculosis* mc^{2}7000 was treated with the LysB proteins (Figure 7). These results are in agreement with recent publications, in which LysBs—from other mycobacteriophages—were tested against mycobacteria[74,76].

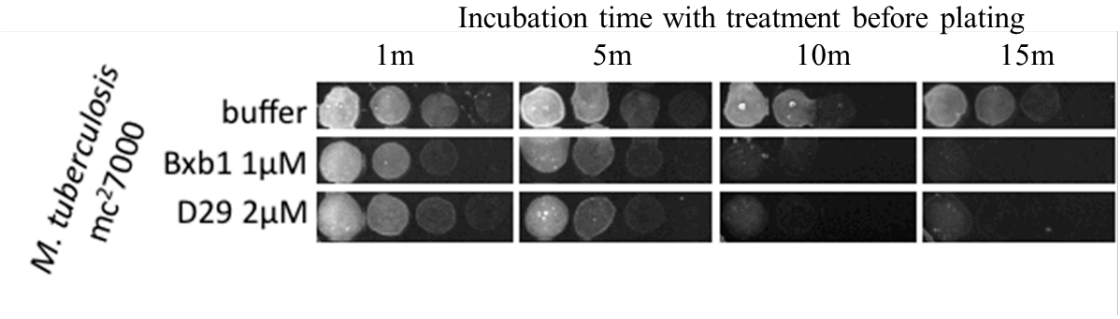


Figure 7. *M. tuberculosis* is susceptible to LysB antimicrobial activity

Cells were incubated with LysB or buffer control until the indicated timepoints, at which aliquots were removed, serially diluted, and spotted onto 7H11 agar plates. After two weeks, cell growth was compared across conditions. Both D29 and Bxb1 lysterases were also able to reduce the amount of *M. tuberculosis* survival. Cells needed to be incubated for at least ten minutes to see this effect.

3.2.2 Development of lysterase resistance

The development of antibiotic resistance is a major concern when treating any bacterial infection and some experts believe we are rapidly progressing toward a post-antibiotic era[83,84]. Fortunately, bacteria susceptible to endolysin treatment have never demonstrated the ability to develop resistance to the enzyme[14]. It is hypothesized that, because of the millennia of coevolution between the phages and their hosts, phage lysis enzymes have evolved to target immutable bacterial cell wall components[14]. Therefore, we were also interested in whether the mycobacteria could develop resistance to lysterase activity.

M. smegmatis cells that had survived the Bxb1 LysB or control treatment in an initial killing assay were used to seed new cultures. These cultures were then used in another round of killing assay. The original *M. smegmatis* culture was also passaged and used as a negative control. Therefore, for each generation of killing assay after the initial assay, three sets of *M. smegmatis* cells were used: naïve, buffer-treated, and Bxb1 LysB-treated. This method was then repeated for a total of three generations of killing assay. Survival after Bxb1 LysB or buffer treatment were compared across cell type. If LysB resistance developed, we would expect to see cells that had previously survived LysB treatment to be less susceptible to LysB in later generations (Figure 8A). Whereas, all cells, regardless of previous treatment, should be comparably susceptible to later-generation LysB treatment if no resistance has developed (Figure 8B).

After two generations, no evidence of Bxb1 LysB resistance was observed, as all cells showed similar survival against LysB treatment (Figure 8C). Our experimental method was modified from standard endolysin resistance assay[12], but it is possible that we did not run this

experiment for enough generations. Repetition alongside a positive control—*i.e.* an antibiotic toward which *M. smegmatis* is known to develop resistance—is needed.

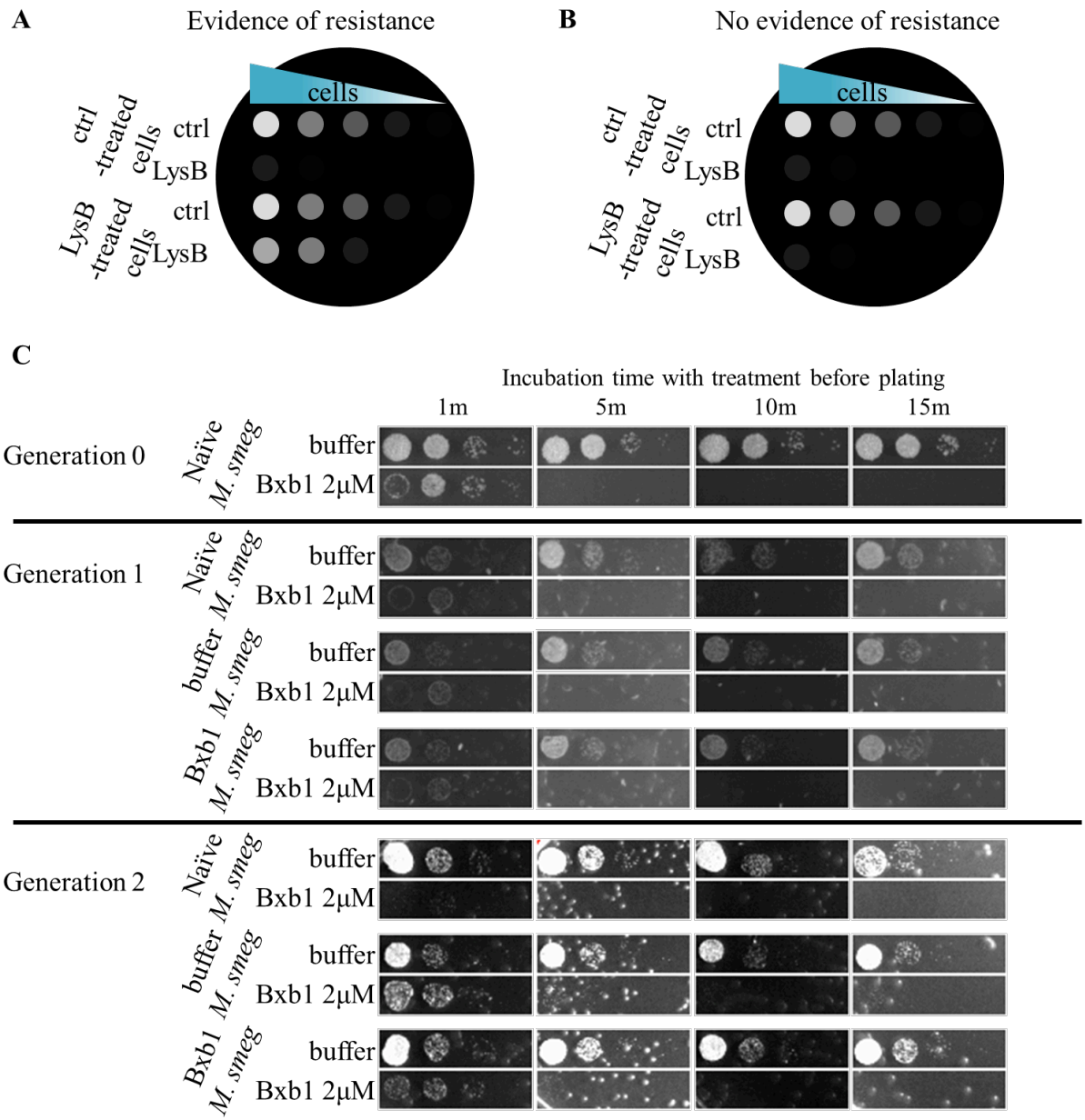


Figure 8. *M. smegmatis* did not develop resistance to Bxb1 LysB.

Models of expected results if cells pretreated with LysB do (**A**) or do not (**B**) develop resistance to LysB treatment. All *M. smegmatis* cells showed the same susceptibility to Bxb1 LysB, regardless of pretreatment with Bxb1 LysB or protein storage buffer (**C**). The original (naïve) *M. smegmatis* cells were passaged and subjected to killing assays to control for any effects of extended culturing.

3.2.3 Lysterase activity against other mycolata

While most endolysins have antimicrobial activity only against the natural host bacteria of the phages that encode them, little is known about the susceptibility range of lysterases. We hypothesized that other mycolic acid-containing bacteria might be susceptible to a protein that hydrolyzes mycolic acids from the mycobacterial cell wall. Some species of the genera *Corynebacterium, Nocardia,* and *Rhodococcus* are known to have mycolic acids in their cell walls, but with fewer carbons per chain[31]. Together with the mycobacteria, these taxa are known as the mycolata. Furthermore, unlike in mycobacteria, the biosynthesis of these mycolic acids is not essential[31]. That is, these species can grow in the presence of mycolic acid synthesis inhibitors such as isoniazid[85,86].

Killing assays were performed similarly for the mycolata as with the mycobacteria, except that the mycolata were incubated with LysB at 30ºC with agitation. With the exception of *Corynebacterium glutamicum*, all members of the mycolata tested were susceptible to both D29 and Bxb1 LysB proteins (Figure 9). Interestingly, the Bxb1 LysB had an antimicrobial activity on *C. glutamicum*, while the D29 enzyme did not have any effect. The reason for this difference in activity is unknown, but an interesting hypothesis is that the N-terminal domain of Bxb1 LysB plays a role in its ability to kill *C. glutamicum.* Therefore, *C. glutamicum* could provide a model system for investigating the differences between the two lysterases.

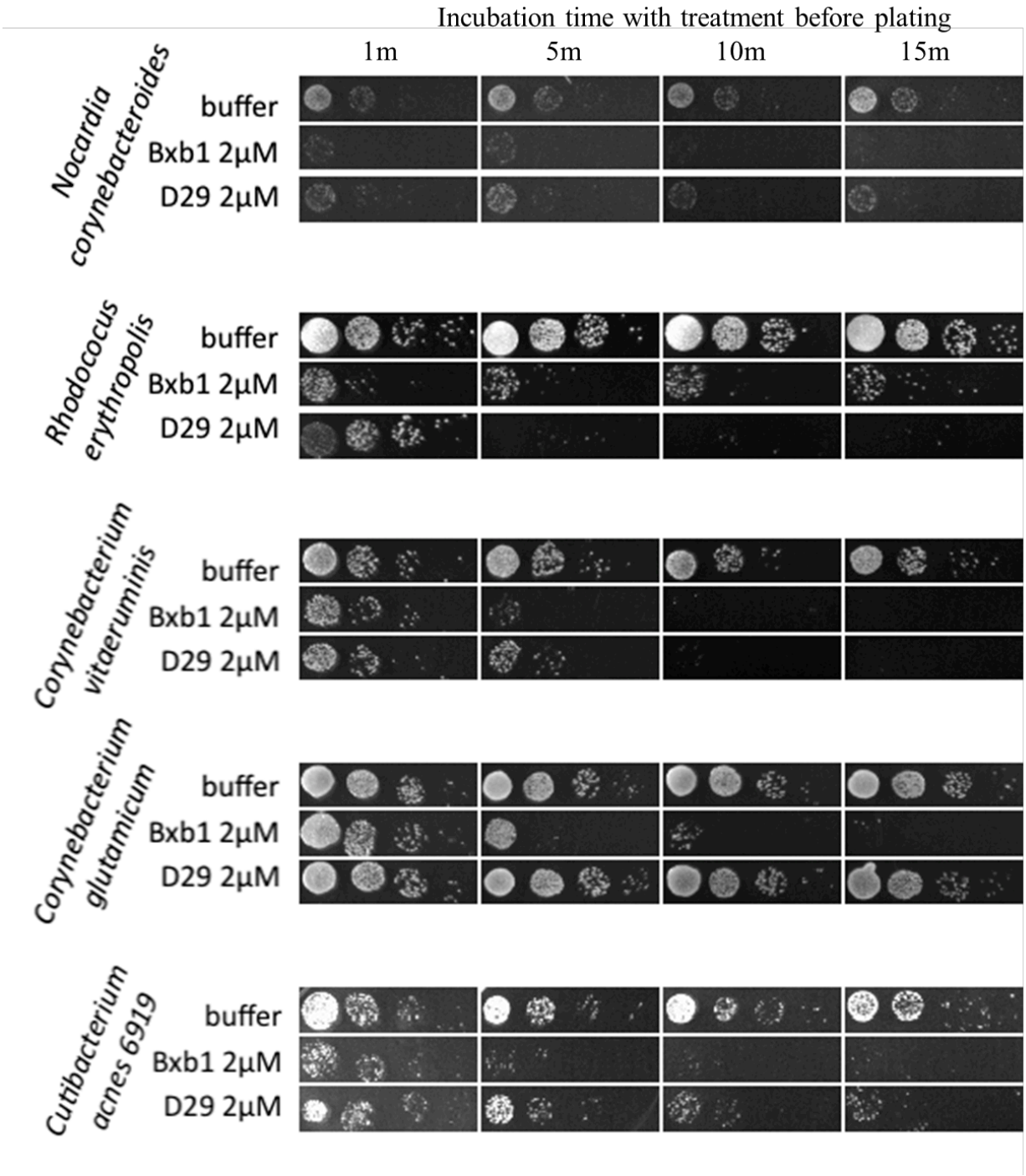


Figure 9. LysB antimicrobial activity against members of the mycolata.

For the mycolata, killing assays were performed as above for *M. smegmatis*, except that incubation and growth took place at 30ºC. Both D29 and Bxb1 LysB had antimicrobial effect on *N. corynebacteroides*, *R. erythropolis*, and *C. vitaeruminis.* Some of these strains saw reduction of survival in as little as one minute of incubation. Only Bxb1 LysB affected *C. glutamicum*. No *C. glutamicum* killing was seen with D29 LysB even if the incubation time was extended to 60 minutes (data not shown).

3.2.4 Resistance of other Gram-positive and Gram-negative bacteria to lysterases

We then tested LysB susceptibility on Gram-positive *Staphylococcus epidermidis* and *Bacillus cereus* and Gram-negative *Serratia marcescens* and *E. coli*. Because none of these bacteria contain mycolic acids in their cell walls, we hypothesized that they should all be resistant to LysB treatment. Indeed, none of the Gram-positive or Gram-negative bacteria selected were susceptible to either LysB (Figure 10).

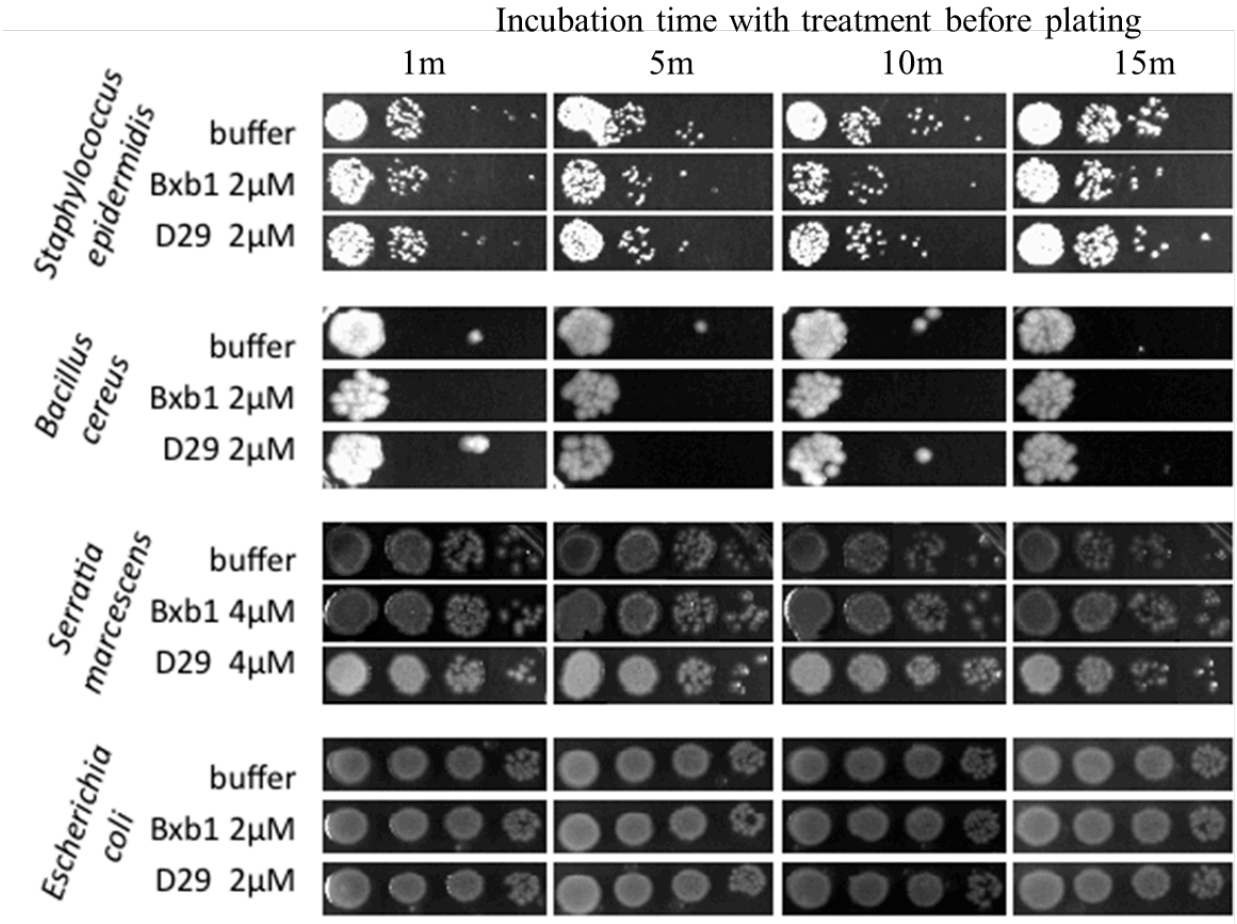


Figure 10. Other Gram-positive and Gram-negative bacteria are not susceptible to LysB treatment.

Killing assays were performed as above. *S. epidermidis* and *E. coli* were incubated and grown at 37ºC, while 30ºC was used for *S. marcescens* and *B. cereus*. None of the bacteria from outside the Order Actinomycetales were affected by treatment with either LysB. In some cases, like *S. marcescens*, a range of LysB concentrations were used to determine if resistance was dose dependent, but the proteins had no effect even at higher concentrations. Increasing incubation times to 60 minutes also did not have any effect on survival (data not shown).

4.0 LYSTERASE ACTIVITY AGAINST *CUTIBACTERIUM ACNES*

We also wished to test the LysB susceptibility of bacteria that 1) do not contain mycolic acids, but 2) are closely related to the mycobacteria. *Cutibacterium acnes* fit this description, as it is a member of the Order Actinomycetales, which includes the mycolata, but mycolic acids have never been isolated from their cell walls[87].

Originally placed in the genus *Corynebacterium*[88], the acne bacillus was later recategorized as a *Propionibacterium* when it was discovered that it produced propionic acid as a major product of glucose fermentation[89]. Within the last year, a phylogenetic analysis showed the genus *Propionibacterium* to be non-monophyletic, leading to the creation of the novel genus *Cutibacterium*[90]. *Cutibacterium* species are differentiated from other former *Propionibacterium* species by their peptidoglycan amino acids (discussed below), their common presence on human skin, and their lower G+C content[90]. *C. acnes* is the implicated causative pathogen in the skin disease acne vulgaris. While acne vulgaris lacks a model organism, *C. acnes* cannot definitively be named the causative pathogen, but the bacterium is present at about 100-fold greater concentrations in acne patients than age-matched healthy individuals[91]. Acne vulgaris is the single most common cutaneous disorder, affecting 80% of adolescents and 50% of adults worldwide[92]. Although it is a nonfatal disorder, the United States alone spends approximately four billion dollars annually treating acne[93]. The most common treatments involve topical and/or oral antibiotics and retinoids or topical astringents, but many of these have worrying side

effects[93]. Additionally, as antibiotic resistance increases worldwide[93], alternative therapeutics—especially with fewer side effects—are always in demand.

4.1 *C. ACNES* CELL WALL

Unfortunately, like the vast majority of bacteria, little is known about the specifics of the *C. acnes* cell wall. The cell-wall sugars of this Gram-positive, aerotolerant anaerobe are glucose, mannose, and—in some strains—galactose, with peptidoglycan amino acids alanine, glutamic acid, glycine, LL-2,6-diaminopimelic acid (LL-A_2PM), and—in some strains—*meso*-2,6-diaminopimelic acid (*meso*-A_2PM)[87,90]. Additionally, part of their historical differentiation from other members of the Order Actinomycetales relies on *C. acnes* lack of mycolic acids[88]. While *C. acnes* bacteriophages share some genes with the mycobacteriophages, they have low genetic diversity, and no lysterase gene has been identified in any *C. acnes* phage[35,94–96].

4.2 RESULTS

In the Hatfull laboratory, *C. acnes* is grown in small, closed anaerobic chambers using AnaeroPack sachets or in sealed glass tubes with Oxyrase, an anaerobic media additive. Therefore, the killing assays could not be performed entirely anaerobically. Anaerobically grown cells were prepared and treated with LysB or buffer in aerobic conditions. Once the timepoints were spotted onto A Media plates, the plates were placed in anaerobic chambers to grow. We did

not expect nor did we see any evidence that the brief aerobic handling of the cells affected the experiment, as *C. acnes* is aerotolerant.

Due to their lack of mycolic acids, we predicted that the LysB proteins would have no effect on *C. acnes* 6919. Therefore, we were surprised to find that *C. acnes* 6919 was susceptible to both D29 and Bxb1 LysB (Figure 11).

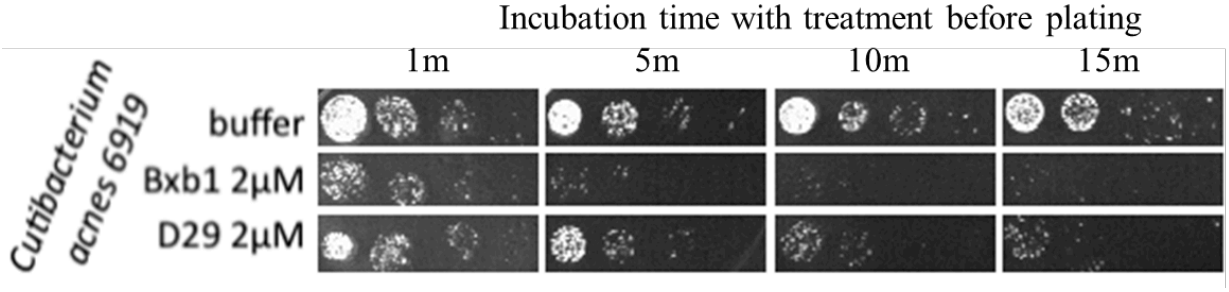


Figure 11. LysB antimicrobial activity against *C. acnes*.

Both D29 and Bxb1 LysBs had killing activity against *C. acnes* despite the lack of mycolic acids produced by the bacterium. Bxb1 LysB dramatically reduced the number of surviving cfus within five minutes of incubation, while it took ten minutes for the D29 enzyme to achieve comparable killing

4.2.1 Lysterase treatment releases a lipid from *C. acnes* cells

To investigate the effect of LysB on *C. acnes*, we treated whole *C. acnes* 6919 cells with each LysB, performed total lipid extraction, and visualized any changes in lipid profile using TLC (Figure 12) as we have previously done for *M. smegmatis*. Clearly LysB treatment releases at least one unidentified lipid from the *C. acnes* 6919 cell wall (Figure 12, Bxb1 or D29 lanes vs buffer lanes). Due to the dearth of information on *C. acnes* cells wall components, appropriate standards could not be run. Instead, we utilized the same running conditions[77] as above with *M. smegmatis* (Figure 4), which have been optimized to visualize free mycolic acids (Figure 12A) and trehalose dimycolate and trehalose monomycolate (Figure 12B). This way, even though *C. acnes* lack mycolic acids, any lipids with similar properties should be visible. Scraping the

band(s) in question from the silica TLC plate, purifying the lipid(s), and analysis with mass spectrometry is a logical next step, but the carbon- and hydrogen-rich nature of lipids complicates definitive identification.

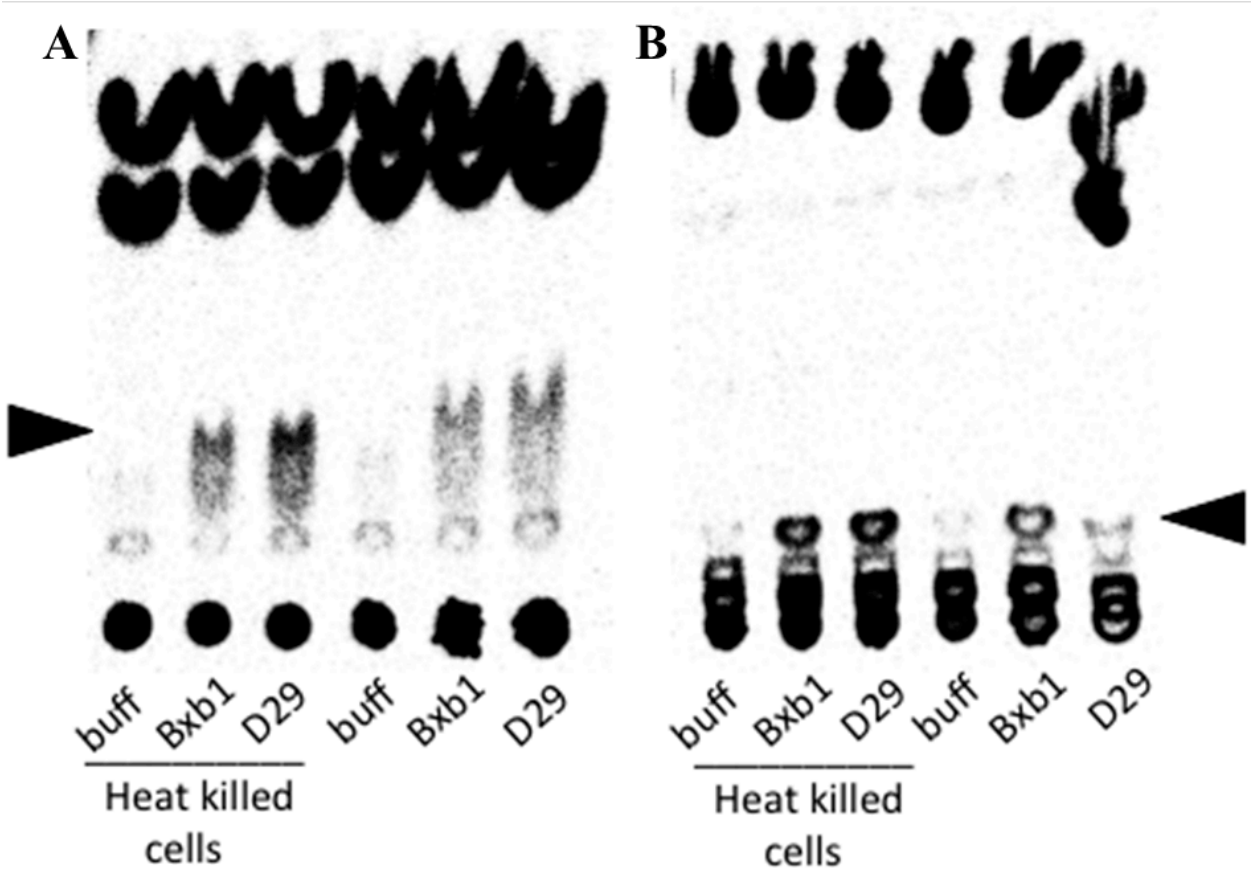


Figure 12. LysB treatment releases at least one lipid from whole *C. acnes* cells.

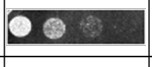
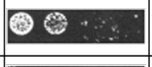
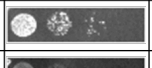
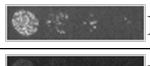
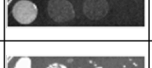
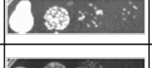
C. acnes cells were treated with either D29 or Bxb1 LysB and analyzed as with *M. smegmatis* above. Because little is known about the *C. acnes* cell wall, appropriate standards could not be run, but the same running conditions were used to enable qualitative comparisons. At least one unidentified lipid (arrows) is released from *C. acnes* cells during LysB treatment as compared to the buffer control when TLC is performed under optimized conditions[87] to visualize free mycolic acids (**A**) and the difference between trehalose di- and monomycolate (**B**).

4.2.2 Lysterase activity against *C. acnes* clinical isolates

Not only is *C. acnes* the implicated causative agent in acne vulgaris, it is also an opportunistic pathogen, particularly in surgical settings. *C. acnes* strains involved in different infections have been correlated with specific genetic markers[97–100]. Ribotypes (RT) 1, 4, 5, and 8 are associated with acne, RT 3 with eye infections, and RT 6 with blood and medical device infections[97]. There are exceptions to these associations; for example, *C. acnes* strain KPA 171202 is RT 1, but is associated with blood and medical device infections rather than acne[100].

Because of the variety of *C. acnes* clinical isolates, we were interested in testing our LysBs against a range of *C. acnes* genetic markers, such as ribotype (Table 1). Killing assays for *C. acnes* clinical isolates were carried out in the same manner as *C. acnes* 6919. While some of our clinical isolates were susceptible to one or both of our LysBs, others were entirely resistant. At present, no clear pattern has emerged from the clinical isolate data, and more testing is needed.

Table 1. LysB effect on *C. acnes* clinical isolates.

Strain	Phylotype	Ribo-type	MLST4	Skin Assoc.	Disease Assoc.	Buffer by 15 min	Bxb1 LysB by 15 min	D29 LysB by 15 min
HL036PA1	IA-1	532	IA1 (CC1)	Neutral	Acne		 **S**	 **R**
ATCC 6919	IA-2 plasmid -	1	IA1 (CC3)	Neutral	Acne		 **S**	 **S**
HL043PA1	IA-2 plasmid +	5	IA1 (CC3)	Acne	Acne		 **R**	 **R**
HL013PA1	IB-2	3	IA2	Neutral	Eye Infection		 **R**	 **R**
HL067PA1	IB-2	3	IA2	Neutral	Eye Infection		 **R**	 **R**
KPA 171202	IB-3	1	IB	Neutral	Medical Device & Tissue Infections		 **R**	 **R**
HL042PA3	II	6	II (ST7)	Healthy Skin	Medical Device & Blood Infections		 **S**	 **S**
HL110PA4	II	6	II (ST7)	Healthy Skin	Medical Device & Blood Infections		 **S**	 **S**

C. acnes clinical isolate strains HL036PA1, HL042PA3, and HL110PA4 were susceptible to treatment by either LysB protein. Strains HL043PA1, HL013PA1, HL067PA1, and KPA171202 were resistant to both enzymes. *C. acnes* ATCC 6919 is included for comparison. S and R indicate susceptibility or resistance, respectively, to each LysB.

Phylotype, ribotype, and multi-locus sequence type (MLST) are three methods that have been used to categorize *C. acnes* stains. *C. acnes* strains were originally classified into distinct phylotypes[98] based on serological agglutination tests and cell wall sugar analysis[87]. Ribotype refers to the 16s rDNA sequence of each *C. acnes* strain; RT1 is the most abundant, and all other ribotypes have 99% or greater sequence similarity to RT1[97]. MLST relies on analyzing seven core housekeeping genes[99].

5.0 DISCUSSION, CONCLUSIONS, AND FUTURE DIRECTIONS

Our data clearly show that lysterases from D29 and Bxb1 have antimicrobial activity against members of the Order Actinomycetales, and other Gram-positive and Gram-negative species are not susceptible to LysB activity. This is in partial agreement with recently published results[74,76]. Both Grover *et al* and Lai *et al* saw antimicrobial activity against mycobacteria, but Lai did not see activity against any other bacteria they tested. This discrepancy in our results is most likely due to sampling, as Lai and colleagues did not test any other members of the Actinomycetales[67]. It could be valuable to continue testing strains within the Order Actinomycetales and less closely-related species in the Phylum Actinobacteria to find the limit of LysB susceptibility, especially as there are other bacteria within the Actinomycetales that lack mycolic acids such as *Streptomyces* species. Such results could help elucidate the target of LysB activity in *C. acnes*, since LysB's *in vitro* ability to hydrolyze ester-linked carbon chains of varying length from *p*-nitrophenyl[34,35,74] and kill *C. acnes*, which lacks mycolic acids—the only known biologically relevant substrate of LysB activity—suggest that LysB has broader specificity than previously anticipated.

Additionally, it has been previously hypothesized that lysterases could only be useful therapeutics when used in combination with endolysins[101]. While combination therapies many increase the range and/or efficacy of LysB therapies, it is clear from our results that—at least in the conditions tested—LysBs have antimicrobial activity in the absence of an endolysin. Indeed,

in the two instances of endolysin and lysterase combination treatments, LysB-only treatment had significantly more antimicrobial activity than endolysin treatment alone, and combined the two enzymes did not kill mycobacteria more effectively than the LysB alone[21,76].

Finally, while more investigation is needed, lysterases appear to be a promising enzyme for use in the fight against mycobacterial and *C. acnes* infections. The ability of LysB to quickly degrade the mycobacterial outer membrane and the ease with which the proteins can be overexpressed and purified suggest that lysterases could be a potentially useful tool in diagnosing Mtb infections, as they could replace harsh chemicals and lengthy sonications during DNA extraction. Whether LysBs could be useful as an Mtb therapeutic is still unknown, as the question of delivery through granulomas is unanswered. Lai *et al.*[76] found that exogenous lysterase treatment of Mtb-infected macrophages resulted in the reduction of the bacteria, but this result should be viewed with a healthy dose of skepticism until more research is conducted. Our results are more promising for LysB use against *C. acnes*, as most acne vulgaris treatments are applied topically. Furthermore, topical application eliminates the concern of potential antibody generation against LysB that comes with intravenous administration. In fact, the University of Pittsburgh has filed a patent for the use of lysterases against *C. acnes*, and Dermalytica Inc. has optioned the patent for development.

6.0 MATERIALS AND METHODS

6.1 PLASMIDS

The original Bxb1 LysB expression plasmid (pDB1) was created by cloning the Bxb1 LysB gene PCR product into the pET21a+ vector using *NdeI* and *EcoRI* restriction enzyme sites. The catalytically inactive mutant Bxb1 LysB expression plasmid (pDB2) was created from pDB1 using Q5 (New England Biolabs) site-directed mutagenesis (SDM) to change the catalytic serine 158 to alanine. The N-terminal 77 amino acids of the Bxb1 LysB were deleted from pDB1 using Q5 SDM to create pDB3. Gibson assembly was utilized to create the Bxb1 LysB-fluorescent protein fusion constructs, pDB4 and pDB5, which have the lysterase fused to Clover and super-folder GFP (sfGFP), respectively. The DNA templates for the fluorescent proteins were provided by Jenna Zalewski from the Andrew Van Demark lab (University of Pittsburgh) in the form of separate plasmids containing Clover-hRock and sfGFP-thrombin. Q5 SDM was used delete the 77 N-terminal amino acids of Bxb1 LysB from pDB4 to create a ΔN-Bxb1-Clover construct, pDB6, and all Bxb1 LysB amino acids **except** for the first 77 to create a ΔC-Bxb1-Clover protein: pDB7. Catalytically inactive mutants of pDB4 and pDB6 were constructed in the same manner as pDB2 and were named pDB8 (catalytically inactive full-length Bxb1-Clover) and pDB9 (catalytically inactive ΔN-Bxb1-Clover), respectively. The D29 LysB expression plasmid (pLAM3) was created by Dr. Laura Marinelli during her time as a graduate student in the Hatfull

lab. All plasmids used a pET21a+ backbone; therefore, the origin was *oriE*, the selection marker was ampicillin/carbenicillin, and each protein had a C-terminal His-6 tag. A list of these plasmids and the primer required for their construction can be found in Tables 2 and 3, respectively.

Table 2. Expression plasmids used in these studies.

Plasmid	Gene of Interest	Parental Plasmid	Insert Source	Restriction Sites	Primers	Cloned by
pDB1	Bxb1 gp9	pET21a+	Bxb1 gDNA	NdeI, EcoRI	DBO1, DBO2	Dominque Barbeau
pDB2	Bxb1 gp9 S158A mutant (catalytically inactive)	pDB1		NdeI, EcoRI	DBM3, DBM4	Dominque Barbeau
pDB3	Bxb1 gp9 N-terminal deletion mutant	pDB1		NdeI, EcoRI	DBM5, DBM6	Dominque Barbeau
pDB4	Bxb1 gp9 and Clover fluorescent protein fusion	pDB1	Clover-hRock plasmid	NdeI, EcoRI	DBG17, DBG18, DBG21, DBG22	Dominque Barbeau
pDB5	Bxb1 gp9 and superfolderGFP fusion	pDB1	sfGFP-thrombin plasmid	NdeI, EcoRI	DBG17, DBG18, DBG25, DBG26	Dominque Barbeau
pDB6	Bxb1 gp9 N-terminal deletion mutant and Clover fusion	pDB4		NdeI, EcoRI	DBM5, DBM6	Dominque Barbeau
pDB7	Bxb1 gp9 C-terminal deletion mutant and Clover fusion	pDB4		NdeI, EcoRI	DBM9, DBM10	Dominque Barbeau
pDB8	catalytically inactive Bxb1 gp9 mutant and Clover fusion	pDB4		NdeI, EcoRI	DBM3, DBM4	Dominque Barbeau
pDB9	catalytically inactive Bxb1 gp9 N-terminal deletion mutant and Clover fusion	pDB6		NdeI, EcoRI	DBM3, DBM4	Dominque Barbeau
pLAM3	D29 gp12	pET21a+	D29 gDNA			Laura Marinelli

Table 3. Cloning primers used in these studies.

Primer	Sequence	Restriction Site	Tm	Description
DBO1	CTA GGT CAT ATG CCG CTT AAG CTC GGC GA	NdeI	58C	Forward cloning primer for Bxb1 gp9 (LysB)
DBO2	TCT GAT GAA TTC CAA GTC ACA GAG CGC AGA AAG TCC GTC	EcoRI	58C	Reverse cloning primer for Bxb1 gp9 (LysB)
DBM3	/5Phos/ GCG CCT TGC GGG TAG CCG ATC		68C	Forward Q5 primer for mutating the Bxb1 gp9 catalytic serine to alanine
DBM4	/5Phos/ GAT CGT CAC CTC CGA GCT GTG		68C	Reverse Q5 primer for mutating the Bxb1 gp9 catalytic serine to alanine
DBM5	CAT ATG TAT ATC TCC TTC TTA AAG TTA AAC AAA ATT ATT TCT AGA G	NdeI	68C	Forward Q5 primer for deleting the first 77 amino acids of Bxb1 gp9
DBM6	GCC CGT CCG GTA CTG CTC		68C	Reverse Q5 primer for deleting the first 77 amino acids of Bxb1 gp9
DBG17	TGG AAT TCG AGC TCC GTC	EcoRI	65C	Linearizes pDB1 directly after (but does not include any of the amino acids of) Bxb1 gp9 to create Gibson Assembly template
DBG18	GCC ACC TCC GCC ACC AGT CAC AGA GCG CAG AAA G		65C	Linearizes pDB1 directly after (and includes the final amino acids of) Bxb1 gp9 and adds a 3' 5 glycine linker to create Gibson Assembly template
DBG21	ACT TTC TGC GCT CTG TGA CTG GTG GCG GAG GTG GCA TGG TGA GCA AGG GCG AG		69C	Forward primer to create Gibson Assembly template for Clover FP with a 5' 5 glycine linker
DBG22	TCG ACG GAG CTC GAA TTC CAG GCG GCG GTC ACG AAC TC	EcoRI	69C	Reverse primer to create Gibson Asesembly template for Clover FP
DBG25	ACT TTC TGC GCT CTG TGA CTG GTG GCG GAG GTG GCA TGA GCA AAG GTG AAG AAC		58C	Forward primer to create Gibson Assembly template for sfGFP with a 5' 5 glycine linker
DBG26	TCG ACG GAG CTC GAA TTC CAG CTA CCT TTA TAC AGT TCA TC	EcoRI	58C	Reverse primer to create Gibson Asesembly template for sfGFP
DBM9	ATC CTC GGG AGC CGG AAT C		70C	Forward Q5 primer for deleting the final 245 amino acids of Bxb1 gp9
DBM10	GGT GGC GGA GGT GGC ATG		70C	Reverse Q5 primer for deleting the final 245 amino acids of Bxb1 gp9 from either FP fusion

6.2 PROTEIN OVEREXPRESSION AND PURIFICATION

Protein induction and purification was carried out similarly for all of the constructed proteins. BL21 (DE3) *E. coli* cells (Invitrogen/Life Technologies) carrying the LysB plasmid were grown to OD_{600} 0.5 to 0.7 at 37°C in LB containing carbenicillin (50 μg/mL), followed by induction with 2 mM IPTG overnight (18 h) at 18°C with shaking. Cells were pelleted and suspended in 1mL lysis buffer (500 mM NaCl, 250 mM Tris pH 8, 10% glycerol, 1 mM BME) per gram of pelleted cells and then frozen at -80°C. Pellets were thawed and sonicated 30x 30s bursts on ice. Lysate was separated from the pellet by centrifugation at 13,000 rpm for 30 min at 4°C. The lysate was added to 2 mL per original 500 mL culture of nickel-NTA agarose beads (Qiagen) and rocked at 4°C for at least 1 hour. The beads were then washed sequentially by pipetting within the column at 4°C with 10 mL of lysis buffer (3 times), 15 mL of lysis buffer with 10 mM imidazole (3 times), and 15 mL of lysis buffer with 50 mM imidazole (4 times). The beads were allowed to settle in the column and bound protein was eluted with 10 mL of 150 mM imidazole in lysis buffer. A supplemental elution of 5 mL of lysis buffer with 250 mM imidazole was used to remove any additional bound protein. Samples of each elution and supplemental elution fraction were analyzed via SDS-PAGE and fractions containing significant amounts of protein were concentrated using dialysis against storage buffer (50 mM Tris pH 8, 50 mM NaCl, 50% glycerol). Protein was stored at -20°C. Concentrations were determined via Bradford colorimetric assay (Bio-Rad).

6.3 KILLING ASSAY: COLONY GROWTH AFTER LYSTERASE TREATMENT

Cells and treatment conditions were prepared in 1.5 mL Eppendorf tubes according to the formula: 50 µL x (# of timepoints + 1). Bacterial cells were grown to log-phase and then pelleted and suspended in phosphate buffered saline with Tween (PBST) at a concentration of $1x10^6$ cfu/mL; one tube of cells per treatment condition. For treatment conditions, like 2 µM Bxb1 LysB, the specified protein was added to additional storage buffer so that the total volume of protein and buffer was equal to the largest volume of protein needed. PBST was then added to reach the final volume as calculated by the formula above. Cells were then pelleted again and suspended in their treatment condition. Mixtures were incubated at 37°C with agitation. At each timepoint, 50 µL of each mixture was removed to a 96 well plate and serially diluted by a factor of 10. 5 µL of each dilution was then spotted onto an agar plate of appropriate growth media, and the cells were allowed to grow under normal growing conditions. All strains tested can be found in Table 4. After several days—long enough for each cell type to form single colonies—surviving colony forming units were counted. These experiments have also been conducted on stationary-phase cells, with similar results.

Table 4. Bacterial strains used in these studies.

Strain	Growth media	Selection marker
Mycobacterium smegmatis mc^2155	7H9, 7H10	CB, CHX
Mycobacterium tuberculosis mc^27000	7H9, 7H11	CB, CHX
Nocardia corynebacteroides ATCC31130	PYCa	
Rhodococcus erythropolis	PYCa	
Corynebacterium vitaeruminis	PYCa	
Corynebacterium glutamicum	PYCa	
Propionibacterium acnes ATCC 6919	RCM, A media	KAN
Propionibacterium acnes HL036PA1	RCM, A media	KAN
Propionibacterium acnes HL043PA1	RCM, A media	KAN
Propionibacterium acnes HL013PA1	RCM, A media	KAN
Propionibacterium acnes HL067PA1	RCM, A media	KAN
Propionibacterium acnes KPA171202	RCM, A media	KAN
Propionibacterium acnes HL042PA3	RCM, A media	KAN
Propionibacterium acnes HL100PA4	RCM, A media	KAN
Staphylococcus epidermidis	LB	
Bacillus cereus	LB	
Serratia marcescens	LB	
Escherichia coli NEB5a	LB	

6.4 LYSTERASE RESISTANCE

A new *M. smegmatis* culture was resurrected and labeled "naïve," as they had never seen previous treatment with LysB or protein storage buffer. These "Generation 0" cells were used in an initial killing assay as described above. New liquid cultures were started from Generation 0 cells that survived one minute of incubation with buffer or Bxb1 LysB and from an aliquot of the Generation 0 naïve cells. These "Generation 1" cells were used in a subsequent killing assay, and

the method was repeated for a final generation. Survival was then compared to assess whether susceptibility to LysB changed if cells were previously treated with LysB. This method was modified from Loeffler *et al*.[12] who assayed for resistance over as little as three and as many as sixteen generations.

6.5 LYSTERASE EFFECT ON WHOLE CELL LIPIDS

M. smegmatis or *C. acnes* cells were grown in the presence of ^{14}C-acetate for three or twenty-four hours, respectively. Bacterial cells were grown to log-phase and then pelleted and suspended in PBST a concentration of $1x10^9$ cfu/mL. Half of the cells were incubated at 100°C for 10 minutes. Cells were then mixed with 8 μM of either LysB or an equivalent volume of protein storage buffer. Cell mixtures were incubated at 37°C with agitation for 15 minutes. Total lipids were extracted and analyzed by thin layer chromatography, as described in Besra 1998[77].

Briefly, cell mixtures were transferred to glass tubes and pelleted. The supernatant was discarded. The pellet was suspended in chloroform:methanol (2:1), a magnetic stir bar was added, and the extractions were incubated overnight at 37°C with stirring. The following day, another 2 mL of chloroform:methanol (2:1) were added and evaporated off. Four milliliters of chloroform:methanol:water (4:2:1) were added to the lipids and centrifuged to separate the aqueous and organic layers. The bottom layer was removed to a new glass tube and the solvents were evaporated off. Finally, the lipids were suspended in 200 μL of chloroform:methanol (2:1), the radioactivity of the lipids was measured using a scintillation counter, and samples were spotted on the TLC silica plate at equal radioactivity. To separate free mycolic acids, the samples were analyzed using a running buffer of chloroform:methanol (97:3). A running buffer of

chloroform:methanol:ammonium hydroxide (40:10:1) was used to visualize trehalose di- and mono-mycolate. Radiographs were captured using a FujiFilm FLA-5100 imager.

6.6 CELL LABELING AND IMAGING

Bacterial cells were grown to log-phase and then pelleted and suspended in PBST at a concentration of $1x10^8$ cfu/mL in 1.5 mL Eppendorf tubes. Cells were then pelleted and suspended in 3 μM of each Clover fusion protein or an equivalent volume of protein storage buffer. After 10 minutes of incubation at 37°C with agitation, the cells were pelleted and the supernatant containing any unbound Clover fusion proteins was discarded. Cells were washed three times with and then suspended in PBST. 7.5 μL of each sample was spotted onto microscope slides, coverslips were attached, and the cells were observed on a fluorescent microscope. Meanwhile, 50 μL of each sample was moved to a 96-well plate and fluorescence was recorded using a FujiFilm FLA-5100 imager.

APPENDIX A CELL LABELING USING LYSB-FLUORESCENT PROTEIN FUSIONS

A.1 INTRODUCTION

As discussed in Chapter 2, cell wall binding domains from endolysins of Gram-positive phages have been used to selectively label their specific phage's host bacteria[14,16,40,43,44]. While a definitive CBD has not been identified in any lysterase, the N-terminal domain of the Bxb1 LysB—which is present in 83% of all LysB proteins—has HHpred predicted structural similarity to the CBD of the *Pseudomonas* phage phiKZ. Additionally, it is hypothesized that all LysB proteins should bind quite tightly to their substrates, as it would be detrimental for phages to release lytic proteins into their environment, potentially lysing new host cells for the progeny phages. Investigating whether all or part of a lysterase could bind and label cells, with the help of a fluorescent protein, could not only lead to a diagnostic tool, but also potentially elucidate the role of the N-terminal domain.

A.2 RESULTS AND DISCUSSION

Clover fluorescent protein was fused to 1) full-length Bxb1 LysB, 2) Bxb1 LysB with the N-terminal domain deleted (ΔN), and 3) only the N-terminal domain of Bxb1 LysB (ΔC). Storage

buffer and Clover fused to human Rho Kinase (hRock, provided by Jenna Zalewski of the Andrew Van Demark laboratory) were used as negative controls. Whole *M. smegmatis* cells were incubated with 3 μM of each Clover fusion protein or an equivalent volume of protein storage buffer. After 10 minutes, the cells were pelleted, and the supernatant—with any unbound Clover fusion proteins—was removed. The cells were washed three times with PBS before being spotted onto microscope slides or into 96-well plates for fluorescent imaging.

The full-length Bxb1 LysB-Clover fusion protein was able to stain *M. smegmatis*, while there was somewhat reduced labeling with the ΔN-Bxb1-Clover and ΔC-Bxb1-Clover fusions (Figure 13), suggesting that both domains are necessary for efficient LysB binding. This result is not without caveats though, as catalytically active versions of the full-length Bxb1- and ΔN-Bxb1-Clover fusions were used. At the very least, we expect this to affect the level of fluorescence detected in the full-length Bxb1-Clover treated sample, because as cells are lysed, their cell debris—presumably still with Bxb1 bound—would be removed during the wash steps. Catalytically inactive versions of both proteins have been made, but unfortunately there was not time to repeat the experiment with these proteins. It would also be worthwhile to repeat this experiment with D29-Clover fusions, including a mutant with the N-terminal domain from Bxb1 LysB attached to the N-terminus of the D29 LysB-Clover fusion protein.

Furthermore, these experiments could also be repeated using different bacterial species. It would be interesting to see if a LysB-Clover fusion can bind to *C. acnes* or if differential binding can be observed between D29 LysB- and Bxb1 LysB-Clover fusion proteins on *C. glutamicum*. *E. coli* could be used as a negative control, as *E. coli* is not susceptible to LysB activity and should not contain a LysB target for the fusion protein to bind.

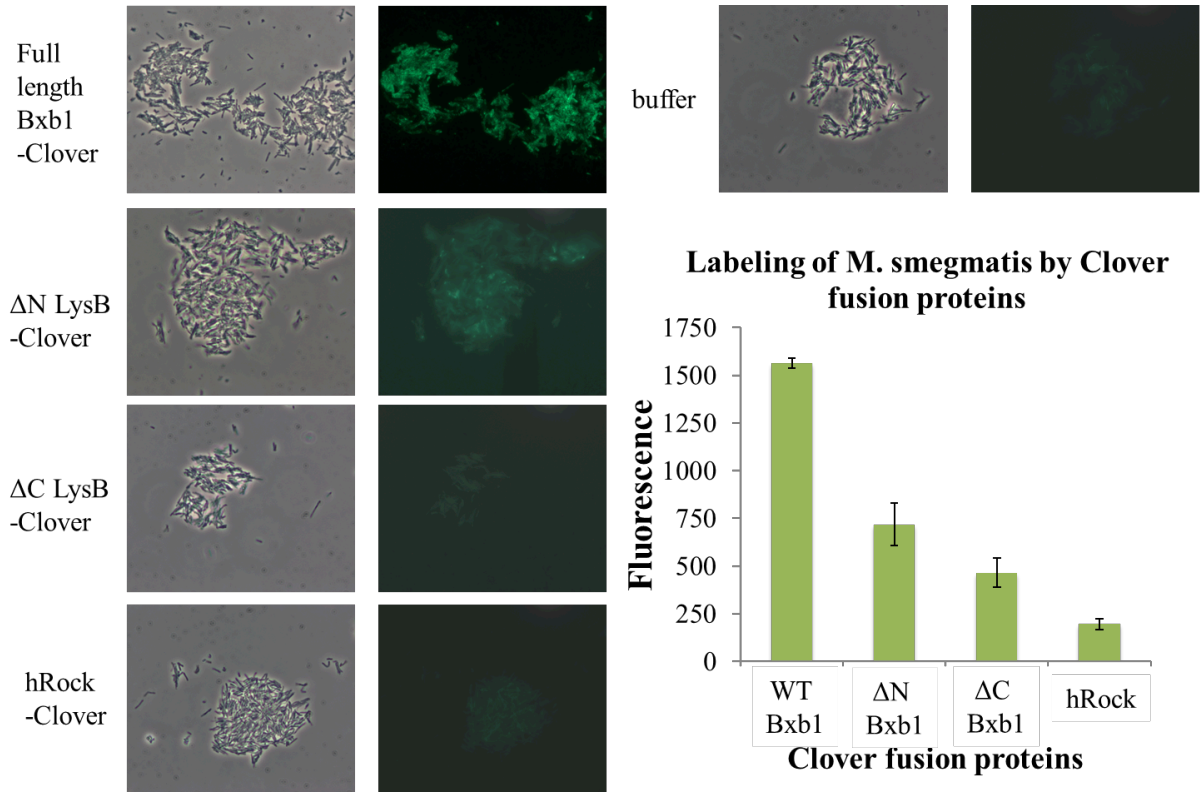


Figure 13. Clover fusion protein labeling of live *M. smegmatis* cells.

Fluorescent microscopy shows that only the full length Bxb1 LysB-Clover fusion protein was retained by the cells after several washes. Buffer and hRock-Clover were used as negative controls. Samples were also measured using a fluorimeter. Values were normalized to the buffer-treated sample to control for artifacts like autofluorescence.

BIBLIOGRAPHY

1. Salmond, G. & Fineran, P. A century of the phage: past, present and future. *Nature Reviews Microbiology* **13,** 777–786 (2015).
2. Casjens, S. R. & Hendrix, R. W. Bacteriophage lambda: early pioneer and still relevant. *Virology* 310–330 (2015). doi:10.1016/j.virol.2015.02.010
3. Petrovski, S., Dyson, Z., Seviour, R. & Tillett, D. Small but Sufficient: the Rhodococcus Phage RRH1 Has the Smallest Known Siphoviridae Genome at 14.2 Kilobases. *Journal of Virology* **86,** 358–363 (2012).
4. Ptashne. ISOLATION OF THE lambda PHAGE REPRESSOR. *Proceedings of the National Academy of Sciences of the United States of America* **57,** 306–13 (1967).
5. Young. Bacteriophage lysis: mechanism and regulation. *Microbiological reviews* **56,** 430–81 (1992).
6. Pimentel, M. in (American Society of Microbiology Press, 2014).
7. Wang, I.-N., Smith, D. L. & Young, R. Holins: The Protein Clocks of Bacteriophage Infections. *Annu. Rev. Microbiol.* **54,** 799–825 (2000).
8. Young, Wang & Roof. Phages will out: strategies of host cell lysis. *Trends in microbiology* **8,** 120–8 (2000).
9. Lella, M., Kamilla, S., Jain, V. & Mahalakshmi, R. Molecular Mechanism of Holin Transmembrane Domain I in Pore Formation and Bacterial Cell Death. *ACS Chemical Biology* **11,** 910–920 (2016).
10. Schmidt, Velleman & Arber. Three functions of bacteriophage P1 involved in cell lysis. *Journal of bacteriology* **178,** 1099–104 (1996).
11. López, García, García & García. The pneumococcal cell wall degrading enzymes: a modular design to create new lysins? *Microbial drug resistance (Larchmont, N.Y.)* **3,** 199–211 (1997).
12. Loeffler, Nelson & Fischetti. Rapid killing of Streptococcus pneumoniae with a bacteriophage cell wall hydrolase. *Science (New York, N.Y.)* **294,** 2170–2 (2001).
13. Yoong, P., Schuch, R., Nelson, D. & Fischetti, V. Identification of a broadly active phage lytic enzyme with lethal activity against antibiotic-resistant Enterococcus faecalis and Enterococcus faecium. *Journal of bacteriology* **186,** 4808–12 (2004).
14. Fischetti, V. Bacteriophage endolysins: A novel anti-infective to control Gram-positive pathogens. *Int J Med Microbiol* **300,** 357–362 (2010).
15. López, R. & García, E. Recent trends on the molecular biology of pneumococcal capsules, lytic enzymes, and bacteriophage. *FEMS microbiology reviews* **28,** 553–80 (2004).
16. Loessner, M. Bacteriophage endolysins--current state of research and applications. *Current opinion in microbiology* **8,** 480–7 (2005).

17. Pang, T., Savva, C., Fleming, K., Struck, D. & Young, R. Structure of the lethal phage pinhole. *Proceedings of the National Academy of Sciences of the United States of America* **106,** 18966–71 (2009).
18. Pang, T., Park, T. & Young, R. Mapping the pinhole formation pathway of S21. *Molecular microbiology* **78,** 710–9 (2010).
19. Xu, M., Struck, D., Deaton, J., Wang, I.-N. & Young, R. A signal-arrest-release sequence mediates export and control of the phage P1 endolysin. *Proceedings of the National Academy of Sciences of the United States of America* **101,** 6415–20 (2004).
20. Xu, M. *et al.* Disulfide isomerization after membrane release of its SAR domain activates P1 lysozyme. *Science (New York, N.Y.)* **307,** 113–7 (2005).
21. Payne, K. M. Mycobacteriophage lysins: bioinformatic characterization of Lysin A and identification of the function of Lysin B in infection. (2010).
22. Shockman & Barrett. Structure, function, and assembly of cell walls of gram-positive bacteria. *Annual review of microbiology* **37,** 501–27 (1983).
23. Schäffer, C. & Messner, P. The structure of secondary cell wall polymers: how Gram-positive bacteria stick their cell walls together. *Microbiology (Reading, England)* **151,** 643–51 (2005).
24. Matias, V., Al-Amoudi, A., Dubochet, J. & Beveridge, T. Cryo-transmission electron microscopy of frozen-hydrated sections of Escherichia coli and Pseudomonas aeruginosa. *Journal of bacteriology* **185,** 6112–8 (2003).
25. Schleifer & Kandler. Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriological reviews* **36,** 407–77 (1972).
26. Berry, J., Summer, E., Struck, D. & Young, R. The final step in the phage infection cycle: the Rz and Rz1 lysis proteins link the inner and outer membranes. *Molecular microbiology* **70,** 341–51 (2008).
27. Summer, E. *et al.* Rz/Rz1 lysis gene equivalents in phages of Gram-negative hosts. *Journal of molecular biology* **373,** 1098–112 (2007).
28. Zhang & Young. Complementation and characterization of the nested Rz and Rz1 reading frames in the genome of bacteriophage lambda. *Molecular & general genetics : MGG* **262,** 659–67 (1999).
29. Kedzierska, Wawrzynów & Taylor. The Rz1 gene product of bacteriophage lambda is a lipoprotein localized in the outer membrane of Escherichia coli. *Gene* **168,** 1–8 (1996).
30. Brennan & Nikaido. The envelope of mycobacteria. *Annual review of biochemistry* **64,** 29–63 (1995).
31. Barry *et al.* Mycolic acids: structure, biosynthesis and physiological functions. *Progress in lipid research* **37,** 143–79 (1998).
32. Takayama, K., Wang, C. & Besra, G. Pathway to Synthesis and Processing of Mycolic Acids in Mycobacterium tuberculosis. *Clinical Microbiology Reviews* **18,** 81–101 (2005).
33. Jankute, M., Cox, J., Harrison, J. & Besra, G. Assembly of the Mycobacterial Cell Wall. *Annual review of microbiology* **69,** 405–23 (2015).
34. Gil, F. *et al.* The lytic cassette of mycobacteriophage Ms6 encodes an enzyme with lipolytic activity. *Microbiology* **154,** 1364–1371 (2008).
35. Payne, K., Sun, Q., Sacchettini, J. & Hatfull, G. Mycobacteriophage Lysin B is a novel mycolylarabinogalactan esterase. *Molecular Microbiology* **73,** 367–381 (2009).
36. Russell, D. A. & Hatfull, G. F. PhagesDB: the actinobacteriophage database. *Bioinformatics* **33,** 784–786 (2017).

37. Marinelli, L. *et al.* BRED: A Simple and Powerful Tool for Constructing Mutant and Recombinant Bacteriophage Genomes. *PLoS ONE* **3,** (2008).
38. Loeffler & Fischetti. Synergistic lethal effect of a combination of phage lytic enzymes with different activities on penicillin-sensitive and -resistant Streptococcus pneumoniae strains. *Antimicrobial agents and chemotherapy* **47,** 375–7 (2003).
39. Djurkovic, Loeffler & Fischetti. Synergistic Killing of Streptococcus pneumoniae with the Bacteriophage Lytic Enzyme Cpl-1 and Penicillin or Gentamicin Depends on the Level of Penicillin Resistance. *Antimicrobial Agents and Chemotherapy* **49,** 1225–1228 (2005).
40. Pastagia, M., Schuch, R., Fischetti, V. & Huang, D. Lysins: the arrival of pathogen-directed anti-infectives. *Journal of Medical Microbiology* **62,** 1506–1516 (2013).
41. Obeso, J., Martínez, B., Rodríguez, A. & García, P. Lytic activity of the recombinant staphylococcal bacteriophage PhiH5 endolysin active against Staphylococcus aureus in milk. *International journal of food microbiology* **128,** 212–8 (2008).
42. Rodríguez-Rubio, L. *et al.* Phage lytic proteins: biotechnological applications beyond clinical antimicrobials. *Critical reviews in biotechnology* **36,** 542–52 (2016).
43. Loessner, M., Kramer, K., Ebel, F. & Scherer, S. C□terminal domains of Listeria monocytogenes bacteriophage murein hydrolases determine specific recognition and high□affinity binding to bacterial cell wall carbohydrates. *Molecular Microbiology* **44,** 335–349 (2002).
44. Kretzer, J. *et al.* Use of High-Affinity Cell Wall-Binding Domains of Bacteriophage Endolysins for Immobilization and Separation of Bacterial Cells. *Applied and Environmental Microbiology* **73,** 1992–2000 (2007).
45. Gilman, S. C. From Bench to Bedside: Why Patients Need New Anti-Bacterials. (2016).
46. Pohane, Joshi & Jain. Molecular Dissection of Phage Endolysin: AN INTERDOMAIN INTERACTION CONFERS HOST SPECIFICITY IN LYSIN A OF MYCOBACTERIUM PHAGE D29. *Journal of Biological Chemistry* **289,** 12085–12095 (2014).
47. Vouillamoz, J. *et al.* Bactericidal synergism between daptomycin and the phage lysin Cpl-1 in a mouse model of pneumococcal bacteraemia. *International Journal of Antimicrobial Agents* (2013). doi:10.1016/j.ijantimicag.2013.06.020
48. Daniel, A. *et al.* Synergism between a novel chimeric lysin and oxacillin protects against infection by methicillin-resistant Staphylococcus aureus. *Antimicrobial agents and chemotherapy* **54,** 1603–12 (2010).
49. Schuch, R. *et al.* Combination Therapy With Lysin CF-301 and Antibiotic Is Superior to Antibiotic Alone for Treating Methicillin-Resistant Staphylococcus aureus–Induced Murine Bacteremia. *Journal of Infectious Diseases* **209,** 1469–1478 (2014).
50. Rodríguez-Rubio, L., Martínez, B., Donovan, D., Rodríguez, A. & García, P. Bacteriophage virion-associated peptidoglycan hydrolases: potential new enzybiotics. *Critical reviews in microbiology* **39,** 427–34 (2013).
51. Stockdale, S. *et al.* The lactococcal phages Tuc2009 and TP901-1 incorporate two alternate forms of their tail fiber into their virions for infection specialization. *The Journal of biological chemistry* **288,** 5581–90 (2013).
52. Leiman, P., Chipman, P., Kostyuchenko, V., Mesyanzhinov, V. & Rossmann, M. Three-dimensional rearrangement of proteins in the tail of bacteriophage T4 on infection of its host. *Cell* **118,** 419–29 (2004).

53. Piuri, M. & Hatfull, G. A peptidoglycan hydrolase motif within the mycobacteriophage TM4 tape measure protein promotes efficient infection of stationary phase cells. *Molecular microbiology* **62,** 1569–85 (2006).
54. Delbruck, M. The growth of bacteriophage and the lysis of the host. *J Gen Physiol* **23,** 643–660 (1940).
55. Abedon, S. Lysis from without. *Bacteriophage* **1,** 46–49 (2011).
56. Kao, S. H. & McClain, W. H. Roles of bacteriophage T4 gene 5 and gene s products in cell lysis. *J Virol* **34,** 104–107 (1980).
57. Nakagawa, H., Arisaka, F. & Ishii, S. Isolation and characterization of the bacteriophage T4 tail-associated lysozyme. *J Virol* **54,** 460–466 (1985).
58. Moak, M. & Molineux, I. J. Role of the Gp16 lytic transglycosylase motif in bacteriophage T7 virons at the initiation of infection. *Mol Microbiol* **37,** 345–355 (2000).
59. Rydman, P. s & Bamford, D. H. Bacteriophage PRD1 DNA entry uses a viral membrane-associated transglycosylase activity. *Mol Microbiol* **37,** 356–363 (2000).
60. Kenny, J. G. Bacteriophage Tuc2009 encodes a tail-associated cell wall-degrading activity. *J Bacteriol* **186,** 3480–3491 (2004).
61. Moak, M. & Molineux, I. J. Peptidoglycan hydrolytic activities associated with bacteriophage virions. *Mol Microbiol* **51,** 1169–1183 (2004).
62. Takac, M. & Blasi, U. Phage P68 virion-associated protein 17 displays activity against clinical isolates of Staphylococcus aureus. *Antimicrob Agents Chemother* **49,** 2934–2940 (2005).
63. Rashel, M. *et al.* Tail-associated structural protein gp61 of Staphylococcus aureus phage phi MR11 has bifunctional lytic activity. *FEMS Microbiol Lett* **284,** 9–16 (2008).
64. Paul, V. *et al.* A novel bacteriophage Tail-Associated Muralytic Enzyme (TAME) from Phage K and its development into a potent antistaphylococcal protein. *BMC microbiology* **11,** 226 (2011).
65. Wei, L. *et al.* A mycobacteriophage-derived trehalose-6,6′-dimycolate-binding peptide containing both antimycobacterial and anti-inflammatory abilities. *Faseb J* **27,** 3067–3077 (2013).
66. Teng, Liu & Wei. Anti-Mycobacterial Peptides: From Human to Phage. *Cellular Physiology and Biochemistry* **35,** 452–466 (2015).
67. WHO. The TB disease burden. *Global Tuberculosis Report* 15–53 (2016).
68. Parsons, L. *et al.* Laboratory diagnosis of tuberculosis in resource-poor countries: challenges and opportunities. *Clinical microbiology reviews* **24,** 314–50 (2011).
69. Ling, D., Zwerling, A. & Pai, M. Rapid diagnosis of drug-resistant TB using line probe assays: from evidence to policy. *Expert review of respiratory medicine* **2,** 583–8 (2008).
70. Boehme, C. *et al.* Rapid molecular detection of tuberculosis and rifampin resistance. *The New England journal of medicine* **363,** 1005–15 (2010).
71. Fogel, N. Tuberculosis: a disease without boundaries. *Tuberculosis* **95,** 527–531 (2015).
72. Colebunders, R. & Bastian, I. A review of the diagnosis and treatment of smear-negative pulmonary tuberculosis. . *The international journal of tuberculosis and lung disease : the official journal of the International Union against Tuberculosis and Lung Disease* **4,** 97–107 (2000).
73. Levy, H., Feldman, C., Sacho, H., van der Meulen, H. & Kallenbach, J. A reevaluation of sputum microscopy and culture in the diagnosis of pulmonary tuberculosis. *Chest* **95,** 1193–7 (1989).

74. Grover, N., Paskaleva, E., Mehta, K., Dordick, J. & Kane, R. Growth inhibition of Mycobacterium smegmatis by mycobacteriophage-derived enzymes. *Enzyme and Microbial Technology* **63,** 1–6 (2014).
75. Ojha, A., Trivelli, X., Guerardel, Y., Kremer, L. & Hatfull, G. Enzymatic Hydrolysis of Trehalose Dimycolate Releases Free Mycolic Acids during Mycobacterial Growth in Biofilms. *Journal of Biological Chemistry* **285,** 17380–17389 (2010).
76. Lai, M.-J. *et al.* Antimycobacterial Activities of Endolysins Derived From a Mycobacteriophage, BTCU-1. *Molecules* **20,** (2015).
77. Besra, G. S. in **101,** 91–107 (1998).
78. Söding, J., Biegert, A. & Lupas, A. The HHpred interactive server for protein homology detection and structure prediction. *Nucleic Acids Res* **33,** W244–W248 (2005).
79. Alva, V., Nam, S.-Z., Söding, J. & Lupas, A. The MPI bioinformatics Toolkit as an integrative platform for advanced protein sequence and structure analysis. *Nucleic Acids Res* **44,** W410–W415 (2016).
80. Fokine, A., Miroshnikov, K., Shneider, M., Mesyanzhinov, V. & Rossmann, M. Structure of the Bacteriophage φKZ Lytic Transglycosylase gp144. *J Biol Chem* **283,** 7242–7250 (2008).
81. Šali, A., Potterton, L., Yuan, F., van Vlijmen, H. & Karplus, M. Evaluation of comparative protein modeling by MODELLER. *Proteins Struct Funct Bioinform* **23,** 318–326 (1995).
82. Sambandamurthy, V. *et al.* Mycobacterium tuberculosis DeltaRD1 DeltapanCD: a safe and limited replicating mutant strain that protects immunocompetent and immunocompromised mice against experimental tuberculosis. *Vaccine* **24,** 6309–20 (2006).
83. Martinez, J. General principles of antibiotic resistance in bacteria. *Drug Discov Today Technologies* **11,** 33–39 (2014).
84. Smith, R., M'ikanatha, N. & Read, A. Antibiotic Resistance: A Primer and Call to Action. *Health Commun* **30,** 309–314 (2014).
85. Haynes & Britz. The effect of growth conditions of Corynebacterium glutamicum on the transformation frequency obtained by electroporation. *J Gen Microbiol* **136,** 255–263 (1990).
86. Tomiyasu, I. & Yano, I. Isonicotinic acid hydrazide induced changes and inhibition in mycolic acid synthesis in Nocardia and related taxa. *Arch Microbiol* **137,** 316–323 (1984).
87. Johnson & Cummins. Cell wall composition and deoxyribonucleic acid similarities among the anaerobic coryneforms, classical propionibacteria, and strains of Arachnia propionica. *Journal of bacteriology* **109,** 1047–66 (1972).
88. Gilchrist, T. C. A bacteriological and microscopical study of over three hundred vesicular and pustular lesions of the skin, with a research upon the etiology of acne vulgaris. *Johns Hopkins Hospital Report* **9,** 409–430 (1901).
89. Douglas, H. C. & Gunter, S. E. The taxonomic position of Corynebacterium acnes. *J Bacteriol* **53,** 15–23 (1946).
90. Kilian, M. & Scholz, C. The natural history of cutaneous propionibacteria and reclassification of selected species within the genus Propionibacterium to the proposed novel genera Acidipropionibacterium gen. nov., Cutibacterium gen. nov. and

Pseudopropionibacterium gen. nov. *Int J Syst Evol Micr* (2016). doi:10.1099/ijsem.0.001367
91. Leyden, J. J., McGinley, K. J., Mills, O. H. & Kligman, A. M. Propionibacterium levels in patients with and without acne vulgaris. *The Journal of investigative dermatology* **65,** 382–4 (1975).
92. Sathish, Shayeda & Rao. Acne and its treatment options: a review. *Current drug delivery* **8,** 634–9 (2011).
93. Eady, Cove, Holland & Cunliffe. Erythromycin resistant propionibacteria in antibiotic treated acne patients: association with therapeutic failure. *The British journal of dermatology* **121,** 51–7 (1989).
94. Hatfull, G. Molecular Genetics of Mycobacteriophages. *Microbiology Spectrum* **2,** (2014).
95. Payne, K. & Hatfull, G. Mycobacteriophage Endolysins: Diverse and Modular Enzymes with Multiple Catalytic Activities. *PLoS ONE* **7,** (2012).
96. Marinelli, L. *et al.* Propionibacterium acnes Bacteriophages Display Limited Genetic Diversity and Broad Killing Activity against Bacterial Skin Isolates. *mBio* **3,** e00279–12 (2012).
97. Fitz-Gibbon, S. *et al.* Propionibacterium acnes Strain Populations in the Human Skin Microbiome Associated with Acne. *J Invest Dermatol* **133,** 2152–2160 (2013).
98. Tomida, S. *et al.* Pan-Genome and Comparative Genome Analyses of Propionibacterium acnes Reveal Its Genomic Diversity in the Healthy and Diseased Human Skin Microbiome. *Mbio* **4,** e00003–13 (2013).
99. McDowell, A. *et al.* A novel multilocus sequence typing scheme for the opportunistic pathogen Propionibacterium acnes and characterization of type I cell surface-associated antigens. *Microbiol Read Engl* **157,** 1990–2003 (2011).
100. Brüggemann, H. Insights in the Pathogenic Potential of Propionibacterium acnes From Its Complete Genome. *Seminars in Cutaneous Medicine and Surgery* **24,** 67–72 (2005).
101. Gerstmans, Rodriguez-Rubio, Lavigne & Briers. From endolysins to Artilysin(R)s: novel enzyme-based approaches to kill drug-resistant bacteria. *Biochemical Society Transactions* **44,** 123–128 (2016).