PROTEIN ANTIBIOTIC OF PHAGE M

An Undergraduate Research Scholars Thesis

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ABSTRACT

Protein Antibiotic of Phage M

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Phage mediated lysis of bacteria is one of the most common events in the biosphere, and a greater understanding of phage lysis mechanisms may lead to the development of novel antibacterial therapies. As they encode a single, nonezymatic protein ("protein antibiotic) to effect lysis, small lytic phages offer great promise. The lysis proteins E and A_2 of prototypical small phages ΦX174 (*Microviridae*) and Qβ (*Allolevivirida*e) inhibit cell wall biosynthesis and cause lysis at the septum of a dividing cell. The lysis protein L of prototypical small phage MS2 (*Leviviridae*), conversely, causes lysis at random regions of the cell through an unknown mechanism. Although MS2 and phage M belong to the *Leviviridae* family, their lysis genes *L* and *M lys* have evolved at different locations (figure 1A) and cause different lysis phenotypes (figure 1B).

In this project, we investigated the lysis mechanism of M Lys. Based on similarities in membrane topology and lysis phenotype, we hypothesized that M Lys, like E, inhibits cell wall biosynthesis. Isolation of host mutants resistant to lysis yielded mutations in *murJ*, a proposed *E. coli* lipid II flippase involved in transporting cell wall precursors from the cell interior to the exterior. This suggests that M Lys confers lysis by inhibiting MurJ and thus host cell wall

biosynthesis. This also provides additional support for the identification of MurJ as the *E. coli* lipid II flippase.

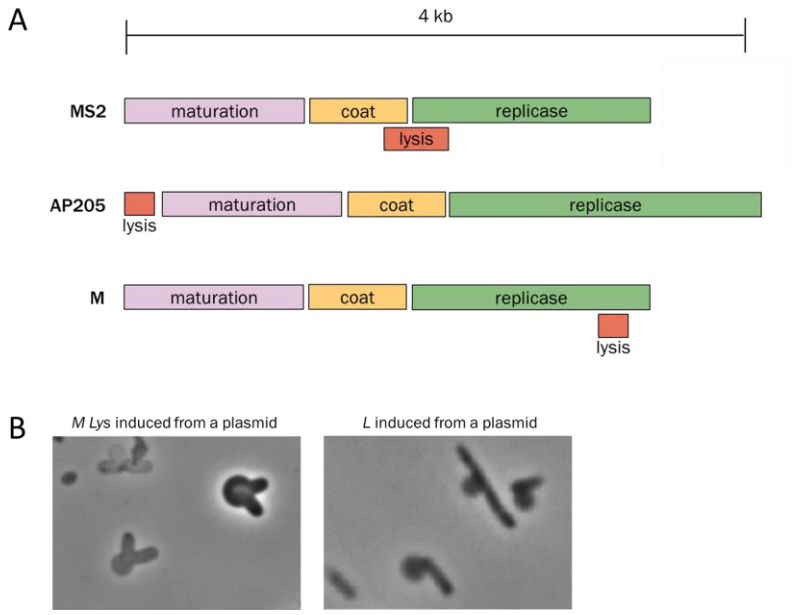

Figure 1. Small lytic phages of the *Leviviridae* genus display a range of lysis phenotypes and genetic architectures.
(A) *Leviviridae* phages have evolved three distinct genetic architectures regarding lysis gene location.
(B) M Lys causes septal lysis in *E. coli* while L causes random blebbing.

DEDICATION

I would like to dedicate this thesis to my parents and friends. I would not be where I am today without their constant support, and I am truly thankful for everything they have done for me.

ACKNOWLEDGEMENTS

I would like to thank the members of the Young lab for everything they do to support myself and my fellow undergraduates. I would like to thank Karthik Chamakura in particular for spending so much time and energy in mentoring me and guiding me through this process. In addition, I would like to thank Dr. Ryland Young for accepting me into his lab with open arms.

CHAPTER I

INTRODUCTION

The rise in antibiotic resistance

The over-prescription of antibiotics has contributed to an increase in antibiotic resistance, and previously easily treatable bacterial infections are now becoming increasingly difficult to treat. *Enterobacteriaceae,* a family of Gram-negative bacteria including many commonly known pathogens such as *Escherichia coli, Salmonella, and Shigella*, have been slow to adapt antibiotic resistance to broad spectrum antibiotics. Even when *Enterobacteriaceae* began exhibiting multi-drug resistance, physicians have previously been able to administer a class of antibiotics called carbapenems as a last resort. Although carbapenems belong to the same class of antibiotics as penicillins and cephalosporins, they affect a wider range of bacteria and have resisted antibiotic resistance. Despite this, *Enterobacteriaceae* resistant to all or almost all of currently known antibiotics, including carbapenems, have become more prevalent and are associated with ever higher mortality rates [1, 2].

As a cell component unique to bacteria and vital to maintaining structural integrity, the cell wall, made of peptidoglycan (PG, also known as murein), is a common target for antibiotics such as penicillin and carbapenems. Indeed, most antibiotics currently in use inhibit PG biosynthesis, causing cells to lyse as their high internal osmotic pressure overcomes the strength of the inner and outer membranes [2]. Pharmaceutical antibiotics, however, are not the only molecules found to target PG biosynthesis; bacteriophages, known in short as phages, are natural predators of bacteria that have evolved proteins targeting PG as well [3].

PG precursors and biosynthesis

The main difference between Gram-negative and Gram-positive bacteria is the presence or absence of an outer membrane (OM). The OM stabilizes the inner membrane (IM) of Gram-negative bacteria, resulting in a relatively thin PG meshwork (<10 nm). In contrast, Gram-positive bacteria rely on a thick PG layer (30-100 nm) to compensate for the absence of an OM. As the cell wall is vital to structural integrity, PG biosynthesis must be strictly regulated to avoid breaches during cell growth and division [4].

PG is a polymer made of repeating units of cross linked disaccharide polypeptides, and its biosynthetic pathway can be divided into three stages. The first stage occurs in the cytoplasm and begins with the synthesis of the UDP-*N*-acetylmuramyl-pentapeptide (UDP-MurNAc-PP) precursor from UDP-*N*-acetylglucosamine (UDP-GlcNAc) by MurA. In Gram-negative bacteria, this pentapeptide consists of L-alanine-γ-D-glutamate-diaminopimelate(meso-DAP)-D-alanine-D-alanine (L-ala-D-glu-mDAP-L-lys-D-ala-D-ala) (Sobhanifar). From here, the integral IM protein MraY catalyzes formation of the first IM lipid intermediate, lipid I, from the carrier lipid undecaprenyl (also known as bactoprenol) and UDP-MurNAc-PP, and the peripheral membrane protein MurG catalyzes formation of the second inner membrane lipid intermediate, lipid II, from lipid I and UDP-GlcNAc. Lipid II is then transported from the cytoplasmic leaflet of the IM to the periplasmic leaflet, where the intact PG layer is degraded by autolysins and the transglycosylase and transpeptidase activities of various penicillin-binding proteins (PBPs) add the GlcNAc-MurNAc disaccharide pentapeptide PG precursor to the growing PG layer [5].

Lipid II and its flippase

As the final intermediate before the addition of PG precursors to the PG layer, lipid II is essential to PG biosynthesis and is targeted by many antibiotics. Bacitracin, for example, prevents the carrier lipid undecaprenyl phosphate from being recycled, preventing lipid II synthesis. Glycopeptides like vancomycin, on the other hand, bind to lipid II terminal D-alanine residues, preventing crosslinking. Additionally, lipid II modifications are slow to develop, leading to relatively low rates of antibiotic resistance. Vancomycin resistance, for example, only developed 30 years after clinical use when bacteria began utilizing a terminal D-alanine-D-lactate in lipid II instead of D-ala-D-ala [2].

The enzyme catalyzing the transport of lipid II from the cytoplasm to the periplasm, then, is also vital to PG biosynthesis as it connects the cytoplasmic synthesis of PG precursors to the addition of precursors to the PG layer. Lipid II is an amphipathic molecule -- the undecaprenyl moiety is a long hydrophobic molecule and the attached disaccharide pentapeptide is hydrophilic -- and cannot simply diffuse across the membrane. An IM enzyme containing a hydrophilic cavity must therefore transport lipid II across the IM. In the current model of transport, a flippase catalyzes the “flipping” of the hydrophilic disaccharide pentapeptide through the hydrophilic cavity while the hydrophobic undecaprenyl phosphate remains in the hydrophobic core of the IM. Controversy surrounds the identity of this flippase in *E. coli*, though, with two main contenders: FtsW and MurJ [6].

FtsW as the lipid II flippase

It has been recognized that the lipid II flippase must be an IM protein, conserved among gram negative bacteria synthesizing PG, absent in bacteria not synthesizing PG, and essential to cell survival. Based on these criteria, FtsW was proposed to be the missing lipid II flippase. FtsW and its two homologs RodA and SpoVE are members of the SEDS (shape, elongation, division and sporulation) family, members of which can be found in bacteria synthesizing PG. FtsW, RodA, and SpoVE (found in *Bacillus subtilis*) are vital to cell division, elongation, and sporulation respectively, and can be found complexed with various PBPs. In addition, FtsW of *E.coli* is located in the same operon as PBP3 and MurG, the PG biosynthesis enzyme catalyzing lipid II synthesis, and is known to localize to the cell septum during cell division. This suggests that FtsW may act as the lipid II flippase during cell division and, by homology, RodA and SpoVE may act as the lipid II flippase during cell elongation and sporulation respectively [7].

One impediment in identifying the lipid II flippase is the lack of an assay to determine flipping activity. Mohammadi *et al*., 2011 developed an *in vitro* fluorescence-transfer assay in which a decrease in fluorescence of a lipid II derivative and a subsequent increase in fluorescence of a vancomycin derivative indicated lipid II flipping activity. Vancomycin is an antibiotic that inhibits PG biosynthesis by binding to the terminal D-alanine-D-alanine residues on the disaccharide pentapeptide PG precursor of lipid II. As vancomycin cannot penetrate the IM, the observation of such fluorescence is indicative of lipid II flipping [7].

Mohammadi *et al*., 2011 showed that overexpression of FtsW in *E. coli* membrane derived vesicles led to an increase in the change in fluorescence indicative of lipid II flipping activity.

Furthermore, deletion of FtsW from membrane derived vesicles largely resulted in a deletion of lipid II flipping associated fluorescence; residual fluorescence was attributed to RodA activity. Additionally, a dithionite reduction assay in which dithionite reduced fluorescence of flipped lipid II derivatives showed that the presence of purified FtsW in large unilamellar vesicles resulted in a decrease of fluorescence. Neither of these assays displayed lipid II flipping activity in the presence of MurJ, leading the authors to conclude that FtsW alone is the *E. coli* lipid II flippase [7].

Several factors in experimental design may have affected the results of this *in vitro* assay, though, including the introduction of lipid II precursors through a freeze-thaw method, the reliance on a lipid II derivative that may display different flipping activity than wild type lipid II, and the fact that this assay was conducted at 14°C to prevent loss of lipid II fluorescence by transglycosylase activity not indicative of lipid II flipping and vancomycin binding [8]. Additionally, this assay did not address the selectivity of FtsW lipid transport [6].

MurJ as the lipid II flippase

Ruiz *et al*., 2008, on the other hand, initially identified MurJ as the sole lipid II flippase in *E. coli* using a reductionist bioinformatics search. MurJ sequence homologs are present in non-bacterial organisms producing PG and archaea producing PG-like molecules, supporting this hypothesis. MurJ is also a member of the multidrug/oligosaccharidyl-lipid/polysaccharide (MOP) exporter superfamily, sharing greatest sequence homology to the Wzx flippases of the polysaccharide transporter (PST) family that flip lipid II-like undecaprenyl-linked saccharide precursors across the IM to the periplasm. FtsW was ruled out as a possible lipid II flippase due to its presence in

several PG-less bacteria and absence in other PG-synthesizing bacteria. Additionally depletion of MurJ was shown to lead to a decrease in cell density indicative of cell lysis, and incorporation of ^{3}H-mDAP, the third amino acid of the disaccharide pentapeptide PG precursor, decreased in MurJ-depleted cells after several generations [9].

A subsequent structural functional analysis of MurJ *in silico* using the I-TASSER server revealed structural similarity between MurJ and members of the multidrug and toxic compound extrusion (MATE) family, also members of the MOP superfamily. Proteins of the MATE family are Na^{+} and H^{+} driven antiporters that transport hydrophobic and amphiphobic molecules across the IM and contain 12 TMDs bundled into two groups of 6 helices. These proteins contain a central cavity through which molecules are transported and exist in two V-shaped conformations, one with the central cavity open to the cytoplasm and one with the central cavity open to the periplasm. Through *in silico* modeling and *in vivo* experiments, MurJ was shown to have 14 TMDs with an N-in, C-in topology and TMDs 1, 2, 7, and 8 surrounding a positively charged, solvent-exposed central cavity, suggesting a possible function transporting the negatively charged disaccharide pentapeptide of lipid II [10].

The development of an *in vivo* assay linking MurJ to lipid II flippase activity further cemented the identification of MurJ as the *E. coli* lipid II flippase. In this assay, cells were treated with the protein toxin colicin M (ColM). Upon treatment, ColM is transported into periplasm where it cleaves the disaccharide pentapeptide moiety from lipid II. This disaccharide pentapeptide is then further processed by periplasmic carboxypeptidases to yield disaccharide tetrapeptides. As the IM is impermeable to ColM, ColM activity is specific for periplasmic lipid II, and the

appearance of disaccharide tetrapeptides is indicative of lipid II flipping. Lipid II flipping was eliminated upon MurJ inactivation, but continued despite FtsW reduction in *ΔrodA* mutants, indicating that FtsW and RodA play no part in lipid II flipping [11].

Still, critics of MurJ argue, although *in vivo* assays can help pinpoint the function of a protein, they do not necessarily demonstrate a direct relationship between a protein and its putative function; there are numerous additional variables that may affect the observed activity. The lack of observed lipid II flippase activity upon MurJ inactivation in the *in vivo* assay, for example, may simply arise from the inherent insensitivity of the assay. Only *in vitro* systems expressing a single protein can demonstrate a direct relationship between protein and function, and the lack of lipid II flippase activity upon MurJ expression in the *in vitro* assay developed suggests that FtsW is the flippase instead [6].

Alternatively, MurJ and FtsW may both act as lipid II flippases, and the results of the ColM assay may simply demonstrate that FtsW exists in a large protein complex with MurG and PBP3 while MurJ exists as a lone IM protein. As such, lipid II would be more inaccessible to ColM due to the size of the protein complex and perhaps the quicker transfer of lipid II from FsW to PBP3. The essentiality of both MurJ and FtsW, however, seems to suggest that the two serve two non-redundant functions [6].

Lytic bacteriophage life cycle

Phages are viruses that infect solely bacteria. Lytic phages can be divided into two classes based on genome size: large phages that utilize double stranded DNA (dsDNA) anywhere from 20 kb

to 300+ kb long; and small phages that utilize single stranded genomes about 4 kb long. Although these phages differ drastically in genome size, they follow the same general life cycle in which they inject their genetic material into a bacterial host, use host machinery and resources to reproduce themselves, and eventually lyse their host and release progeny into the environment (figure 2). This last step of lysis is particularly important to phage propagation as it allows progeny phages to escape the confines of the bacterial host [3].

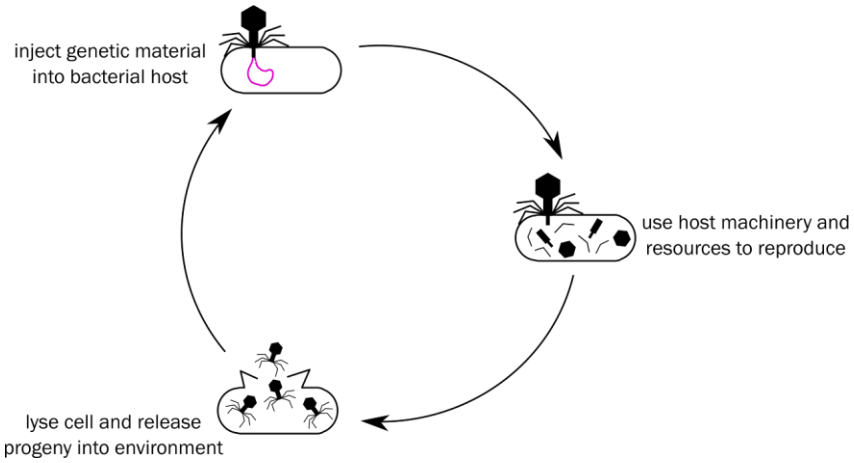

Figure 2. Typical bacteriophage life cycle.

Lysis in large double stranded DNA lytic phages

Canonical holin-endolysin system

Large phages have evolved two systems of degrading the cell wall to effect host lysis. In the canonical holin-endolysin system, largely studied in phage lambda, holin and endolysin proteins are expressed about 8-10 minutes after infection during late gene expression. Homodimers of holins accumulate harmlessly and randomly in the inner membrane while monomers of active endolysins accumulate in the cytoplasm. This occurs simultaneously with morphogenesis of

progeny phages. About 50 minutes after infection upon reaching a critical concentration, holins are triggered and begin aggregating into 1-3 micron sized rafts. These rafts are thought to create holes in the inner membrane (IM), allowing endolysins to escape the cytoplasm and unleash their muralytic activity on the cell wall. The cell membrane bulges out at the site of holin raft formation as the internal osmotic pressure overcomes the integrity of the outer membrane (OM), and the cell eventually lyses [3].

Interestingly, disturbing the proton motive force (PMF) across the IM causes premature holin triggering. It is thought that aggregation of holins into rafts disturbs PMF, and that this disturbance of PMF in turn encourages further holin aggregation and eventual hole formation [3].

Pinholin-SAR-endolysin system

The pinholin-SAR-endolysin system has been studied largely in the coliphage P1 and the lambdoid phage 21. SAR endolysins from phage P1 and phage 21 contain a SAR (signal-anchor-release) domain that allows them to be secreted to the periplasm in their inactive form through the host *sec* system. This initial inactivity allows SAR endolysin muralytic activity to be regulated by pinholin triggering. Unlike other proteins secreted through the *sec* system, the signal domain of SAR is not cleaved upon secretion. SAR endolysins therefore remain anchored to the periplasmic leaflet of the IM until the SAR domain disengages from both the enzyme and the IM, either slowly and spontaneously or quickly upon disruption of PMF. With the help of the free SAR domain, the released enzyme refolds into its active form and begins degrading the cell wall. Because SAR endolysins are distributed evenly through the IM, activation results in even degradation of the cell wall. The cell then appears to shorten before eventually lysing [3].

Unlike in the canonical holin-endolysin system, pinholins from phage 21 do not play a role in transporting SAR endolysins from the cytoplasm to the periplasm. Instead, they accumulate harmlessly in the IM until they are triggered, when they aggregate in heptamers to form about 10^3 small channels that disrupt PMF. Pinholins, much like holins, then, regulate the timing of lysis [3].

Degradation of the cell wall by coordinated action of holins and spanins in lambda is regulated by a dual-start motif in which the holin encoding gene is translated at two sites, one yielding a holin and one yielding a slightly longer antiholin. In lambda, anti-holins heterodimerize to holins, preventing homodimerization of holins and subsequent aggregation. The ratio of holin:antiholin is about 2:1, and heterodimerization of anti-holins and holins controls the timing of lysis [3].

Regulation of timing of lysis

Dual-start motifs have been also been found in other holins, like the pinholin of phage 21 and the holin of T4. Although the anti-holins encoded by lambda and phage 21 act similarly, the antiholin of T4 acts by a completely different mechanism. T4 encodes a holin consisting of one transmembrane domain (TMD) and a periplasmic domain. If the antiholin is activated, presumably in conditions where there are few or no surrounding hosts to infect, it binds to this periplasmic domain and prevents hole formation. This is the only known form of holin inhibition that actively inhibits holin function [3].

Spanins

Although the cell wall is a large barrier imposed by the host, simply degrading the cell wall is not to effect lysis. A third group of proteins called spanins, so named because they span the periplasm, is necessary for lysis (Young 2014). Spanin deficient mutants round up into spheres instead of lysing as the cell wall responsible for cell shape is degraded. Only the OM is intact at this point in time, suggesting that spanins must disrupt OM integrity in lysing the cell [12].

I-spanins and o-spanins

There are two classes of spanins: spanins consisting of an inner membrane subunit (i-spanins) and an outer membrane subunit (o-spanins); and unimolecular spanins (u-spanins); Lambda encodes this first spanin where the i-spanin Rz is an inner membrane protein with an N-in, C-out topology and the o-spanin Rz1 is an N-terminus outer membrane lipid-associated protein.. *Rz1*, interestingly enough, is embedded in the +1 reading frame of *Rz*. The discovery of *Rz1* in *Rz* was the first time one gene was found in a different reading frame of another gene participating in the same biological function. The lysis genes *E* and *L* of ssDNA phage ΦX174 and ssRNA phage MS2, respectively, were previously found embedded in different reading frames of other gene, but these genes were of different functions separate from lysis [3].

The identification of *Rz1* as the o-spanin was supported by the presence of a strong Shine-Delgarno sequence and a strong signal peptidase II cleavage motif, or lipobox. Members of the signal peptidase II class cleave the signal domain from lipoprotein precursors at and modify the cleaved cysteine residue with a lipid (Inderjit), indicating that Rz1 is a lipoprotein. The presence of threonine and serine residues in the +1 and +2 positions after the lipid-modified cysteine in

particular suggests that Rz1 is transported to the OM by the Lol system, further supporting the identification of Rz1 as the o-spanin [3].

Upon further analysis of *Rz* and *Rz1* homologues in other phages, three distinct genetic architectures have been discovered: embedded, as with *Rz* and *Rz1*; overlapping, in which the *Rz1* reading frame begins in the *Rz* reading frame but extends past *Rz* termination; and separate, in which *Rz1* is immediately downstream of *Rz*. Embedded i-spanins and o-spanins are proposed to have evolved from separate genes that, over time, evolved to interact with each other in order to effect lysis. It is thought that, over time, overlapping and embedded i-spanins were favored because of the minimal probability of recombination associated which this gene structure; many phages undergo a hyper-recombinational state during their infection cycle [3].

Rz and Rz1 are synthesized and inserted into their respective membranes throughout late gene expression. Rz and Rz1 interact with each other at their C termini and thread through spaces in the PG in order to span the periplasm. Rz and Rz1 each homodimerize through the formation of disulfide bonds; Rz contains two cysteine residues, and Rz1 contains one cysteine residues. These disulfide bonds are formed by the periplasmic Dsb system upon secretion through the *sec* system. DsbA in particular promotes homodimerization of either Rz or Rz1, which then promotes spontaneous homodimerization of Rz1 or Rz respectively. Homodimers of Rz and Rz1 then dimerize as well to form a heterotetramer spanin complex. DsbA, however, also promotes the formation of intramolecular disulfide bonds in Rz. These intramolecular disulfide bonds in Rz must then be reduced by DsbC before Rz homodimerization can occur, whether catalyzed by DsbA or homodimerization of Rz1. Similar to the discovery of the *RzRz1* genetic structure, this

is the first time the Dsb system has been found to promote formation of intermolecular disulfide bonds; Dsb had been previously implicated in the formation of intramolecular disulfide bonds [3].

U-spanins

U-spanins, such as gp11 of phage T1, share characteristics of both i-spanins and o-spanins. Gp11 has a TMD at one terminus, like i-spanins, and an N-termial lipobox in which the lipid-modified cysteine is followed by serine and threonine residues, like o-spanins. Furthermore, gp11 capable of rescuing lysis in lambda *RzRz1* deficient mutants, supporting the identification of proteins such as gp11 as u-spanins [3].

Proposed mechanisms of action for spanins

Three mechanisms of action have been proposed for spanins: enzymatic activity, pore formation, and membrane fusion. Due to its simplicity and other factors that will be discussed below, membrane fusion seems to be the most likely mechanism [3].

At the very least, enzymatic spanin activity in which spanins cleave PG peptide crosslinking or PG peptide links to the OM seems impossible. None of the known RzRz1-like and gp11-like proteins share homology to any proteolytic or lipolytic domains. Additionally, assuming that i-/o-spanin complexes and u-spanins operate in a similar fashion, it is difficult to imagine that either is capable of refolding to form a globular enzymatic domain in the periplasm. The length of both i-/o-spanin complexes and u-spanins seems to negate this possibility, as both are merely capable of spanning the periplasm; the length of the soluble portion of the RzRz1 complex, for

example, is on par with the width of the periplasm. The secondary structures of Rz and Rz1 are problematic as well. Rz is predicted to contain coiled coil α-helices, and Rz1 is predicted to have an unorthodox structure due to its numerous proline residues, neither of which are conducive to the fomation of a globular domain [3].

A second mechanism of action has been proposed in which i-spanins couple to holins, resulting in spanin-mediated micron pore formation upon holin triggering [3]. Several problems exist with this theory as well, though. P1 lysis, which implements a combination of holins and SAR endolysins (as opposed to the canonical holin-endolysin system or the pinholin-SAR-endolysin system), has been shown to occur independent of holins [13]. Additionally, assuming that spanins function similarly in both phages utilizing holins and phages utilizing pinholins, the size of the holes formed by pinholins (about two nm in diameter for phage 21) seems to negate the legitimacy of this theory, as the smallest dsDNA phages are about 50 nm in diameter [3].

By process of elimination and its innate simplicity, the third proposed mechanism in which spanins promote fusion of the IM and OM seems the most likely. Purified Rz1 has been found to localize in membrane fractions of intermediate density between the IM and OM [14]. Additionally, the periplasmic region of Rz consists of two α-helices connected by a hinge, and formation of the heterotetramer complex seems to promote α-helical structure and the formation of coiled-coils [15]. A mechanism has been proposed, then, in which PG degradation releases spanins from the confinement of the PG mesh and allows for intramolecular coiled coil formation in spanins. This brings the two membranes together, allowing a catalytic domain in the o-spanin to catalyze membrane fusion. Although the periplasmic domain of u-spanins is

predicted to consist of several β-sheet structures instead of α-helices, u-spanins are proposed to function in a similar manner with intramolecular or intermolecular interactions between β-sheets [3].

Lysis in small single stranded genome lytic phages

Small lytic phages have an average genome size of 4 kbp. Given that the average gene is 1 kb long, most small lytic phages encode only four proteins by which they infect a bacterial host, reproduce, and cause lysis. As a result, small lytic phages employ a different lysis mechanism than large lytic phages do, instead utilizing single gene lysis (SGL) systems encoding a single lysis protein in the amurin class, so named for their lack of muralytic (PG-degrading) activity [16].

There are three prototypical small lytic phages, each encoding a lysis protein with a unique mechanism: the ssDNA phage ΦX174 of the *Microviridae* genus encodes ten proteins, including the lysis protein E; the ssRNA phage Qβ of the *Allolevivirdae* genus and the ssRNA phage MS2 of the *Leviviridae* genus each encode a mere four proteins, including the lysis proteins A_2 and L respectively (figure 3) [16].

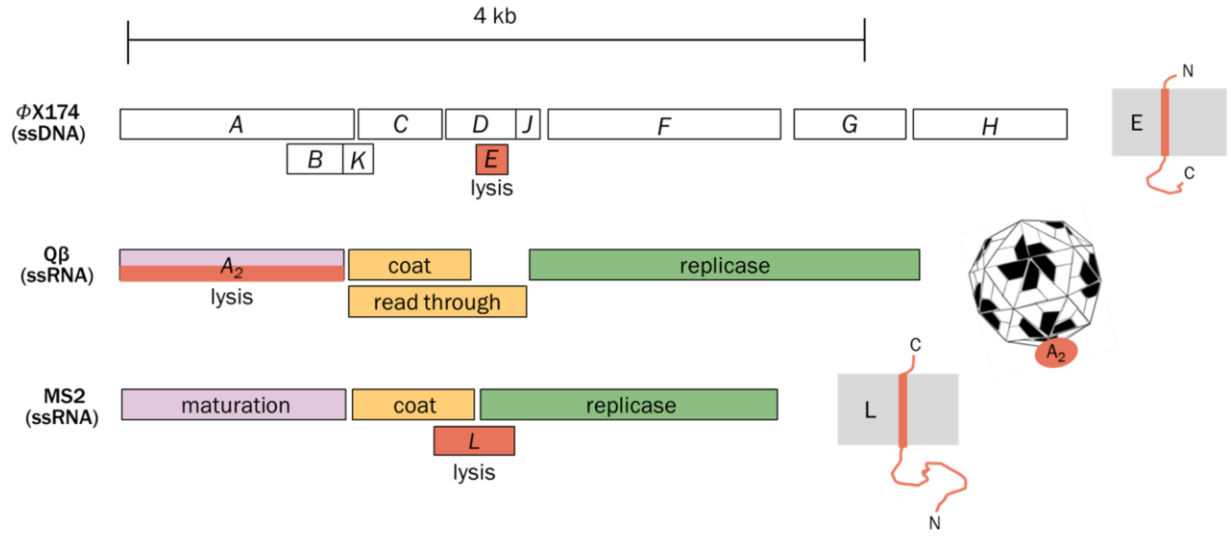

Figure 3. Genetic architectures and lysis proteins for prototypical small lytic phages.

The lysis protein E of phage ΦX174

Phage ΦX174 lysis of *E. coli* was first studied in Markert and Zillig, 1964 who observed lysis to take place over one generation of *E. coli* as opposed to the quick and abrupt lysis observed in dsDNA phage T-even infections. Additionally, the timing and speed of lysis was observed to depend on the the growth phase of infected bacteria. Lysis of bacteria infected during stationary phase occurred later and at a slower rate than lysis of bacteria infected during log phase, a phase indicative of cell growth and division. These observations can be explained by the later deduced lysis mechanism [17].

The lysis gene *E* is 91 codons long and is embedded in the morphogene *D* in a +1 reading frame. E is an inner membrane protein with an N-out, C-in topology (figure 3) and has been shown to be necessary and sufficient for ΦX174 mediated lysis. Before elucidation of the E lysis mechanism, several hypotheses existed. E-mediated lysis shares characteristics to penicillin-mediated lysis; both require cell growth for lysis to occur and cause septal lysis. As penicillin

targets the peptide crosslinking step of PG biosynthesis (Ciak), it was hypothesized that E also targets PG biosynthesis. Alternatively, E-mediated lysis in hosts with impaired autolytic systems was also impaired, giving rise to the hypothesis that E activates the host autolytic system normally involved in degrading the cell wall during PG biosynthesis [18]. And the discovery of 50 nm - 200 nm holes in the cell septum and, occasionally, cell poles, prompted the hypothesis that E aggregates to form tunnels spanning the cell envelope [19].

Roof *et al*., 1994 isolated an *E. coli* host mutant named *slyD* (sensitivity to lysis) which displayed a complete block to lysis. This mutant was determined to contain a mutation in a novel protein, thereafter named SlyD, which shares homology with the FKBP family of peptidyl-prolyl cis-trans isomerases, a protein family shown to interconvert proline residues between the *cis* and *trans* conformations. E contains five proline residues [20], three of which lie in the TMD (Bernhardt 2000), and *slyD* mutants display decreased levels of E in the IM. It was thus hypothesized that SlyD folds E into its active configuration (Roof 1994), stabilizes E, or is involved in inserting E into the IM [21].

E mutants, deemed *Epos* (plates on *slyD*), capable of overcoming the *slyD* block to lysis and lysing host cells were found thereafter. To determine the protein targeted by E, host mutants resistant to *Epos*-mediated lysis, named *eps* (*Epos* sensitivity), were therefore isolated. Although most *eps* mutants developed lysis resistance by decreasing expression of *Epos*, several *eps* mutants expressing wildtype levels of *Epos* were found to contain mutations in the PG biosynthesis enzyme MraY [21]. E was thus found to act as a noncompetitive inhibitor of MraY, interacting with the enzyme and the enzyme-substrate complex with equal affinity. The location

of *eps* mutations indicates that the TMD of E interacts with MraY at TMD 5 and 9, causing a conformational change that decreases MraY activity [22]. This discovery of the protein target of E explains the necessity of cell growth and the observance of septal lysis. Cell growth and division are marked by increased PG biosynthesis, especially at the septum where new cell wall material must be laid down before cell division. Cells are therefore more sensitive to inhibition of PG biosynthesis during the log phase and display septal lysis upon PG biosynthesis inhibition. Interestingly, even a slight increase in MraY levels blocks E-mediated lysis [23], indicating that *E* expression remains constant at about 500 molecules per cell no matter the conditions [22].

Further studies by Tanaka *et al*., 2012 showed that E interacts with MraY specifically in the periplasmic leaflet of the inner membrane. The length of the TMD in E and its location in the inner membrane are vital to its inhibition of MraY. More specifically, the location of P21 is vital to E function. Proline residues produce kinks in the α-helices commonly found in TMD and are thought to allow for tighter packing between membrane proteins. This, however, does not to seem the function of P21 as moving the proline residue one turn towards the periplasm results in decreased function. P21, then, seems to confer a structure necessary for function [24].

The lysis protein A_2 of phage Qβ

The cytosolic lysis protein A_2 has also been shown to be necessary and sufficient for Qβ-mediated lysis. Interestingly, A_2 serves a dual function as the maturation protein (figure 3), enabling phage Qβ to adsorb to the host sex pilus and protecting phage genetic material from degradation. Like E, A_2 also causes septal lysis, supporting the hypothesis that A_2 also inhibits PG biosynthesis. As ^{3}H-DAP is not incorporated into PG or even the last cytosolic precursor

UDP-MruNAc-PP, A_2 was proposed to block one of the cytosolic steps of PG biosynthesis. Selection of host mutants resistant to lysis yielded two *rat* (resistance to A-two) mutants, both of which contained the same mutation in *murA* where A_2 putatively binds. MurA catalyzes the conversion of UDP-GlcNAc and phosphoenolpyrvuate (PEP) to UDP-GlcNAc-enolpyruvate (UDP-GlcNAc-EP), the first committed step of PG biosynthesis, and exists in one of three conformations: open; closed and bound to UDP-GlcNAc (singly liganded); and closed and bound to UDP-GlcNAc and PEP (doubly liganded). Subsequent analysis of the sugar-nucleotides that accumulate in cells expressing A_2 revealed an accumulation of the MurA substrate UDP-GlcNAc, further supporting the identification of MurA as the A_2 target [25].

Hatfull 2001 postulated that A_2 does not assume MurA inhibitory activity until assembled on a complete phage. Such a mechanism would solve the problems of lysis timing and the insolubility of free A_2. However, Reed *et al*., 2013 has since found that MurA inactivates fully assembled Qβ phages and promotes degradation of Qβ genomic RNA. Instead, Reed argues that the location of *rat* mutations suggests A_2 binds to the opening of the "mouth" of MurA when MurA is closed and singly liganded, locking MurA in a closed conformation and preventing further synthesis of UDP-GlcNAc-EP. The presence of a hydrophobic patch surrounded by hydrophilic residues on the proposed A_2-MurA binding site and binding assays showing that A_2 binds preferentially to the singly liganded state support this argument [26].

The timing of lysis is instead regulated by the amount of Qβ RNA. A_2 is synthesized in levels about three-fold that of MurA and largely exists bound to Qβ RNA, limiting the amount of free A_2 available to bind to MurA [26].

The lysis protein L of phage MS2

The lysis gene *L* overlaps with MS2 coat and replicase genes and encodes a 75 amino acid IM protein with a C-out, N-in topology (figure 3). Unlike the lysis proteins of other prototypical small lytic phages, L does not cause septal lysis. Instead, L causes random blebbing in which the cell bulges out at random locations before lysing (figure 1) [27]. This is the first indication that L-mediated lysis occurs through a mechanism other than inhibition of PG biosynthesis. Additionally, the average glycan strand length and extent of crosslinking decrease upon L-mediated lysis, suggesting that L functions by activating host autolysins normally involved in PG degradation during PG biosynthesis [28]. This mechanism continues to be investigated.

Phage M

The continued increase in antibiotic resistance necessitates the discovery of novel antibiotics and their targets. As the three prototypical small lytic phages display great diversity in their lysis mechanisms, studying the lysis proteins of other small phages may lead to the discovery of novel lysis mechanisms that can be applied to the development of antibacterial therapies. The lysis proteins of *Leviviridae* phages have evolved in three genetic architectures: separate from all other genes as in AP205, overlapping with the coat and replicase genes in a +1 reading frame as in MS2, and embedded in the +1 reading frame of the replicase gene as in M. We therefore chose to study the lysis proteins of *Leviviridae* phages, reasoning that the wide range of genetic architectures suggests a similar range in lysis targets [29].

In particular, we studied M Lys, the lysis protein of phage M. Phage M was isolated from sewage in South Africa in the early 1980s and adsorbs to pili encoded by the IncM plasmid. As the IncM plasmid can be found in multiple bacterial strains, phage M can infect various IncM-containing strains of *Escherichia, Klebsiella, Serratia, Salmonella, and Proteus.* M Lys is an IM protein 37 amino acids long [29] with an N-out, C-in topology. Because of its similarities in both membrane topology and lysis phenotype to the lysis protein E, we hypothesized that M Lys inhibits PG biosynthesis.

CHAPTER II

METHODS

Bacterial strains, plasmids and growth conditions

XL1-Blue (*recA endA1 gyrA96 thi hsdR17 supE44 relA1 lac* [F'::Tn10 *proA*$^{+}$*B*$^{+}$ *lacIq* Δ(*lacZ*)M15]) was used for the isolation of insensitive to M Lys (*iml*) mutants and nonfunctional *M lys* mutants. Additional strains used in this work are listed in Table A1 in Appendix A.

Plasmids used in this work were constructed by a graduate student in the laboratory and are listed in Table A1 in Appendix A.

Cultures were grown in lysogeny broth (LB) supplemented with 100 μg/ml ampicillin (Amp; RPI), 10 μg/ml chloramphenicol (Cam), 0.4% final arabinose (Ara), 1mM final isopropyl-β-D-thiogalactopyranoside (IPTG; RPI), and 20 μg/ml final 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-Gal) where indicated. All chemicals were supplied by Sigma Aldrich unless otherwise indicated.

Selection and screening for *iml* mutants

A culture of XL1-Blue pBAD24 M Lys was grown to an A_{550} of 0.2, after which M Lys expression was induced with arabinose. Upon the completion of lysis 2 hours after induction, cells were collected from 10 ml of culture through centrifugation, resuspended in 100 μl LB, and plated on LB-Ara-Amp-IPTG-X-gal plates. To ensure lysis resistance and continued M Lys

expression, surviving blue colonies were isolated and subjected to the same selection and screening protocol.

Selection and screening for nonfunctional *M lys* mutants

M Lys was subjected to error-prone PCR using the GeneMorph® II Random Mutagenesis Kit (Agilent Technologies), cloned into the pBAD24 vector, and transformed into XL1-Blue cells. The selection and screening process used to isolate nonfunctional *M lys* mutants is identical to that described for *iml* mutants above.

DNA sequencing

Plasmid DNA was purified from cells using the QIAmp DNA Mini Kit (QIAGEN). The *murJ* locus in *iml* mutants was PCR amplified using the Phusion® High-Fidelity DNA polymerase (NEB) and primers listed in Table A2 in Appendix A. DNA was submitted to Eton Bioscience for Sanger sequencing.

Lysis profiles

Cultures were grown in LB-Amp or LB-Amp-Cam at 37°C to an optical density at 550 nm (OD_{550}) of 0.2 to 0.6, and M Lys expression was induced with arabinose. Growth was monitored at OD_{550} until lysis was complete.

Crosslinking

M Lys was crosslinked to neighboring molecules using maleimidobenzophenone (MBP), a heterobifunctional crosslinking reagent containing a sulfhydryl-reactive group and a photoactive group.

A culture of TB28 was grown to OD_{550} 0.6, induced with arabinose for 10 minutes, and harvested by a 15 minute centrifugation at 10,000 x g at 4°C. The pellet was resuspended in 1 ml minimal A buffer pH 7.2, and 100 µl of resuspension was added to a sterile well dish. 1 µl of 100 mM MBP in dimethylformamide (DMF), or 1 µl of DMF for the mock treatment, was added. The reaction mixture was incubated in the dark for 30 minutes, after which 1 µl 1 M dithiothreitol was added to quench the reaction. The mixture was irradiated with 366 nm light for 20 minutes and subjected to TCA precipitation for further analysis by SDS-PAGE and Western blotting.

SDS-PAGE and Western blotting

Protein samples were precipitated from 1 ml of culture or the crosslinking reaction mixture in 10% TCA on ice for 20 minutes. Precipitated protein was pelleted down by a 10 minute centrifugation at 10,000 x g, washed in cold acetone, and resuspended in 10 µl 2X SDS BMS and 10 µl 0.1 M Tris pH 3 10 M urea. Samples were boiled for 10 minutes before being loaded onto an appropriate gel (16.5% Tris-Tricine for analyzing M Lys expression and 4-20% Tris-Glycine for crosslinking experiments) and subjected to electrophoresis. Proteins were transferred from the gel to a nitrocellulose membrane using the appropriate apparatus (semidry transfer apparatus for analyzing M Lys expression and tank transfer apparatus for crosslinking experiments),

Membranes were incubated first in either anti-His (1:3,000; Sigma Aldrich) or anti-L (5'-TPASTNRRRPFKHEDC; 1:3,000; Sigma Aldrich) and second in peroxidase anti-mouse produced in goat (1:3,000; Thermo Scientific). The SuperSignal® West Femto Maximum Sensitivity Substrate kit (Thermo Scientific) was used to develop the signal, and chemiluminescent imaging with ChemiDoc™ XRS system (Bio-Rad) was used to detect the signal.

In silico modeling

The structure of MurJ was predicted using the I-TASSER server [30-33] based on a previous study showing that I-TASSER accurately predicted MurJ membrane topology and the location of the solvent-exposed central cavity [10].

CHAPTER III

RESULTS

Selection of *iml* (insensitive to M Lys) mutants

To identify the target of M lys, we sought to identify spontaneous mutants resistant to M lys-mediated lysis. Mutations arising in any one or all of the following proteins might confer resistance to lysis: the target protein of M lys; chaperone proteins involved in M lys folding or insertion in the inner membrane; and proteins involved in M lys expression. Proteins involved in M lys expression provide no information on *E. coli* host factors involved in the lysis pathway, so these mutations were selected against (figure 4A).

To avoid mutants that downregulate M lys expression, a modified protocol for alpha complementation was implemented. β-galactosidase normally cleaves glycosidic bonds in β-galactosides such as lactose. This activity can be taken advantage of to visually mark cells displaying β-galactosidase activity; intracellular cleavage of the lactose analog X-gal, for example, yields a blue substituted indole and therefore dyes cells blue. Linking M lys expression to β-galactosidase assembly and plating cells on media containing X-gal, then, dyes cells expressing M lys blue.

Towards this end, *M lys* and l*acZα* were cloned into the arabinose inducible pBAD24 in tandem and transformed into XL1-Blue cells containing *lacZω* on the IPTG inducible F' plasmid. Because the two are genetically linked, *M lys* expression also results in *lacZα* expression. Separately, LacZα and LacZω are nonfunctional fragments of β-galactosidase. In the presence of

each other, though, the two fragments spontaneously assemble to form functional β-galactosidase. Thus cells resistant to M Lys-mediated lysis can be selected and screened for blue colonies (figure 4B).

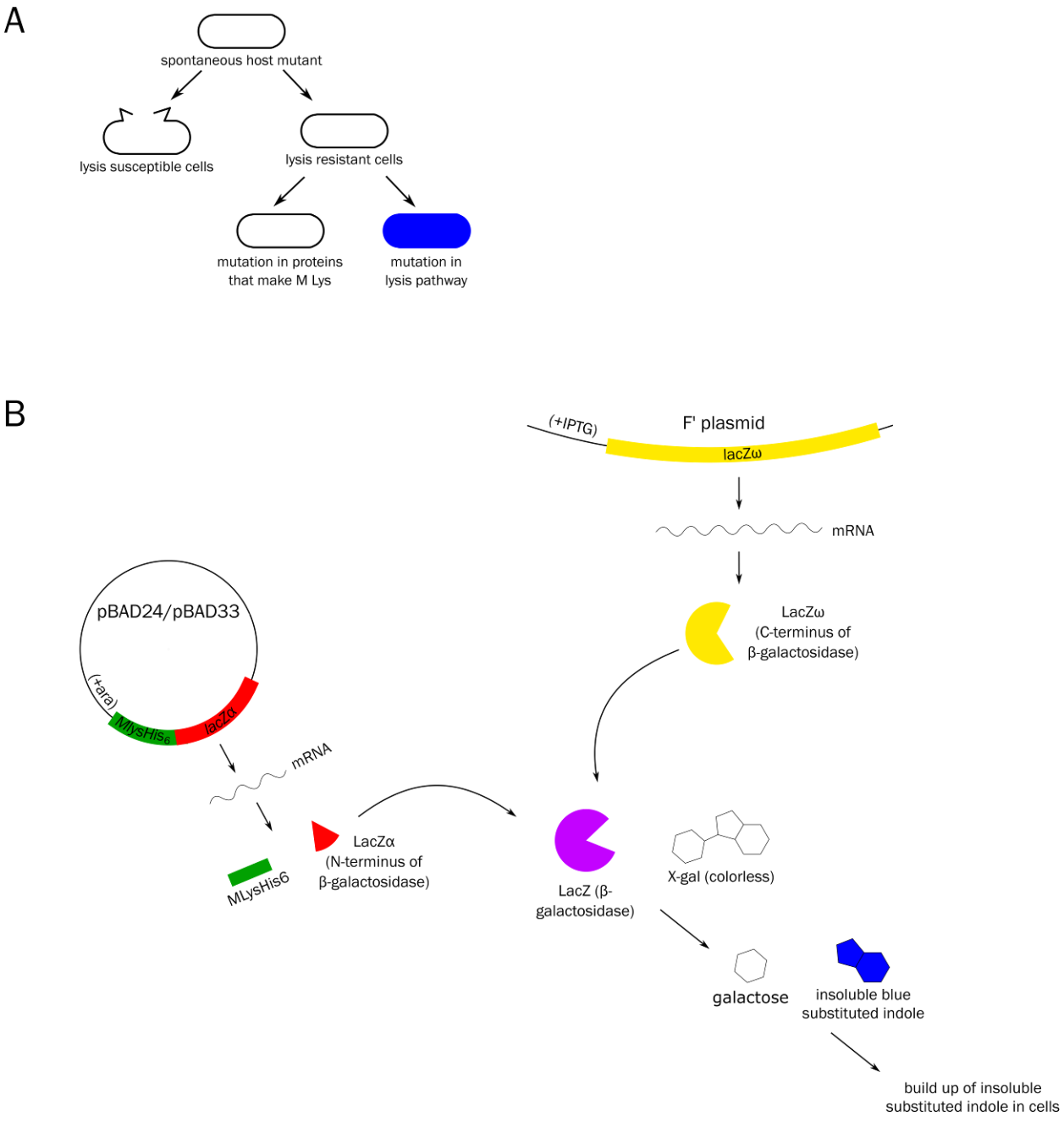

Figure 4. A modified alpha complementation protocol was used to select *iml* mutants
(A) Mutations in two types of proteins can give rise to lysis resistance: proteins involved in M Lys expression and

proteins involved in the lysis pathway. Mutations of proteins involved in the lysis pathway were selected for. (B) LacZα expression was linked to M Lys expression. Subsequent LacZω expression from the F' plasmid resulted in the spontaneous assembly of functional β-galactosidase which, when paired with X-gal, yields blue cells.

Cultures were grown in LB-Ampicillin broth. Lysis was induced with arabinose at A_{550} 0.2 – 0.6 and survivors were collected and plated on LB-Ampicillin-Arabinose-IPTG-X-gal agar plates (figure 5). Eleven blue colonies have been isolated so far.

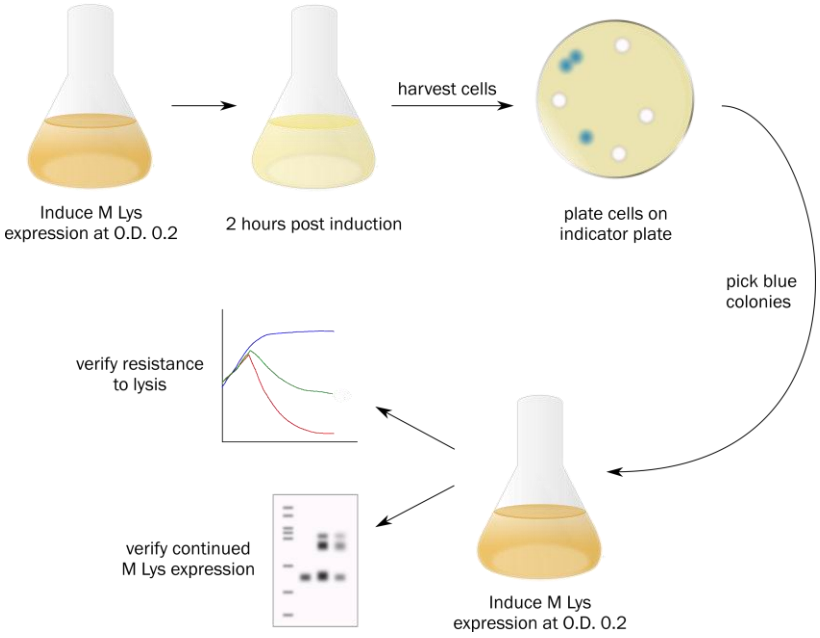

Figure 5. Upon *iml* mutant isolation, lysis resistance and continued M Lys expression were verified.

To verify that *iml* mutants do not downregulate M lys expression, M lys levels in the *iml* mutants were visualized through Western blotting. Mutant lysis profiles were also generated to ensure resistance to M lys-mediated lysis (figure 5). Of the eleven blue colonies selected, M Lys expression and lysis resistance have been verified in *iml* 1, 2, 3, and 4 (figure 6).

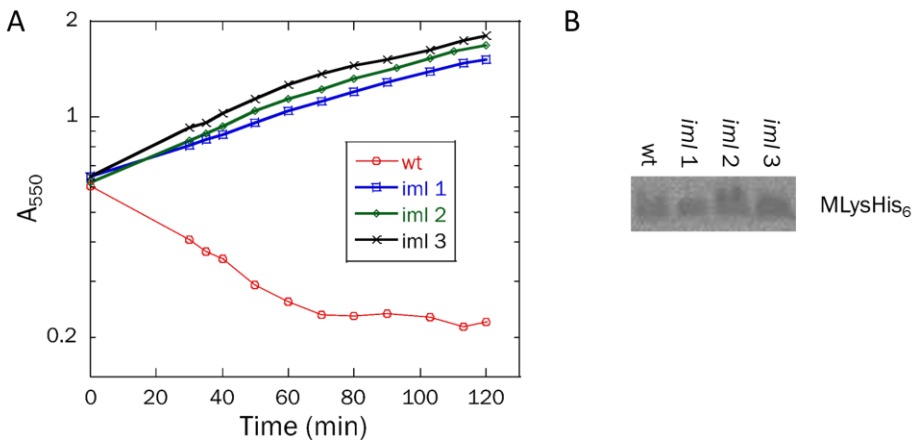

Figure 6. Insensitive to M lysis (*iml*) mutants display resistance to M lys-mediated lysis
(A) Cultures of wild type (red) and *iml* mutants 1 (blue), 2 (green), and 3 (black) carrying *M lys-his*$_6$ in plasmid pBAD24 were induced at A_{550} = 0.6.
(B) Trichloroacetic acid (TCA) precipitation of cultures was analyzed by SDS-PAGE and immunoblotting with anti-His antibody. Mutants accumulated similar levels of M Lys-His$_6$ as the wildtype.

The *iml* mutants contain substitutions mutations in MurJ

Work done in parallel by a graduate student in the lab has indicated that MurJ, a flippase in the peptidoglycan biosynthetic pathway that flips lipid II from the cytoplasm into the periplasm might be the target of M Lys. Briefly, he isolated multiple clones of *murJ* that provided protection against M Lys in a multi-copy suppression assay. Based on this new information, the *murJ* locus from *iml* mutants was therefore amplified using PCR and sequenced.

Each *iml* mutant yielded a *murJ* mutation except *iml* 4 and 11 (table 1). PCR amplification products from these mutants yielded low quality sequencing, so *murJ* in these mutants will be amplified via PCR and sequenced again.

In the mutants that yielded high quality *murJ* sequencing, seven unique substitution mutations were found, two located on TMD 2 and five located on TMD 7 (table 1, figure 7). Furthermore,

TMD 7 mutations are located every four residues, indicating that they map on the same face of the α-helix, thus providing strong evidence for an M Lys interaction interface with the TMD 7 of MurJ.

iml Mutant	MurJ Mutation	TMD 2	TMD 7
1	V299G		X
2	M233L		X
3	F64L	X	
4	n/a		
5	A59G	X	
6	I248S		X
7	I252F		X
8	Q244P		X
9	I248S		X
10	I248S		X
11	n/a		

Table 1. *iml* mutations were found in TMDs 2 and 7 of MurJ.

One issue encountered while selecting spontaneous *iml* mutants is the reappearance of the mutant I248S (table 1). To avoid consistently selecting *iml* mutants with the same mutation, selected *iml* mutants will be subjected to chemical mutagenesis via EMS and other chemical and physical mutagenic agents. The resulting mutants will be subjected to the same selection process as used above.

Selection of *M lys* mutants

To identify the specific M Lys amino acids involved in lytic activity, *M lys* was subjected to error-prone PCR, cloned into empty vectors in tandem with LacZα, and transformed into XL1-Blue cells. Nonfunctional mutants were selected using the same selection process as used above

(figure 5). Lysis profiles will be generated and M Lys expression verified through Western blotting in the future.

Figure 7. MurJ sequence and location of isolated mutations.

Substitution mutations in *M lys* mutants

More than twenty *M lys* mutants have been isolated at this time, five of which contain one of the following substitution mutations: N6K, A16P, I20K, S25L, and V32E (figure 8). As M Lys is a predicted α-helical transmembrane protein, disrupting transmembrane hydrophobicity or structure interferes with M Lys insertion in the membrane and thus enables lysis resistance. This

is exemplified by the mutants N6K, A16P, and I20K. N6K and I20K introduce a positively charged residue to the transmembrane domain. As the inner membrane is a hydrophobic environment, the presence of charged molecules is thermodynamically unfavorable and unlikely to occur. A16P introduces proline to the transmembrane domain. Because proline is a cyclic amino acid, the bond angles in its backbone are fixed and not conducive to forming α-helices. The amine group on proline residues is also incapable of forming the hydrogen bonds necessary to form α-helices. A kink in the α-helix therefore forms when proline is present.

Changing the charge of either terminus relative to the other could also confer lysis resistance to host cells. The cytoplasm is negatively charged compared to the periplasm, so inner membrane proteins tend to orient with the positive terminus located inside the cytoplasm. Both termini in M lys have a +1 charge. The C terminus, however, have two positively charged residues while the N terminus has one. It can therefore be predicted that M lys has an N-out, C-in topology. Assuming this prediction is correct, M Lys variants with mutations like V32E that neutralize the charge of the C terminus will have an inverted membrane topology and thus impaired M lys function. To verify M lys membrane topology, charged tags can be fused to either M lys terminus and lysis profiles established. Tags accentuating the charge differences between the termini should result in continued M lys function, and tags reversing the charge differences should result in impaired M lys function.

The mutant S25L does not affect the transmembrane domain or change the charge of either terminus. This mutation, then, may prevent M lys-mediated lysis by affecting M lys interaction with MurJ.

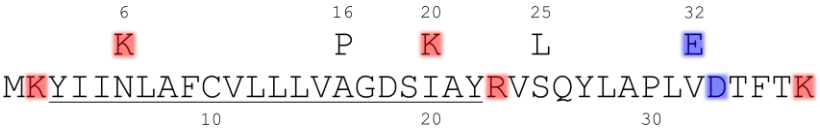

Figure 8. M Lys-His_6 sequence and location of isolated mutations.
Amino acid residues highlighted in red and blue are positively and negatively charged, respectively, at physiological pH. Underlined residues make up the transmembrane domain.

M Lys crosslinking studies

MBP contains a sulfhydryl-reactive group and a photoactive group which allow for its use as a crosslinking agent. M Lys contains one cysteine residue, Cys^{10}, and so can react with MBP at one location. Cys^{10} lies within the transmembrane domain. Site directed mutagenesis was used to create the mutant M Lys C10S, which serves as a negative control. The wildtype M Lys serves as a positive control. All M Lys variants were tagged with 6XHis and L2-36 to allow for visualization during Western blotting.

E. coli of various *iml* mutants were transformed with a pBAD33 vector carrying one of the two *M lys* mutants. After selecting for cells containing pBAD33, cultures were grown to a desired OD and *M lys* expression induced. Samples were collected prior to lysis and subjected to MBP crosslinking, and crosslinked proteins were visualized using Western blotting.

The outcomes from the resulting Western blot are inconclusive. No bands appear in wildtype cells transformed with *M lys* C29S containing vectors, indicating a lack of *M lys* expression. This sample was therefore disregarded from further analysis. Background bands appear in the negative controls (empty pBAD33 vector, incubation with DMF instead of MBP, and *M lys* C10S C29S). Interestingly, the *M lys* C10S C29S negative controls display bands at molecular

weights corresponding to M Lys crosslinked with MurJ. These bands may simply be background bands, though, because the same band appears in the empty pBAD33 sample (figure 9).

To avoid the presence of background bands, FLAG-tagged MurJ will be used instead of His-tagged M Lys in future crosslinking experiments.

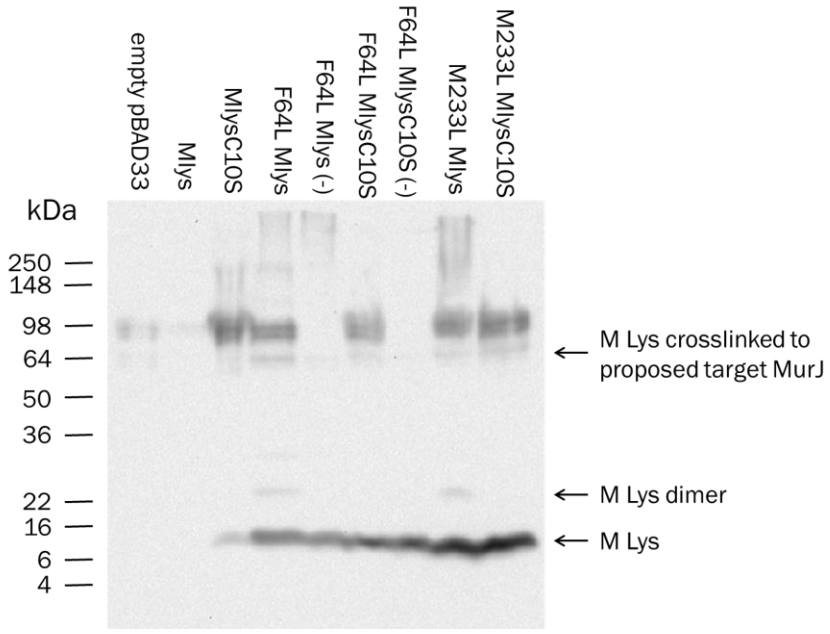

Figure 9. M Lys crosslinking experiments yield inconclusive results.
Trichloroacetic acid (TCA) precipitation of cultures was analyzed by SDS-PAGE and immunoblotting with anti-L antibody. The presence of background bands makes it difficult to draw conclusions from this blot.

CHAPTER IV

DISCUSSION/CONCLUSIONS

As a small lytic phage of the *Leviviridae* family, phage M has evolved a single stranded RNA genome encoding four genes, only one of which is involved in host lysis. Similarities in lysis phenotype and membrane topology between E and M Lys suggested that M Lys, like E, effects lysis by inhibiting PG biosynthesis. Here we have used a genetic approach to identify the M Lys target protein and, in doing so, provide support for the identity of the *E. coli* lipid II flippase.

Using a modified alpha complementation protocol, we identified host mutants resistant to M Lys-mediated lysis, henceforth referred to as *iml* mutants. A multi-copy plasmid suppression expressing SauIIIA1 digests of the *E. coli* genome rescued cells from lysis upon expression of either the arabinose exporter YdeA or the proposed lipid II flippase MurJ (K. R. Chamakura and R. Young, unpublished). We hypothesize that YdeA rescues cells from lysis by exporting arabinose before it can activate the araBAD promoter linked to *M lys* and induce expression. MurJ, on the other hand, rescues cells from lysis through an unknown mechanism. The simplest explanation is that M Lys targets and inhibits MurJ, and overexpressing MurJ from a multi-copy plasmid rescues MurJ function.

Based on these results, we sequenced the *murJ* locus in isolated *iml* mutants and observe single substitution mutations in MurJ, although two mutants yielded low quality sequencing and need to be re-sequenced. Interestingly, two mutations map to the same face of MurJ TMD 2 and five to the same face of TMD 7 (figure 10), outlining the M Lys-MurJ interface essential to M Lys

function. Mutation of this interface presumably weakens M Lys-MurJ interactions, thus lending lysis resistance to the host cell. This in turn suggests a possible lysis mechanism for M Lys function. MurJ has a positively charged central cavity and, as a member of the MATE family, has been proposed exist in two V-shaped conformations with the central cavity exposed to either the cytoplasm or the periplasm. In the current model for MurJ-mediated lipid II flipping, MurJ binds to lipid II in the cytoplasmic leaflet, changes conformations, and releases lipid II into the periplasmic leaflet. The location of the *iml* mutations suggests that M Lys interacts with MurJ along TMDs 2 and 7, locking MurJ into one conformation and preventing further flipping activity.

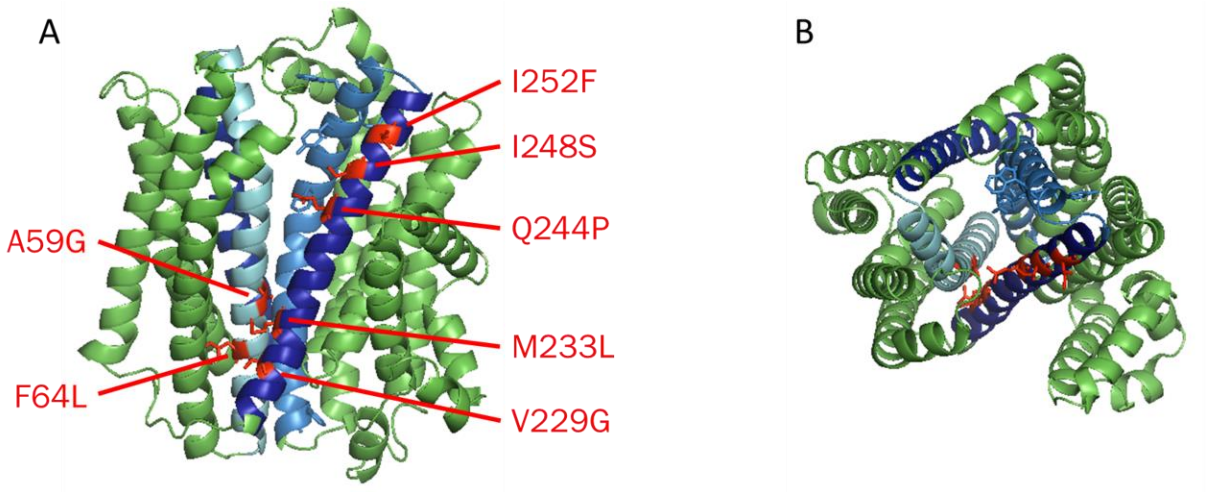

Figure 10. Location of *iml* mutations on I-TASSER model structure of MurJ
(A) Front view from within the membrane plane. The central cavity of MurJ is open to the periplasm in this conformation.
(B) View from the periplasm.

Our results support the identification of MurJ as the *E. coli* lipid II flippase. Based on similarities to the lysis protein E in membrane topology and lysis phenotype, we predicted that M Lys inhibits an IM enzyme involved in PG biosynthesis; both M Lys and E have an N-out, C-in topology, and both cause septal lysis, a lysis phenotype also seen in penicillin-mediated lysis and

associated with inhibition of PG biosynthesis. We have shown here that M Lys targets MurJ, implicating MurJ in PG biosynthesis. The only proposed function of MurJ in PG biosynthesis is that of the lipid II flippase. Additionally, we address the concerns of background noise obfuscating FtsW-mediated lipid II flipping in the colM assay developed by Sham *et al*., 2014. M Lys expression from one plasmid either causes cell lysis in wildtype *E. coli* cells or doesn't affect cell density in *iml* mutants with no intermediary phenotypes (figure 6)

M Lys overexpression from two plasmids, interestingly enough, causes reduced lysis in *iml* mutants (data not shown), suggesting that a single mutation in the M Lys-MurJ interaction interface is not enough to completely block M Lys-MurJ interaction. While the M Lys-MurJ interaction is weak enough in *iml* mutants to allow for continued MurJ function when M Lys is expressed from a single plasmid, overexpressed M Lys overwhelms the amount of MurJ present in a host cell through sheer numbers and inhibits MurJ function, leading to reduced lysis.

Future Work

We now have genetic evidence suggesting that M Lys interacts with and inhibits MurJ. However, biochemical evidence for the M Lys-MurJ interaction remains to be elucidated. Two common methods of demonstrating protein interaction are *in vivo* crosslinking and co-immunoprecipitation. We began the process of identifying interacting proteins using MBP, a heterobifunctional crosslinking reagent with a sulfhydryl-reactive group and photoactive group, The presence of background bands interfered with interpretation, however, and reaction conditions remain to be optimized. The use of reagents similar to MBP but with differing arms lengths will allow the interaction distance between M Lys and MurJ to be deduced.

Alternatively, appropriate affinity columns can be used to purify the 6XHis-tagged M Lys or FLAG-tagged MurJ variants used.

Our work here has also established a new class of antibiotics that target flippases, of which M Lys is the first known member. Furthermore, the presence of MurJ homologs in all cell wall synthesizing bacteria implicates M Lys in a possible role as a broad spectrum antibiotic. M Lys function must first be tested in other bacteria to verify that function is indeed conserved among a range of MurJ homologs, and M Lys specificity to MurJ must be determined. M Lys itself, however, cannot be used as a clinical antibiotic due to its inability to diffuse across the cell membrane, and so small molecules capable of diffusing into a cell and acting along the same mechanism must first be developed. Toward this end, a greater understanding of the MurJ mechanism and how M Lys inhibits it is necessary. The use of various mutagenesis protocols, such as error-prone PCR and site-specific mutagenesis, to identify nonfunctional M Lys mutants could aid in the identification of M Lys residues critical for function. Additionally, various M Lys truncations could be tested to determine the minimal inhibitory domain.

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APPENDIX A

Table A1. Strains and plasmids

Strain	Relevant genotype [a]		Source or Reference
MG1655	*ilvG rfb*50 *rph*1		[34]
TB28	MG1655, *lacIZYA*<>*frt* [wt]		[35]
Construct	Relevant genotype [a]	ori	Source or Reference
Plasmids:			
pBAD33	*cat araC* P_{BAD}::	pACYC	[36]
pRY34353	*cat araC* P_{BAD}::*M lys-his*$_6$ *lacZα*	pACYC	This work
pBAD24	*bla araC* P_{BAD}::	ColE1	[36]
pRY34354	*bla araC* P_{BAD}:: *M lys-his*$_6$	ColE1	This work
pRY34355	*bla araC* P_{BAD}:: *M lys-his*$_6$ *lacZα*	ColE1	This work

Table A2. Primers used to amplify the *murJ* locus in *iml* mutants.

Primer name	Sequence
MurJ forward	5’ GAA TAA ACC CTG TAA CAT CTG GCG G 3’
MurJ int forward 1	5’ ATC ATG GTG ACC GCG CCA GGC TTC GCT GAC 3’
MurJ int forward 2	5’ CTT GCT TCC GGT TCG GTG TCT TGG ATG 3’
MurJ int reverse 1	5’ CGG GGT AAA GAT TTT CTG CTT ACG C 3’
MurJ reverse	5’ ACC GCA TCA GGA ACC GAC GCT ATA TG 3’