BACTERIOPHAGE MS2 L PROTEIN: GENETIC AND BIOCHEMICAL CHARACTERIZATION

A Dissertation

by

BRENLEY KATHLEEN McINTOSH

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2008

Major Subject: Microbiology

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Approved by:

Chair of Committee,	Ryland F. Young
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ABSTRACT

Bacteriophage MS2 L Protein: Genetic and Biochemical Characterization.

(May 2008)

Brenley Kathleen McIntosh, B.S., University of Lethbridge

Chair of Advisory Committee: Dr. Ryland F. Young

In order to release progeny, bacteriophages must lyse the host cell by compromising the peptidoglycan layer. There are two known strategies of lysis: the holin-endolysin system and single gene lysis (SGL), which are dependent on the genome size. Large phages encode multiple proteins, including a holin and endolysin, for lysis. In contrast, small ssRNA phages (*Leviviridae* and *Alloleviviridae*) and ssDNA phages (*Microviridae*) do not encode a muralytic enzyme and accomplish lysis with a single gene. The cellular target of the lysis gene E from the prototypic microvirus, ϕX174, and A_2 from the prototypic allolevivirus, Qβ, has been elucidated. In both cases, these proteins were demonstrated to inhibit specific enzymes within the peptidoglycan biosynthetic pathway and infected cells lyse as a result of septal catastrophes. The prototype Levivirus MS2 encodes L, a 75 aa polypeptide that effects lysis without inhibiting murein synthesis.

The purpose of the work described in this dissertation was to characterize MS2 L using genetic and biochemical strategies. Using a genetic approach, PcnB was shown to be important to the entry of the MS2 RNA into the cytoplasm. L accumulation during

infection was quantified by comparison to purified, oligohistidine-tagged L. Biochemical experiments demonstrated the L protein behaved as a periplasmic, membrane-associated protein. The morphologies of cells undergoing L-mediated lysis are significantly different from cells lysing due to A_2 expression, since L-lysing cells do not show septally localized membrane protrusions.

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CHAPTER I

HISTORICAL OVERVIEW AND INTRODUCTION

Bacteriophages, or bacterial viruses, are the most common organism on earth, totaling an estimated 10^{31} (Brüssow and Hendrix, 2002). As such, phages have a massive, though entirely understudied role in every life process and elemental cycle. After identification, phages rapidly became a desirable agent in the field of biology. In their simplicity, bacteriophages were seen as a model for understanding more complex organisms. Many advances in biology and even the creation of entirely new fields of study, from genetics to genomics, are a direct result of studying these simple, yet elegant organisms. The intellectual force behind this research was Max Delbrück. Although trained as a physicist with Niels Bohr, Delbrück moved into the field of biology and after a brief stint with *Drosophila*, chose the simplest organism he could find, bacteriophages. Delbrück believed that studying the simplest system available was a more straightforward conduit to understanding similar processes in higher organisms (Luria and Delbrück, 1943). He vigorously promoted specific phages, known as the T-set, as the leading model system for studying any biological phenomenon (Demerec and Fano, 1945). In doing so, he worked closely with a number of influential biologists, including Salvador E. Luria and Alfred Hershey. Many of the most important discoveries about the nature of genes, the definition of a codon, transcription and

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This dissertation follows the style of Cell.

translation through recombination were originally characterized in phages (Benzer, 1955; Crick et al., 1961; Hershey and Rotman, 1949).

Max Delbrück envisioned bacteriophages as organisms that would act as a simple model for higher processes and organisms. Based on his assumption of how uncomplicated phages were, he presumed that all of the basic mysteries of bacteriophages would be rapidly identified and solved. Considerable progress has been made in the field of phage biology, but many questions still exist. The topics of this chapter have been the subject of numerous reviews, though it will emphasize research involving MS2 (see Lawley et al., 2003; Young et al., 2000; Zinder, 1975).

Infection

F^+ (male) *E. coli* cells carry the conjugative plasmid F, which encodes a DNA transfer apparatus. The F DNA is approximately 100 kilobases (kb), including the *tra* genes, and is maintained either as an extrachromosomal, single-copy plasmid or integrated directly into the *E. coli* chromosome. The F genes code for the "sex pilus" that protrudes from the surface of the cell and after contacting a F^- cell, allows for the transfer of DNA to the recipient F^- upon mating pair formation. In addition, the F plasmid carries genes that function to exclude F transfer from another male cell and others that enable the host to shed the pilus under suboptimal mating conditions, a phenomenon referred to as "phenocopying." F^+ bacteria generate the sex pilus, constructed out of roughly 10,000 monomers of the product of the *traA* gene, pilin.

Initially, the gene product of *traA*, known as propilin, is 130 amino acids in length. After an N-terminal cleavage event that removes a 50 amino acid leader sequence, the N-proximal residue is acetylated, and the remaining C-terminal 70 amino acids form the mature pilin protein. These monomers oligomerize into a long, helical structure that is more than twice the length of the cell at 2-4 μm. It has a diameter of about 8 nm and a central, hollow core that is 2 nm in diameter. When the pilus comes into physical contact with a recipient, or F^- cell, the pilus retracts to bring the cells into direct contact, which leads to the transfer of F DNA. It remains unclear whether the F DNA is transported into the recipient through the pilus or by some other mechanism after the formation of a mating pair causes fusion of the cell walls (Harrington and Rogerson, 1990; Lawley et al., 2003).

Infection by filamentous bacteriophages

Loeb (1960) identified the requirement for an F-specific pilus, when he demonstrated the ability of certain phages to infect F^+ bacteria, but not F^- bacteria. Filamentous, single-strand (ss) DNA bacteriophages, including M13 and f1, use one of their ten gene products for recognition of an F^+ host cell. The amino-terminus of gene product III (Gp III) identifies and is responsible for binding to the tip of the pilus (Armstrong et al., 1981; Gray et al., 1981). Most mutations in TraC, a component of the pilus assembly complex, eliminate production of a pilus, conjugation and plating of all F-specific phage (Willets and Achtman, 1972). However, a *traC* mutant, *traC1044*, has been characterized that fails to elaborate a F pilus, but remains sensitive to filamentous

phage, f1. These mutants transfer genetic markers 10,000 fold less efficiently than wild-type cells even though mature pilin is produced. It was proposed that this particular *traC* mutation caused the cells to produce a short pilus tip without the extended polymeric structure that allowed for binding of f1. Upon binding, the pilus retracts, drawing the phage to close proximity with secondary receptors (Jacobson, 1972). Upon nearing the cell surface, three secondary receptors, TolQ, TolR and TolA, allow the phage to integrate into the cell envelope (Sun and Webster, 1986, 1987). The exact mechanism through which the genome enters the cell remains a topic of debate. Currently, there are three models proposed: transport of the genome into the central pore of the pilus followed by retraction and penetration; binding of the phage at the tip causes retraction and brings the phage to the cell surface, where it can inject its genome; binding occurs at the tip followed by movement of the phage itself down the pilus to the cell surface (Model and Russel, 1988). At this time, there has not been an experiment that clearly distinguishes between these models. Nevertheless, it appears from the *traC1044* mutant that filamentous phage do not require the retraction event for infection, rather it may merely be responsible for bringing the phage in close proximity to the cell surface and TolQRA proteins. Interestingly, after the genome enters the cytoplasm of the cell, radiolabeled monomers of the major coat protein (GpVIII) from the infecting phage can be found in the inner membrane of the cell and are reutilized to complete assembly of progeny phage (Smilowitz, 1974).

Filamentous phage adsorb to the tip of the pilus to prevent formation of mating pairs between an F^+ and F^- cell, thus acting as a competitive inhibitor of conjugation.

This suggests that the tip is essential for conjugation (Ou, 1973). Addition of 1 mM Zn^{2+} prevents the formation of mating pairs and binding by filamentous phages to the tip of the pilus, but the ability of ssRNA to bind to the sides of the pili remains intact (Ou and Anderson, 1972).

Infection by ssRNA bacteriophages

In contrast to filamentous ssDNA phage, ssRNA phages adsorb to the sides of the pilus and do not cause its retraction (Brinton, 1965). Early on in this field, it was noted that when a culture of F^+ bacteria was infected with 500 phage per cell, the sites of attachment were not saturated along the length of the pilus (Paranchych et al., 1970). Mutations in *traA*, the gene encoding the pilin subunits, providing additional positive charges near the C-terminus of the protein are resistant to both ssRNA and ssDNA phages (Frost, 1985). These mutations decrease the ability of the phage to interact with the pilus, though these mutants assembled an F pilus normally. Manchak et al. (2002) performed mutational analysis of pilin and showed a cluster of mutations from residue 11-27 are particularly important to ssRNA phage binding and are proposed to form the exposed sides of the pilus. These mutations did not drastically affect the function of the pilus, allowing for near normal mating efficiency and pilin production (Manchak et al., 2002). After binding to the pilus, the ~3.5-4.5 kb ssRNA molecule gains entry into the cell accompanied by at least one phage protein through a mechanism that remains unclear (Paranchych, 1975). The RNA genome exits the phage capsid and enters the pilus unidirectionally, beginning at the 3' end of the genome (Wong and Paranchych,

1976). It is thought that the ssRNA genomes travel down the pilus, since ssRNA phage do not require retraction of the pilus to bring the phage in closer proximity to the cell surface for infection (Brinton, 1965; Paranchych, 1975; Paranchych et al., 1970). It remains unknown whether the RNA-A protein complex is transported through the central pore of the pilus, or possibly slides down the exterior surface of the pilus to the cell envelope before penetrating the cytoplasm (Brinton, 1965; Brinton, 1971).

Other host mutants resistant to ssDNA and ssRNA phages

A number of F mutants have been characterized that provide varying degrees of resistance to ssRNA and/or ssDNA phages. Specific temperature-sensitive mutations in *traD*, which encodes a protein involved in conjugal DNA metabolism, are resistant to MS2, R17, f2 (Achtman et al., 1971; Paranchych, 1975; Schoulaker and Engelberg-Kulka, 1978). At the non-permissive temperature, these $traD^{ts}$ mutants remain sensitive to Qβ and elaborate more pili per cell than wild-type cells, but have dramatically reduced mating efficiencies: 1% of that occurring at the permissive temperature (30°C) (Achtman et al., 1971). By using radiolabeled RNA, Schoulaker and co-workers showed that the genome of MS2 penetrated into the $traD^{ts}$ cells. These cells were capable of producing coat protein and maturation protein at the non-permissive temperature, presumably by using the incoming genome as an mRNA template for translation. Further characterization showed that the $traD^{ts}$ cells produced significantly less MS2-specific replicase protein at the non-permissive temperature. Additionally, these mutants failed to convert the genome into the double-stranded form during replication at the non-

permissive temperature, though Qβ replication occurred normally (Schoulaker and Engelberg-Kulka, 1981, 1983). This led the authors to suggest that the mutation in TraD affects the ability of the *Leviviridae* to assemble an active replicase complex (Schoulaker and Engelberg-Kulka, 1983).

Mutations were also identified in *traV* that reduce the plating efficiency of both ssRNA and ssDNA bacteriophages by nearly 6 orders of magnitude (Harris and Silverman, 2002). TraV is an outer membrane lipoprotein component of a complex of Tra proteins, including TraK and TraB, which spans the envelope of *E. coli* and are responsible for anchoring the F pilus (Harris et al., 2001). As would be expected, these mutations also affect the ability of the strain to conjugate, reducing the transfer of selectable markers by 6 to 7 orders of magnitude (Harris and Silverman, 2002). The reduction in both plating for both types of F-specific phage and conjugation is likely because the cells fail to construct a stable pilus and apparently do not even produce a stable tip, unlike some *traC* mutants.

There are several host mutants for which the genes affected were not identified, though the effects of the mutations have been more thoroughly characterized. Silverman et al. (1968) isolated an Hfr mutant that was resistant to ssRNA phage, but remained sensitive to ssDNA phage. Additionally, another mutant was resistant only to a Levivirus, f2 (Silverman et al., 1967). The mutants were shown to affect an early step in the infection process, the RNA injection stage. Although the genes involved were never identified, the phenotypes of these mutants were described.

It becomes obvious, collectively, from these studies that there exist numerous steps early in the life cycle of MS2 and other F-specific bacteriophages that are not well understood. The processes between the time at which the ssRNA or ssDNA genome leaves the capsid to the time at which it enters the cytoplasm remain unknown. Since resistant hosts can be isolated, it is straightforward to conclude that there are host proteins involved in these steps and it is simply not a matter of a phage binding to the F pilus and the genome appearing in the cytoplasm a short time later.

Genes and gene expression

The two families of ssRNA bacteriophages, *Alloleviviridae* and *Leviviridae*, produce a total of four proteins each, making them the simplest of all bacteriophage (Figure 1.1). The classes are separated based on genome organization, genome size, and serological differences (Furuse, 1987). Using these proteins and a few host proteins, the bacteriophage completes an infection cycle that results in the production and release of progeny virions. The bacteriophage lifecycle begins as the phage adsorbs to the pilus of an F^+ *E. coli* cell and injects its ssRNA genome, which penetrates into the cytoplasm. Once there, the genome is replicated, and progeny phage are produced and assembled. After completion of these tasks, lysis of the host is required to release the progeny phages into the environment. MS2, the prototypic Levivirus, completes its entire lifecycle in about an hour, generating five hundred to one thousand plaque-forming units (p.f.u.) per cell (Paranchych et al., 1970; van Duin and Tsareva, 2006). Both families of

ssRNA phages produce high titers of viable phage that are able to form plaques during each infective cycle. However, approximately 50% of the total phages produced are non-infectious and do not bind to the F pilus. The remaining 50% consists of phages

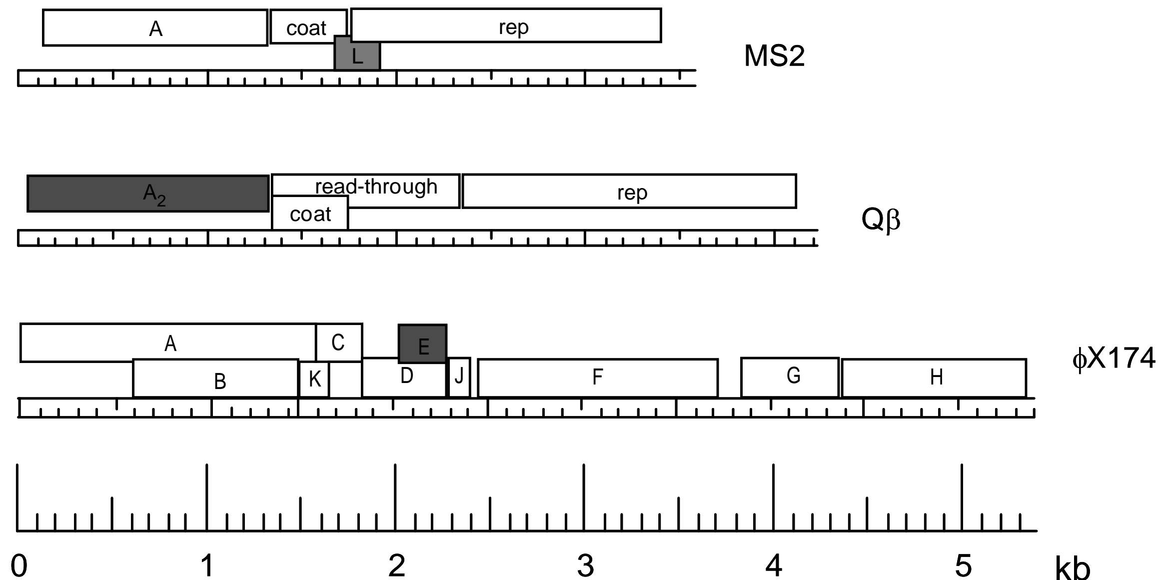

Figure 1.1. Genome organization of the three prototypic small genome phages. These phages include MS2 from *Leviviridae* (ssRNA), Qβ from *Alloleviviridae* (ssRNA) and ϕX174 from *Microviridae* (ssDNA). The genome of Microviridae is circular, but is shown as a linear ssDNA here. The gene responsible for lysis is darkly shaded grey. The scale represents kilobases of sequence.

that adsorb normally to the cell and fall into one of two classes. The first class accounts for nearly two-thirds of phages with the ability to bind to the pilus, but they fail to fully inject their ssRNA genome into the cell. The second class of bound phages makes up the remaining one-third and consists of viable phages that are able to support further rounds of infections (Paranchych et al., 1970). Thus, ssRNA phages are exceptionally

inefficient at producing infectious particles, with only 10-15% of total phage being viable.

MS2 has a small, linear ssRNA genome of 3569 nucleotides. The sequence is almost entirely protein coding, with a total of four genes (Figure 1.1). Starting from the 5' end of the genome, there is a 130 nucleotide stretch that is untranslated, then the first gene, which is the maturation gene, or A. It is followed by another short non-coding span of less than thirty nucleotides, then the coat gene. The third gene from the 5' end is the lysis, or L, gene, which is responsible for host lysis. This extraordinary gene overlaps the 3' end of the coat gene and the 5' end of the replicase coding sequence, which is the last gene. The 3' end of the genome has the longest stretch of non-coding nucleotides, a length of 174 nucleotides.

The translation of phage proteins is maintained by a set of complex folding events within the genome, including numerous long distance interactions, local stem-loops and positive and negative regulation events (Klovins et al., 1998; Min-Jou et al., 1972). One of the best-studied long distance interactions is the MJ (Min-Jou) interaction shown in Figure 1.2, which is involved in control of replicase translation (Min-Jou et al., 1972). Interestingly, the coat gene and its gene product have two, opposing regulatory functions. If the coat gene is being translated, it acts as a positive regulator of translation of the replicase gene, allowing for expression of Rep. On the other hand, the coat protein behaves as a negative regulator of replicase translation after reaching a threshold level, thereby preventing overexpression of Rep in the host cell (Berkhout and van Duin, 1985).

A remarkable characteristic of all ssRNA phage is that the entering genome is treated as a messenger RNA by the cell, though the expression of each gene appears to be regulated by a number of mechanisms. The gene product of the first gene, *A*, is the

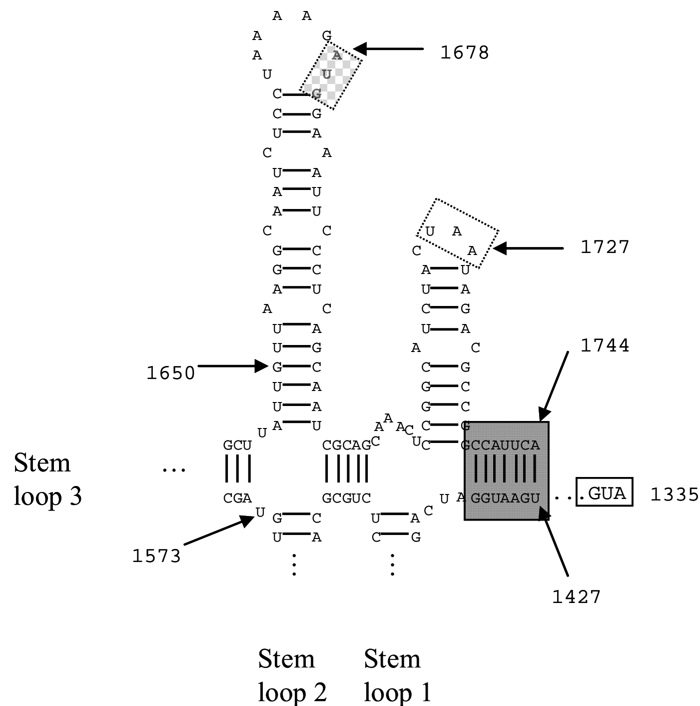

Figure 1.2. Secondary structure of nucleotides 1417-1744, including the coat gene. The solid-line box indicates the start codon of the *coat* gene, the grey box highlights the Min-Jou interaction, the hash grey box surrounds the start codon of the *L* gene and the hash-line box indicates the stop codon of *coat*.

only MS2 protein with multiple functions for the phage. The A, or maturation, protein is 393 amino acids and approximately 44 kDa in molecular mass. Curtiss and Krueger (1974) were able to detect A protein on the surface of the virion using MS2 phage that were conjugated to dintirophenol. Given that dintirophenol cannot penetrate into the

interior of the phage, the A protein was shown to be exposed on the surface of the capsid, therefore making it accessible to modification. In the absence of A, the phage particles are non-infectious since they are unable to adsorb to the pilus of F^+ cells (Heisenberg, 1966; Lodish et al., 1965; Steitz, 1968; Verbraeken and Fiers, 1972). Since the A protein was detected on the surface of the virion and is essential for adsorption of ssRNA phage to the F pilus, it was proposed to act as a "single molecule tail" (Curtiss and Krueger, 1974). Second, the A protein protects the encapsulated ssRNA genome from degradation by RNases present in the environment (Heisenberg, 1966; Paranchych et al., 1970). Third, the A protein escorts the ssRNA genome into the cell and is thought to help protect the genome during entry and penetration into the cytoplasm (Krahn et al., 1972; Paranchych et al., 1970; Steitz, 1968). Since the coat protein of Levivirus R17 does not contain histidines, the only protein associated with assembled phage particles containing His residues is the maturation protein. Phage were grown in the presence of radiolabeled His, purified and allowed to infect F^+ cells. After fractionation to separate empty capsids from the pili of the cells, it was observed that the radioactivity was retained by the cellular fraction, not the phage capsid (Steitz, 1968). This radioactivity was further characterized to show that the A protein goes through a cleavage event after entering the pilus that produces two fragments, both of which are transported into the cytoplasm with the RNA (Krahn et al., 1972). Interestingly, the lysis function for Alloleviviruses is also encoded within the maturation protein, referred to as A_2, which will be discussed later.

The RNA molecule of both Leviviruses and Alloleviviruses is maintained in complex secondary structures that controls the expression of all of the genes in the genome. It is estimated that as much as 75% of the ssRNA genome is retained secondary structures (Skripkin et al., 1990). Secondary structures are present not only in vivo in the cytoplasm of the cell, but are also present after packaging of the genome into the capsid. Surprisingly, these secondary structures remain intact during infection and penetration of the ssRNA into the cell. Wong and Paranchych (1976) cleaved radiolabeled genomes into multiple fragments and showed that these fragments maintained their gross secondary structures as well as the ability to be transported into the cell. The authors concluded that its is doubtful that the genome could be transported through the central pore of the F pilus while folded into secondary structures, suggesting that the sliding model for RNA-A complex transport is more likely (Wong and Paranchych, 1976).

One important secondary structure, produced by one of several long distance interactions that exist in the genome, is responsible for repression of expression of A protein. A cloverleaf structure is formed by pairing of the Shine-Dalgarno sequence of the A gene with a non-coding region 80 nt upstream. In this way, the ribosome binding site is occluded from access by ribosomes, thus preventing expression of A. Mutations that decreased the strength of this structure increased accumulation of maturation protein. Two models have been proposed to explain expression of A protein (Groeneveld et al., 1995). In the equilibrium model, expression of A results from breathing of the cloverleaf structure, at which time ribosomes are able to bind and

initiate translation. The breathing is thought to occur at too infrequent an interval to allow for sufficient translation of A protein (Groeneveld et al., 1995; Poot et al., 1997). Alternatively, the kinetic model suggests that expression only occurs on nascent strands prior to the formation of the long distance interaction (Groeneveld et al., 1995). In this case, the nascent strands being synthesized are the only substrate for translation as the formation of the secondary structure prevents further translation of the A protein, with the exception of a rare breathing event. As such, the kinetics of RNA folding are proposed to control the expression level (Groeneveld et al., 1995). This model hinges on the rate of RNA folding being slow, thereby allowing sufficient time for all of the required A protein to be translated from the nascent strands. This has been shown for this particular area of the ssRNA since mutations in the sequence of the long distance interaction to strengthen or weaken the structure did not have the expected consequences (de Smit and van Duin, 1990). Poot et al. (1997) measured the rate at which ribosomes could bind to a denatured RNA molecule in comparison to the ability of the RNA to refold into a LDI that excluded the binding of ribosomes. These researchers were able to show that though the RNA molecule went through a number of intermediate structures before forming the LDI, there was sufficient time for the ribosomes to bind to the RNA well in advance of the formation of the occluding structure (Poot et al., 1997).

The coat protein of MS2 is 129 aa (14kDa) and responsible for comprising the capsid of the bacteriophage. The completed capsid consists of 180 copies of coat protein, along with a single molecule of A protein. The characteristics of the coat protein were used to delineate Alloleviviridae and Leviviridae by their chemical,

immunological and serological characteristics (Scott, 1965; Nishihara et al., 1969; Watanabe et al., 1967). Translation of coat protein is carefully controlled by LDI structures, but coat protein translation also positively regulates the expression of the replicase (*rep*) gene. The synthesis of coat and replicase is controlled by a LDI, the MJ interaction. The MJ structure is melted by the ribosomes translating the early portion of the coat gene, and in so doing allows translation of the replicase gene from the exposed start codon (Licis et al., 1998; Min-Jou et al., 1972).

Translational polarity was shown in RNA phage after the isolation of certain amber mutants in the coat protein. These mutations were located in sequences directly upstream of the MJ sequence and dramatically reduced the amount of a second protein, Rep. However, since the MJ structure was never disrupted in these mutants, this still allowed for exposure and recognition of the start codon of the *rep* gene (Berkhout and van Duin, 1985; Gussin, 1966; Lodish et al., 1964; Lodish and Zinder, 1966,). In an additional level of control, coat protein binds to the region of RNA near and at the start codon of the replicase gene after this region of RNA has become a stem-loop structure (Bernardi and Spahr, 1972). The binding of coat only occurs after sufficient replicase has been produced to allow for progeny production and essentially eliminates translation of the *rep* gene twenty minutes post infection (Housman, 1971).

The *rep* gene is the largest gene in Leviviridae and encodes Rep, a 61 kDa catalytic subunit with RNA-dependent RNA polymerase activity. Replicase protein is translated using the infecting positive RNA strand as a template mRNA. In Leviviruses, the RNA polymerase holoenzyme is a complex of Rep with the host elongation factors,

EF-Ts and EF-Tu, and ribosomal protein S1 (Blumenthal et al., 1972; Kamen, 1970; Kondo et al., 1970; Wahba et al., 1974). Alloleviviruses require a fourth host protein, Hfq, to complete the holoenzyme. For RNA phage replication, S1 mediates binding of the holoenzyme to the phage RNA to two regions called the S-site and the M-site, located at the beginning of the coat gene and in the early portion of the *rep* gene, respectively (Boni et al, 1991; Goelz and Steitz, 1977; Klovins et al., 1998; Miranda et al., 1997; van Duin, 1988). EF-Tu is required for binding the phage ssRNA genome (Brown and Gold, 1996; Hori et al., 1967). Karring et al. (2004) recently proposed that EF-Ts is responsible for locking EF-Tu in a stable conformation suitable for binding phage RNA, thereby preventing the interaction of EF-Tu with ribosomes. Lodish and Zinder (1966) proposed a mechanism of coupled transcription and translation for the replication and protein production during infection. This system would allow the growing positive strand to be removed from the double-stranded complex by the binding of ribosomes. The ribosomes would then be free to translate the nascent strand prior to completion of all long distance interactions. After a threshold level of coat protein was synthesized, the production of replicase would presumably decrease as coat protein bound to the stem-loop near the replicase gene start and begin the process of phage particle production as the genome is encapsulated by the coat protein (Hung et al., 1969).

Expression of lysis gene L

The fourth gene product of MS2 is the lysis protein, L. This protein is responsible for the lytic event that completes the infection cycle of Leviviruses.

Although Norton and Zinder identified F-specific ssRNA phage in 1961 and the sequence of the entire MS2 genome was published in 1976, the *L* gene was not identified for another three years (Atkins et al., 1979; Beremand and Blumenthal, 1979; Fiers et al., 1976; Model et al., 1979). The other three shared genes (maturation, coat and replicase) of *Leviviridae* and *Alloleviviridae* had been identified and characterized shortly after ssRNA phages were identified, but a dedicated lysis gene was not initially recognized. At the outset, ssRNA phages were thought to cause lysis either by directly encoding a lysozyme function in the coat protein, or by indirectly activating a cellular lysozyme. Zinder and Lyons (1968) were able to show that certain coat amber mutants, known as Op3, were unable to cause lysis; nevertheless, no lysozyme activity was found associated with purified coat protein. They proposed that the coat protein could be causing lysis by some uncharacterized interaction with an unidentified lysozyme. A number of other studies attempted to show a lytic enzymatic activity associated with the ssRNA phages, but ultimately failed to identify a phage-encoded lysozyme (Engelberg and Soudry, 1971; Ozaki and Valentine, 1973; Zinder and Lyons, 1968). The identification of the lysis gene of ϕX174, *E*, set a precedent for the presence of single, overlapping lysis genes in small genome phages (Barrell et al., 1976). Several years later, the identification of the *L* gene was performed by three groups (Atkins et al., 1979; Beremand and Blumenthal, 1979; Model et al., 1979). Shortly thereafter, Coleman et al. (1983) demonstrated the L protein was necessary and sufficient for lysis of *E. coli*. As it turns out, the amber coat mutants studied by Zinder and Lyons (1968) actually mutated the thirtieth codon of L to a stop codon.

Regulation of L

Translation of the *L* gene is regulated primarily by the secondary structure of the ssRNA genome. Kastelein et al. (1982) reported that expression of L from the phage RNA was dependent on translation of the coat protein since L does not have its own Shine-Dalgarno sequence. They proposed that translation of L occurred after a fraction of the ribosomes translating the 3'-end of *coat* underwent a +1 frameshift, resulting in early termination of the coat polypeptide. At that point, it was proposed that these ribosomes would begin translation of *L* (Kastelein et al., 1982). In contrast to the frameshifting ribosome model, another model was suggested to explain the initiation of translation of *L*. According to the lateral diffusion model, ribosomes completed translation of the full-length coat gene and then scanned, or laterally diffused, along the RNA until it encountered the start codon of *L* where it initiated translation of the *L* gene (Adhin and van Duin, 1990; Berkhout et al., 1987; Klovins et al., 1997). Adhin and van Duin (1990) showed that ribosomes could laterally diffuse approximately 40 nucleotides in either direction along a molecule of RNA. They were able to demonstrate that the ribosomes would reinitiate translation at the nearest start codon by introducing false initiation codons between the *L* gene start codon of coat.

Klovins et al. (1997) showed that a stem-loop structure prevents ribosomes from recognizing the start codon of the lysis gene (Figure 1.2). The authors used a set of mutations to disrupt the hairpin structure without mutating the sequence of the coat protein to show that the start codon of L was inaccessible until ribosomes translating the coat gene melted the stem-loop. These mutations did not significantly alter the

accumulation of coat or L protein, but dramatically reduced the fitness of the phage and dropped titers by four orders of magnitude (Klovins et al., 1997). After selecting for suppressor mutations that restored high phage titers, it was found that the revertants and pseudorevertants restored the overall stem-loop structure, indicating the importance of the secondary structure of the RNA in control of the expression of the *L* gene (Klovins et al., 1997; Schmidt et al., 1987).

Synthesis of L during infection

Using a pulse labeling experiment, the amount of L protein was roughly quantified as one-tenth that of coat protein (Beremand and Blumenthal, 1979). It is possible to extrapolate these numbers to the total amount of L based on the production of 500-1,000 plaque-forming units per cell, each with a T=3 morphology. Thus, there would be close to 10,000 – 20,000 molecules of L per cell. The amount of L is strikingly high compared to the amount of A_2 produced during a Qβ infection (McIntosh, B.K., Struck, D.S., and Young, R., unpublished data). This hints at the possibility of a significant difference in the way these proteins cause lysis.

Replication

The study of replication by RNA phages began early in the field of RNA phage biology. Their exquisitely simple systems have been a model for the analysis of RNA synthesis, though there are certain intricacies that remain unexplained today. During

replication, the holoenzyme binds at the M-site, or major binding site and a long distance interaction brings the 3' end of the plus strand into the vicinity (Klovins et al., 1998). This binding signals the production of minus strands, which serve only as a template for the synthesis of many positive strands and are not packaged into phage particles (Blumenthal and Carmichael, 1979). Hfq was initially identified for its requirement during in vitro replication of the Qβ genome, but has since been shown to be necessary during in vivo infections (de Fernandez et al., 1968; Su et al., 1997). Hfq is required for Qβ template switching, or the ability to synthesize minus strands from the plus strand template (de Fernandez et al., 1968). Remarkably, the holoenzyme has some of the highest error/mutation rates of all polymerases at nearly 10^{-3}/bp (Drake et al., 1993). Host mutants have been isolated in the genes for ribosomal protein S1, EF-Tu, EF-Ts and Hfq that are resistant to ssRNA phage (Karring et al., 2004; Lupker et al., 1974; Miranda et al., 1997; Su et al., 1997). All of these host mutants prevent the replication of ssRNA genomes since they are unable to produce viable holoenzyme.

Replication begins with the assembly of the holoenzyme, at which point it recognizes the infecting positive strand. As a result of early studies on replication of Alloleviviruses and Leviviruses, a model for the steps in replication was proposed. This model, known as the butterfly model, suggests that the replication begins with the binding of the holoenzyme at the 3' end of the template, positive strand (Robertson, 1975). Replication results in the synthesis of a reverse complementary, or negative, strand. After completion of the negative strand, the holoenzyme must initiate replication of positive strands, a process called template switching. The trigger for template

switching is currently unknown, though it is proposed to be the result of altered binding affinities of EF-Tu or EF-Ts for the positive and negative strands (Blumenthal et al., 1972). Replication of the positive strands is initiated and completed, and newly synthesized positive strands are packaged to produce progeny phage. One mystery that remains is the exact number of positive and negative strands produced per cell. Although the replication of ssRNA genomes appears operationally straightforward, it is clear that the process is not understood quantitatively.

Morphogenesis

A simple model of morphogenesis was identified immediately with RNA phages since they consist of only three components: coat protein, maturation protein and a ssRNA genome. It was shown early on that purified coat protein could spontaneously encapsulate the RNA to form particles with normal sedimentation and physical properties, and that the coat protein could oligomerize to form capsids without the presence of RNA (Hermann et al., 1968). These early studies indicated that no host proteins were essential for the assembly of the capsids. Interestingly, RNA could not enter the completed capsid (Stavis and August, 1970). This indicated that unlike the dsDNA phages, which assemble a procapsid shell that is then filled with DNA, the RNA phages must assemble the capsid, including the maturation and coat proteins, around the single-strand of RNA. Additionally, these in vitro systems showed that defective (non-infectious) particles could not be made infectious simply by the addition of maturation

protein. If A protein was added before the nucleation of particle formation, infectious particles were obtained that contained coat, maturation protein and encapsulated ssRNA (Hung and Overby, 1969). How is the ssRNA genome packaged? What factors influence the efficiency of encapsulation? Infectious particle formation is proposed to begin with the binding of A protein to the positive strand (Kaerner, 1970; Knolle, 1972). Failure to bind A protein before coat protein nucleates into the capsid around the RNA molecule produces RNase-sensitive, non-infectious particles (Heisenberg, 1966). Binding of coat protein to the replicase stem loop is thought to be the initiator of the nucleation event (Bernadi and Sparr, 1972). The binding of the coat protein to the positive strand also provides specificity, preventing the incorporation of negative strands into virions. It has long been known that RNA phages do not package cellular RNA or the minus strands produced during replication (Blumenthal and Carmichael, 1979). Coat protein then oligomerizes through a series of intermediates to form a shell, or capsid, around the RNA, with the maturation protein protruding from the surface. During the morphogenesis of particles, RNA does not play an entirely passive role and is implicated in initiation of nucleation (Stockley et al., 2007). The RNA was shown to decrease the concentration of coat protein required for nucleation to occur. Factors influencing the efficiency of infectious particle formation are often related to the physiology of the cell. Starvation of the cell for oxygen, amino acids, and certain metabolites result in the presence of more intracellular particles, but also a higher percentage of defective particles. Most often, these defective particles do not contain RNA (Knolle, 1969; Ricciuti and Haywood, 1974; Shimura et al., 1967).

Release of phage progeny

Canonical holin-endolysin lysis

Bacteriophages infect a suitable host and commandeer specific cellular processes to produce progeny. Upon completion of sufficient progeny, all phages face a common obstacle to the release of these phages from the cell – the peptidoglycan layer, also known as cell wall or murein. How do phages conquer this barrier? Evolution has led phages to adapt one of two differing strategies, extrusion (non-lytic) or lysis. Non-lytic, or filamentous, phages have a single stranded ssDNA genome that is encapsulated into the phage capsid during extrusion of the intact particle from the host. In this way, these phages accomplish release of progeny phages without destroying the host cell. The second evolutionary adaptation for crossing the peptidoglycan layer is that used by lytic phages, including the T-set promoted by Delbruck. Lytic bacteriophages encode one or more proteins to accomplish lysis of the host cell. All lytic bacteriophages face a common obstacle to lysis, the peptidoglycan, or cell wall/murein (Figure 1.3). This structure is responsible for the definition and maintenance of cell shape and is composed of repeating disaccharide units crosslinked via a pentapeptide side chain. Normally, the cell carefully expands and maintains this important barrier to prevent unregulated lysis.

Since the cell wall blocks the release of progeny phage, how do lytic phages accomplish this release? First, phages with genomes greater than 10 kb encode multiple proteins involved in carrying out host lysis, including a holin and endolysin. This two-protein motif is the most studied system of host lysis. For many phages, including the

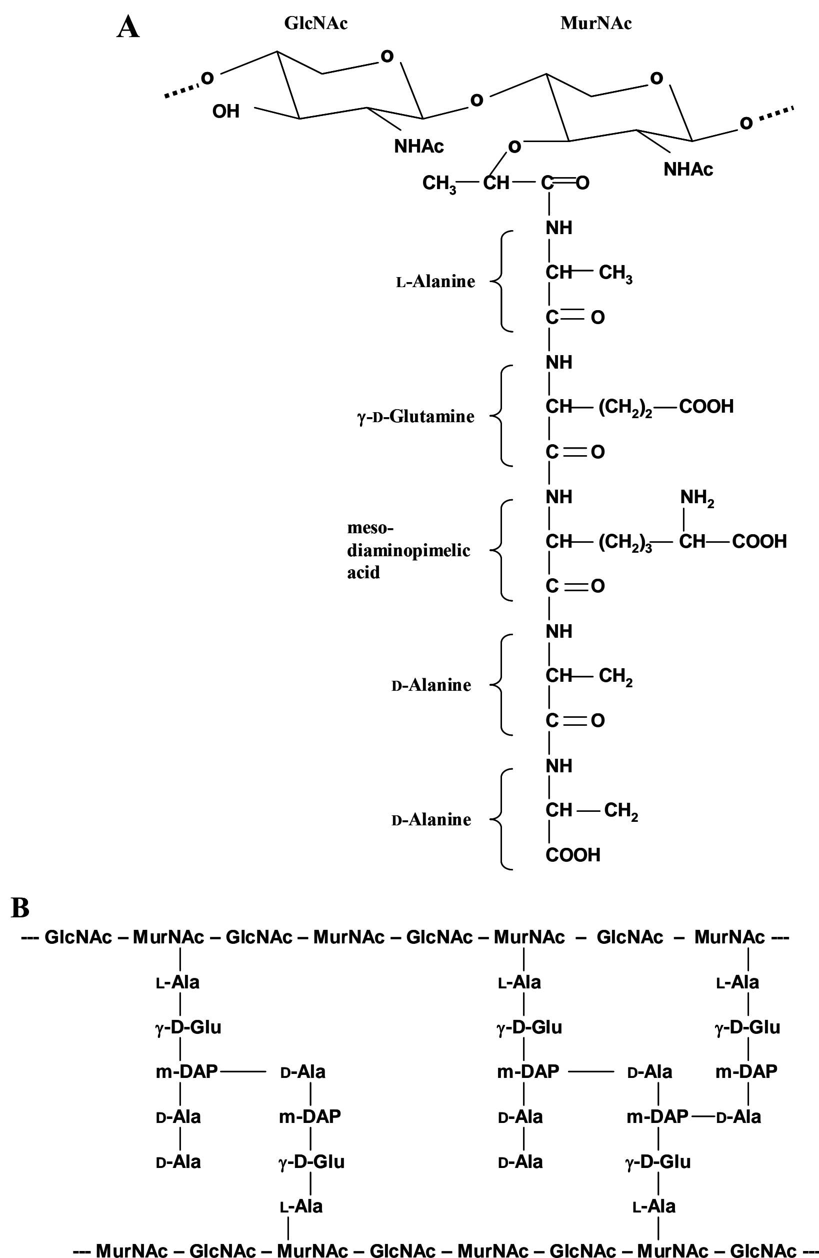

Figure 1.3. Structure of peptidoglycan of *E. coli*. (A) Structure of a monomeric unit of murein, the disaccharide pentapeptide. GlcNAc is the abbreviation for N-acetyl-glucosamine, while N-acetylmuramic acid is abbreviated as MurNAc. (B) Example of cross-linking of the pentapeptide side chains attached to MurNAc. The peptide bond is formed between the *m*-DAP residue and D-Ala. The terminal D-Ala is removed from the second side chain during the reaction.

paradigm phages λ and T4, both the holin and endolysin are essential for lysis (Figure 1.4). The endolysin is responsible for the degradation of the host's peptidoglycan layer, but is sequestered in the cytoplasm of the cell. Since the soluble endolysin is restricted

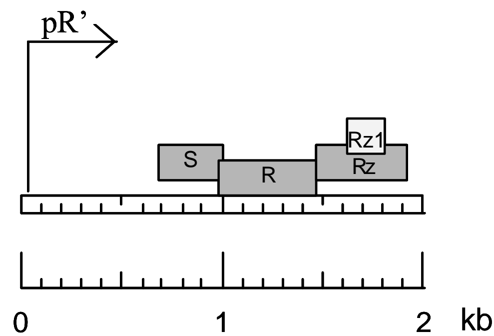

Figure 1.4. Map of the lysis cassette of dsDNA bacteriophage λ. The late promoter, *pR'*, is responsible for expression of the essential lysis genes, *S* and *R*, and the non-essential lysis genes, *Rz* and *Rz1*.

from accessing the cell wall by the inner membrane of the cell, a second protein, or holin, is required. The holin is a small membrane protein that accumulates within the inner membrane until it triggers to form holes at a genetically preprogrammed time. This hole allows release of the endolysin to the periplasm where it degrades the peptidoglycan. In the absence of holin function, lysis does not occur because the endolysin is sequestered in the cytoplasm.

SAR endolysin lysis

Alternatively, P1 and its relatives do not require a holin for lysis. Instead, these phages encode a single essential endolysin capable of bringing about lysis on its own (Xu et al., 2005). This class of endolysins is called SAR, for signal anchor release. SAR endolysins are exported to the periplasm of the cell, but are anchored by a single transmembrane domain, known as a SAR domain, at the N-terminus. This SAR domain is involved in tethering the protein to the membrane in an inactive form. This domain is less hydrophobic than expected for a TMD since about half of the amino acids comprising the SAR domain are serine, threonine, glycine or alanine residues, which allows for its release from the inner membrane (Park et al., 2007; Xu et al., 2005). Upon release of the SAR domain into the periplasm, the SAR endolysin undergoes a rearrangement and initiates degradation of the peptidoglycan. Somewhat surprisingly, P1 and P1-like phages also encode a holin, though it is non-essential for the lytic event. It is thought to assist in causing lysis to be saltatory, since the holin causes depolarization of the membrane. This event is known to increase the rate at which the SAR domain is released from the membrane, subsequently increasing the rate of activation of the SAR lysozyme (Park et al., 2007; Xu et al, 2004).

Single gene lysis: Lysis without an endolysin

Lytic bacteriophages with considerably less codon space (under 10 kb) have evolved another mechanism of lysis (refer to Figure 1.1). They encode a single gene responsible for the production of a lysis protein. These simple phages face the same

obstacle to accomplishing lysis, the host's cell wall, but accomplish lysis without active degradation of the murein layer (Robertson, 1975). As with the large genome phages, single gene lysis phages can also be subcategorized, though by different criteria. First, there are ssDNA phages, such as the prototypic ϕX174, called Microviruses (Sertic and Bulgakov, 1935; Sinsheimer, 1959). These phages encode ten genes, including the *E* lysis gene. The E gene is embedded entirely within the structural *D* gene, though in a +1 reading frame. Second, the ssRNA phages discovered by Zinder and Loeb (1961) are broken into two families, *Leviviridae* and *Alloleviviridae*. ssRNA phages were identified when they described specific phages with the ability to plate on F^+ bacteria, but not on F^- bacteria (Loeb and Zinder, 1961). Leviviruses and Alloleviviruses have been separated from one another based on a number of criteria, including serotyping, genome organization, and coat protein sequence. Alloleviviruses, including the prototypic Qβ, are unusual in that it is the only family of lytic bacteriophages that does not encode a dedicated lysis gene. Instead, Alloleviviruses have a multifunctional protein, A_2, which causes host cell lysis. Leviviruses, on the other hand, including MS2, encode a single gene for lysis called *L*. Strikingly, the *L* gene overlaps two out of the three other genes in the MS2 genome. Both of the families of ssRNA phages are known to require several host proteins for replication. Alloleviviruses and Leviviruses share a requirement for three host proteins: EF-Ts, EF-Tu and ribosomal protein S1. Additionally, Alloleviviruses require another host protein, Hfq (de Fernandez et al., 1968). All of these host proteins have been shown to be involved in assisting the phage's own RNA-

dependent RNA polymerase, known as the replicase, for production of progeny genomes.

A genetic approach was undertaken to identify the target of the lysis protein of ϕX174, E (Bernhardt et al., 2000). *E* was cloned onto a plasmid and expression induced in *E. coli* cells. In this way, host mutants were selected that were resistant to the expression of the E protein. It was expected that the host target gene would be essential, thus requiring a rare missense mutation and not a simple nonsense or deletion mutation. Additionally, it was thought the mutations would be dominant, since the presence of E-resistant target protein would provide protection from lysis by E. Several thousand survivors were screened using ϕX174, which also served to confirm that the host mutants were resistant to expression of E. However, when the mutations were present in trans to a wild-type copy of the gene located on an F prime, the mutations were identified as recessive (Bernhardt et al., 2000). This was the first indication that the *E. coli* mutants initially isolated as E resistant were not in the target gene. Mapping of the host mutation elucidated the mutated gene as *slyD*. *slyD* encodes a peptidylprolyl-*cis*-*trans*-isomerase, or PPIase. PPIases are responsible for folding proteins requiring a peptidyl-prolyl isomerization event. Upon further characterization of these *slyD* mutants, it was shown that E is less stable in the absence of SlyD. It was concluded that E either has an essential peptidyl-prolyl isomerization leading to increased protein stability or that SlyD binds and protects E from degradation (Bernhardt et al., 2001a). Thus, the so-called resistance to E and ϕX174 was actually due to the decreased stability of the lysis protein. Before continuing with the genetic approach to identify the

authentic target of E, an E protein had to be isolated that was capable of lysing a *slyD*$^-$ host. These *E* gene mutants, called Epos for plate on *slyD*$^-$, were shown to overexpress E, thereby compensating for the increased instability in the absence of SlyD (Bernhardt et al., 2001a).

Upon isolation of Epos, the genetic selection and screen were repeated. This time, an *E. coli* mutant in the essential gene, mraY, was isolated that was dominant (Bernhardt et al., 2001a). MraY is a transmembrane protein that catalyzes the addition of UDP-MurNAc-pentapeptide to undecaprenol-phosphate (C_{55}-P) to form lipid I (Ikeda et al., 1991). MurG then modifies Lipid I with the addition of UDP-Glc-NAc to generate lipid II. Both MraY and MurG act in the cytoplasm of *E. coli* and represent the final steps of *de novo* cell wall synthesis to occur in the cytoplasm. The lipid II product is flipped through the inner membrane to be covalently linked to the existing peptidoglycan layer by a penicillin-binding protein (PBP), either PBP1a or PBP1b, which has transglycosylase activity. The pathway of peptidoglycan metabolism is shown in Figure 1.5, the details of which were the subject of a thorough review (Holtje, 1998).

A similar approach was taken to identify the target of the lysis protein, A_2, of ssRNA phage Qβ (Bernhardt et al., 2001b). After expression of a cloned A_2 gene, *E.coli* survivors were isolated that were resistant to expression of A_2 from either a plasmid or Qβ. Mapping of the mutation and subsequent sequencing demonstrated that a single missense change from a leucine at residue 138 to a glutamine in MurA was responsible for resistance to $\underline{A}_2$ (*rat*) (Bernhardt et al., 2001b). MurA is responsible for the first

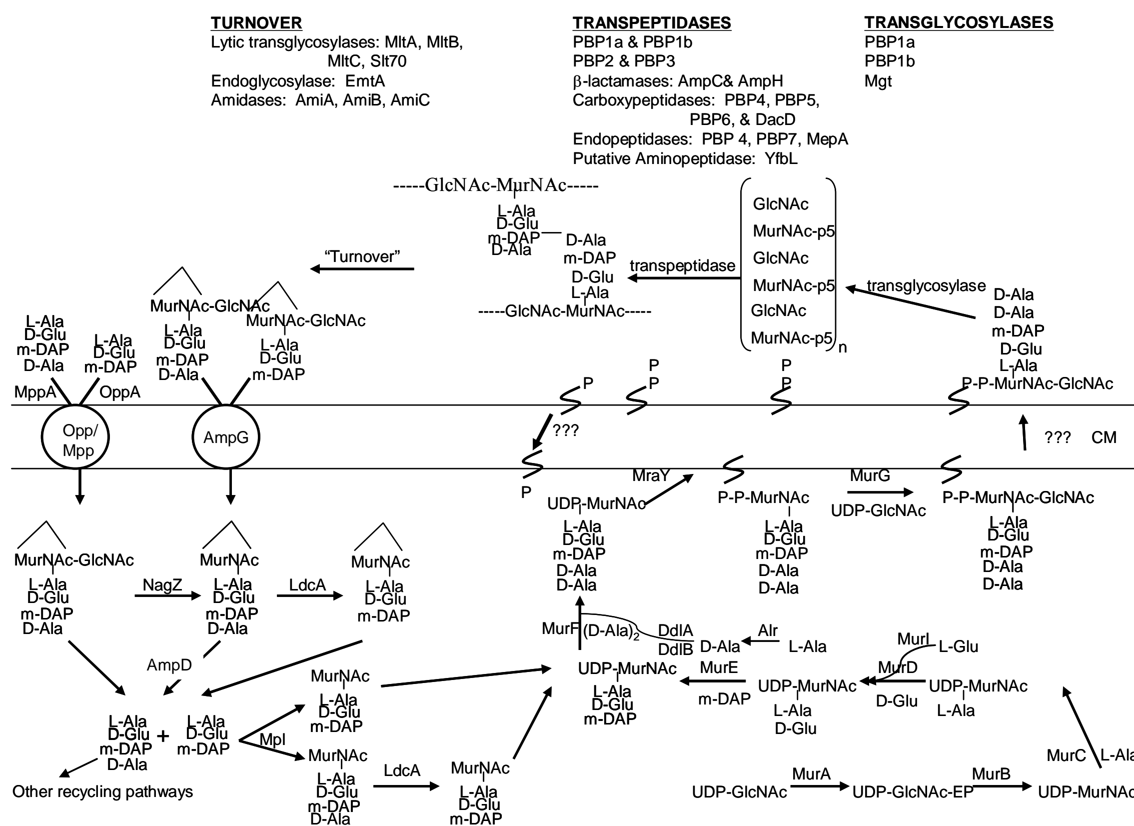

Figure 1.5. Peptidoglycan metabolism in *E. coli*. The S-shaped line in the cytoplasmic membrane (CM) represents the C_{55}-undecaprenol-phosphate. The enzyme responsible for catalyzing each reaction is listed above or beside the arrows. The question marks represent an unknown molecule(s) that flips the lipid-linked precursor into the periplasm and recycles the lipid carrier for use by MraY. Respective enzymatic activities of proteins in the periplasm are listed at the top.

dedicated step in peptidoglycan synthesis and catalyzes the addition of enolpyruvate to UDP-GlcNAc (Marquardt et al., 1992). This product goes through multiple enzyme-catalyzed reactions to produce UDP-MurNAc-pentapeptide, which is one of the substrates for MraY, but inhibition of any of these early steps in cell wall synthesis

would prevent incorporation of radiolabeled precursor into the sacculus, as is seen with A_2 or E expression (Bernhardt et al., 2001a, 2001b; Okazi and Valentine, 1973).

Biochemical characterization of lysis by single gene systems indicated an interesting difference between L and the other prototypic proteins, A_2 and E. In the case of both E and A_2, cell wall synthesis was demonstrated to stop well in advance of the onset of lysis (Bernhardt et al., 2001a, 2001b; Okazi and Valentine, 1973). In contrast, L does not inhibit cell wall synthesis prior to inducing lysis (Holtje and van Duin, 1984; T.G. Bernhardt dissertation, 2001).

The MS2 lysis protein, L, is a 75 amino acid protein that fractionates with the insoluble, membrane fraction of cells (Beremand and Blumenthal, 1979). L is predicted to have a single transmembrane domain (TMD) near the C-terminus of the protein, though this has never been experimentally demonstrated. The C-terminal half of L has some similarity to the N-terminal third of ϕX174 E protein, which is thought to be the TMD of E and has been shown as necessary and sufficient for lysis (Barrell et al., 1976; Beremand and Blumenthal, 1979). The topology of L is predicted to be somewhat of a mirror image of E with the N-terminus of L in the cytoplasm and the C-terminus forming the TMD, whereas the N-terminus of E is the TMD and the remaining portion of the protein is located in the periplasm (Figure 1.6).

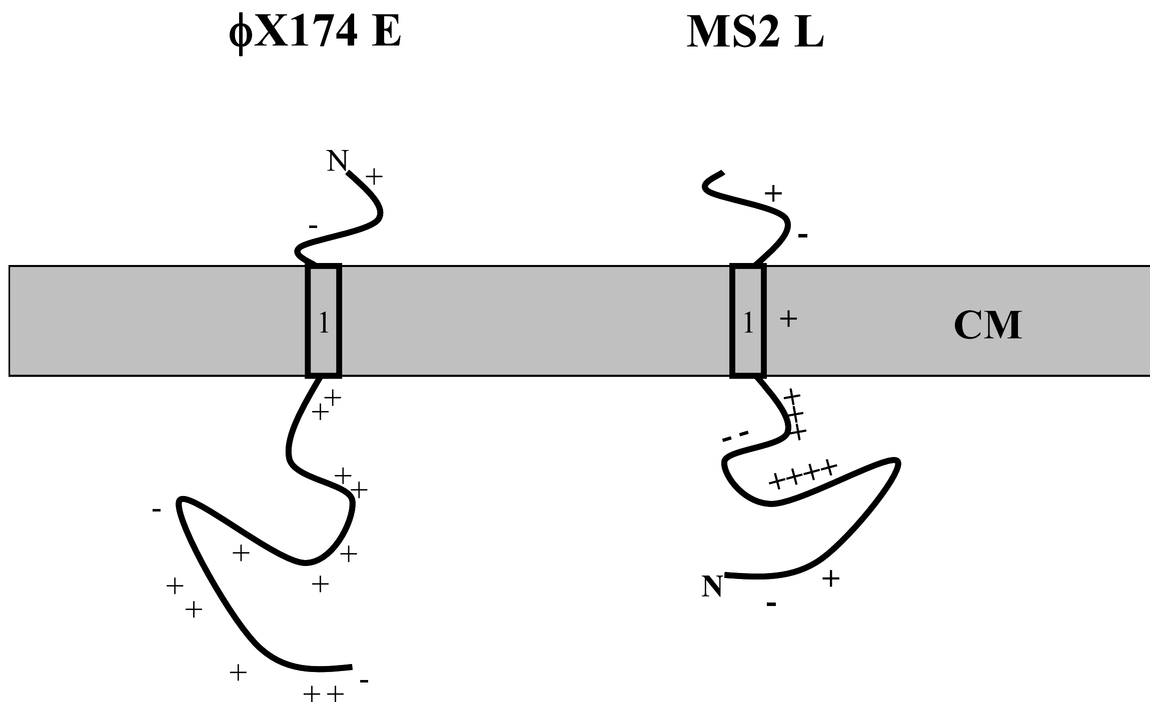

Figure 1.6. Putative topology of the lysis proteins of φX174 and MS2, E and L, respectively. Cytoplasmic membrane is abbreviated as CM.

Lysis mediated by MS2 L

Shortly after failure to identify a phage-encoded lysozyme, two models emerged for the mechanism of lysis induced by MS2 L. The first proposed that lysis was the result of autolytic enzyme activity and the second proposed that lysis resulted from inhibition of peptidoglycan synthesis (Markert and Zillig, 1965). Under normal cellular conditions, cell wall synthesizing and degrading enzymes are carefully regulated during peptidoglycan metabolism. Enzymes that degrade components of the cell wall are

required for specific cleavage events that allow the synthesizing machinery to insert new murein. Autolysis results from the unregulated activity of one or more degradative enzyme, known as autolysins. Autolysins include lytic transglycsylases, endopeptidases, amidases and carboxypeptidases (Holtje, 1998). Lysis by single gene phages, including ϕX174, Qβ and MS2, was proposed to be the result of induction of autolysis (Holtje and van Duin, 1984; Lubitz and Plapp, 1980). Lubitz and co-workers (1980) attempted to show that the cell wall was being rapidly degraded just before the initiation of lysis and concluded that lysis resulted from rapid autolysis, not inhibition of murein synthesis during growth of the cells (Lubitz and Plapp, 1980). Light and electron microscopy along with physiological studies showed similarities with penicillin-induced lysis. Penicillin-mediated lysis had already been attributed to induction of autolytic enzymes, but penicillin was shown to inhibit peptidoglycan synthesis (Tomasz and Holtje, 1977; Tomasz and Waks, 1975). The two models are now not considered mutually exclusive.

A set of experiments were designed to determine the minimum length of L required to cause host cell lysis. Berkhout and co-workers (1985) showed that the N-terminal half of L was dispensable for function. Using a plasmid-based system, they created a set of mutants that contained one to ten amino acids of the coat protein at the new N-terminus of the truncated L gene and provided the Shine-Dalgarno sequence of the coat gene on the plasmid. The most interesting fusion carried ten residues of coat fused to the C-terminal thirty-two residues of L since it was lytic. This small fusion protein was shown to accumulate, though, some longer non-lytic fusions failed to accumulate (Berkhout et al., 1985). Goessens et al. (1988) synthesized the C-terminal

twenty-five residues and characterized the effects of this peptide in vitro. They found that the peptide dissipated the proton-motive force (pmf) of *E. coli* inverted membrane vesicles and reconsititued liposomes. Also, the peptide increased the permeability of the reconsititued liposomes by creating pores/holes that released a self-quenching dye, carboxyfluorescein (CF). The authors proposed a model in which L associated with the inner membrane, causing depolarization, and activating an autolytic enzyme to ultimately cause lysis (Goessens et al., 1988). On the other hand, these experiments were completed entirely in vitro and did not include a negative control, such as a randomized version of the twenty-five residue peptide, which would have shown the peptide interaction was specific for L rather than just an affinity of mostly hydrophobic amino acids for lipids. Thus, applicability of this model to in vivo lysis by the full-length L protein is uncertain.

Lysis inherently requires structural failure of the murein layer and this failure had already been shown to occur after treatment of sensitive cells with penicillin. Strikingly, sensitive cells grown at pH 5 were shown to be tolerant to penicillin, though the catalysis of cell wall stops after penicillin treatment. This penicillin tolerance, as it became known, was thought to delineate another step in autolysis, whereby inhibition of specific peptidoglycan enzymes occurred normally after addition of penicillin, but the triggering of autolytic enzymes was prevented at the lower pH (Goodell et al., 1976; Tomasz and Holtje, 1977, Tomasz and Waks, 1975). When L was expressed in cell grown at pH 5, the localization of L remained unchanged, but lysis of the host cells failed to occur. In the same study, the authors characterized the chemical composition of the murein of

cells expressing L at pH 5 and 7 and showed that the overall length of the strands decreased slightly at pH 5. The overall amount of cross-linking was not significantly different at either pH, though the types of cross-links were altered slightly. This led the authors to conclude that the autolytic enzymes being induced by L to cause lysis were a glycosidase and endopeptidase (Walderich et al., 1988). The ability of L to lyse an *mdoA* mutant was also tested. This strain of *E. coli* fails to synthesize membrane-derived oligosaccharides (MDOs), which are proposed to be important to the spacing of the inner membrane from the murein layer. In an *mdoA* mutant, L failed to cause lysis. The authors proposed this phenotype was due to the inability of L to insert into the inner membrane, since L was not localized to the total membrane fraction (Holtje et al., 1988).

Questions to be addressed

Phages still manage to hide some of the most interesting answers to their riddles. One unknown is the means of genome penetration into the cytoplasm of the cell. Clearly, there are steps involved in the early stages of the MS2 life cycle that remain to be elucidated and early genetic studies indicate host proteins are involved in at least some of these processes. One of the last great mysteries is the mechanism by which *Leviviridae* accomplish host lysis. A precedent exists for inhibition of peptidoglycan biosynthesis by small genome phages that encode a single gene responsible for lysis. However, there are fundamental differences between lysis seen with these proteins and

L, including the effect on the incorporation of newly synthesized cell wall precursor into the murein layer.

CHAPTER II

PHYSIOLOGICAL CHARACTERIZATION OF L-MEDIATED LYSIS

Introduction

Most bacteriophages release their progeny by causing lysis of their host cell. MS2, a lytic ssRNA phage, encodes a single gene for lysis, *L*. Expression of the *L* gene is necessary and sufficient to cause lysis of cells, though the exact mechanism through which it causes lysis has remained elusive. It has long been known that MS2 does not encode a muralytic activity capable of degrading the host cell's peptidoglycan. In some respects, the lysis of MS2 infected cells resembles lysis mediated by penicillin including the requirement for the cells to be growing, sensitivity to low pH, and the apparent accumulation of products of peptidoglycan degradation (Holtje and van Duin, 1984, Walderich et al., 1988). With regard to the latter point, there is a single report claiming that the number of monomeric units (consisting of a GlcNAc-MurNAc and peptide side chain) making up the strands of murein was reduced during L expression (Walderich et al., 1988). After isolating the peptidoglycan from cells expressing L at pH 5 and 7, the samples were digested with muramidase and the products analyzed by HPLC (high performance liquid chromatography). The authors found that there was a decrease of 2.5 monomeric units on average from 19.6 to 16.1 monomers per strand (Walderich et al., 1988). Based on this, it was suggested that L-mediated lysis involved the induction of an as yet uncharacterized host autolytic system (Walderich et al., 1988). In contrast to

penicillin-induced lysis, expression of L does not inhibit incorporation of radiolabeled precursor into the murein layer (Holtje and van Duin, 1984; T. G. Bernhardt dissertation, 2001). This also distinguishes L from the lysis proteins of other small phage which inhibit cytoplasmic steps in the peptidoglycan biosynthetic pathway.

The primary sequences of the known Levivirus L proteins are shown in Figure 2.1. The L proteins all have an N-terminal domain with a high positive net charge. This is followed by a highly conserved central domain, which contains a XLS triad (where X can be the hydrophobic residue F, I, V, or M) that is useful in aligning even distantly related L sequences. In two-thirds of the known L proteins, this is followed by a threonine-rich C-terminal domain which is absent in the remaining L proteins.

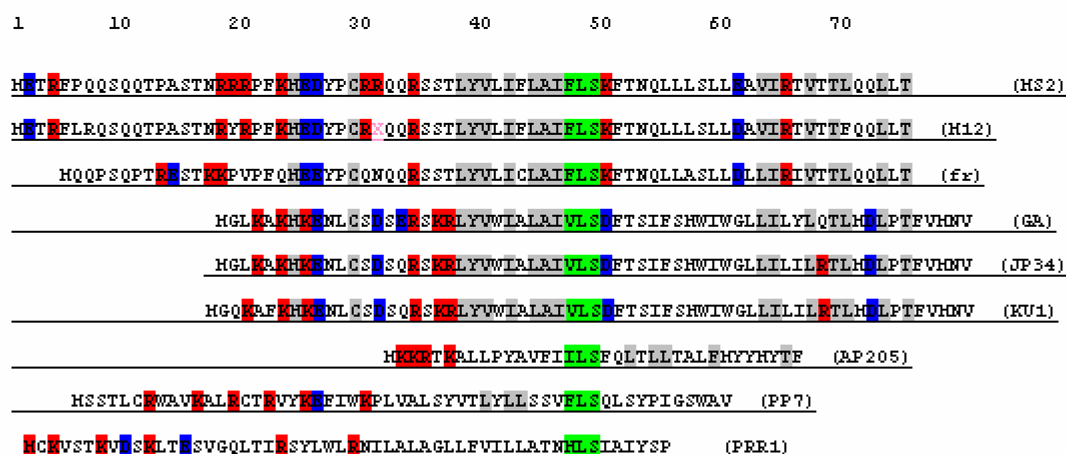

Figure 2.1. L proteins of various F-specific *Leviviridae*. Negatively charged residues are in blue, positively charged residues are in red. Grey and green residues indicate a highly conserved domain.

In the first report of the lytic function of the cloned *L* gene, *L* was placed under the control of the lambda early promoter, *pL* (Remaut et al., 1981). This promoter is normally repressed by the cI protein. The existence of a temperature sensitive allele, cI_{857}, allows for induction of genes under *pL* promoter control by a simple thermal shift. The level of L protein accumulation after induction of this plasmid was never compared to that of L produced during a MS2 infection (Devos et al., 1979; Remaut et al., 1981, Remaut et al., 1982). Moreover, after thermal induction, cultures were left at 42°C instead of being shifted to the more physiologically relevant temperature of 37°C even though this temperature would still allow for expression from the *pL* promoter using this system. Interpretation of these early experiments is further complicated by the fact that these early plasmid constructs also expressed the gene for the MS2 coat protein. This was due to the fact that L does not have a Shine-Dalagarno sequence and its production is coupled to the synthesis of coat protein (Kastelein et al., 1982; Klovins et al., 1997). Finally, *pL* is a strong promoter and it is possible that the lethal effects and other phenotypes attributed to the L protein were artifacts due to the production of coat/L at supraphysiological levels.

In this study, we describe a dual plasmid system that allows L protein to accumulate in induced cells to levels that mirror its production during a MS2 infection. Using this system, the process of L-mediated lysis was partially characterized and a mutational analysis of the *L* gene was initiated.

Materials and methods

Bacterial strains, bacteriophages, plasmids and culture growth

XL1-Blue (*recA endA1 gyrA96 thi hsdR17 supE44 relA1 lac* [F'::Tn10 *proA*$^+$*B*$^+$ *lacIq* Δ(*lacZ*)M15] was used for construction of plasmids. RY3095 (HfrH *lacI*^{q} *lacZ*::Tn5), RY15495 (*lacZ*$^+$ Kan^S derivative of 3095 obtained by plating on MacConkey lactose and screening for the ability to ferment lactose and sensitivity to Kan), ER2738 (F' *proA*$^+$*B*$^+$ *lacI*^{q} Δ(*lacZ*)*M15 zzf*::Tn10 Tet^R)/ *fhuA2 glnV* Δ(*lac-proAB*) *thi*-1 Δ(*hsdS-mcrB*)5) (New England Biolabs, Ipswich, MA), CS840 (K-12 *lac*::Tn5 *relA tonA*::Tn10) (Pugsley et al, 1980), RY15177 (15496 *tonA*::Tn10), RY15194 (15177 pQ pRE-L), RY15602 (MDS12 *tonA*::Tn10) (Tran et al., 2005), 2277 (F$^-$ λ$^+$ *ara leu lacI*^{q} *purE gal his recA srl::Tn10 argG rpsL xyl mtl ilv thi*) (Struck et al., 1984), and RY15071 (*Acinetobacter baumannii*) (kindly provided by Dr. van Duin, University of Leiden) were used where indicated.

Cultures were grown in LB broth supplemented with the appropriate antibiotic: 100 μg/ml ampicillin (Amp), 40 μg/ml kanamycin (Kan), 10 μg/ml chloramphenicol (Cam) and 10 μg/ml tetracycline (Tet). Culture growth and the kinetics of culture lysis were monitored by measuring culture turbidity as previously described (Roof et al., 1997). Plasmid inductions were preformed by addition of isopropyl-β-D-thiogalactoside (IPTG) and arabinose to final concentrations of 1 mM and 0.2%, respectively at time 0 minutes.

KU1 and AP205 were a generous gift from Dr. van Duin (University of Leiden). Phage stocks of MS2 and KU1 were prepared as previously except that RY15476 was used as the host strain for KU1 and MS2 and RY15071 was grown at 30°C as the host for AP205 (Roof et al., 1997).

pRE (Xu et al., 2005), pQ (Grundling et al., 2001), pRW (R. White, unpublished), pZE12-A_2 (Wang and Young, unpublished) and pET DUET (Novagen, Gibbstown, NJ) were used for cloning or expression as described below. Unless otherwise indicated, primers were purchased from Integrated DNA Technologies, Coralville, IA. Automated fluorescent sequencing was performed at the Laboratory for Plant Genome Technology at the Texas Agricultural Experiment Station. Unless otherwise stated, all restriction enzymes were purchased from New England Biolabs (Ipswich, MA) and used according to the manufacturer's instructions.

L-specific antibodies

For immunodetection of L, antisera prepared in a rabbit against the L-specific peptide, 5'-TPASTNRRRPFKHEDC (Sigma Genosys) was used at a dilution of 1:5000. The peptide sequence corresponds to residues 12-26 of MS2 L.

SDS-PAGE and Western blotting

To demonstrate the presence of L protein after phage infection with MS2, 2.5 ml aliquots of the indicated cultures were precipitated by the addition of TCA (trichloroactetic acid) to a final concentration of 10%. The precipitate was collected by

centrifugation, washed with acetone and redissolved in 2X SDS-PAGE sample buffer (Sambrook et al., 1989). Samples were then subjected to SDS-PAGE and Western blotting as described (Bernhardt et al., 2001). Horseradish peroxidase conjugated secondary antibody was from Pierce (Rockford, IL). Blots were developed using the chromogenic substrate 4-chloro-1-naphthol.

L accumulation during MS2 infection

A culture of RY15177 was grown to A_{550} ~0.4-0.5, diluted to A_{550} ~0.1 into pre-warmed LB and infected with MS2 at an MOI (multiplicity of infection) of 10. 2.5 ml samples were removed at the indicated times, added to 260 µl 100% TCA and incubated on ice for at least 30 minutes. Samples were centrifuged at 2,700 x g for 12 minutes, washed once with 5 ml acetone and centrifuged as before. Samples were dried and resuspended in 50 µl sample loading buffer and analyzed by SDS-PAGE and Western blotting (described above).

Quantification of L

Samples of His_6-L protein were purified as described in the Materials and Methods section of Chapter IV. 1 ml of purified protein was precipitated with MeOH (methanol) and $CHCl_3$ (chloroform) essentially as previously described (Wessel and Flügge, 1984). Briefly, samples were mixed with 750 µl of a 1:5:1 solution of water: MeOH: $CHCl_3$ and incubated on ice for 30 minutes. Samples were centrifuged at 13,000 x g for 5 minutes, washed once with 400 µl of 95% MeOH and briefly air dried. The

samples were resuspended, analyzed by SDS-PAGE and Coomassie staining and compared to serial dilutions of a known concentration of HEL lysozyme (2 mg/ml). To estimate the amount of L produced during an infection, samples of His_6-L and L from infected cells 60 minutes after infection were analyzed by Western blot using α-L antibodies described above.

Cloning of the Leviviridae L *genes*

RNA was isolated from 100 µl of lysate containing MS2, KU1 or AP205 phage using an RNeasy kit (QIAGEN Inc., Valencia, CA). RNA was reverse transcribed using the RETROscript kit (Ambion Inc., Austin, TX) and random decamer primers provided according to the manufacturer's instructions. 1 µl of this reaction was used as template for the PCR amplification of the L gene using the primer pairs listed in Table 2.1.

Table 2.1. Primers used to amplify or sequence respective *L* genes from various *Leviviridae*. The restriction site, *EcoRI*, is underlined, the sequence from upstream of the λR gene is in italics and the L gene sequence is bolded. The λR sequence includes a moderate Shine-Dalgarno sequence.

Primer name	Sequence
L with EcoRI Forward	5' ATATAT**GAATTC**_AAGCGGGAGTAGAAG_**ATGGAAACCCGATTCCCTCAG** 3'
L with EcoRI Reverse	5' ATATATGAATTCAGCTAAGGTACGACGGGTCGC 3'
AP205 L with EcoRI Forward	5' ATATATGAATTC_AAGCGGGAGTAGAAG_**ATGAAGAAAAGGACAAAAGCC** 3'
AP205 L with EcoRI Reverse	5' ATATATGAATTCCTAAAAGGTATAATGGTAATA 3'
KU1 with EcoRI Forward	5' ATATATGAATTC_AAGCGGGAGTAGAAG_**TTGGGTCAGAAAGCATTTAAA** 3'
KU1 L with EcoRI Reverse	5' ATATATGAATTCAGTTCCTTACGGAGTGCG
pR' Seq For	5'-GTC ATC GCC GCC CAA CAA CAG-3'

All PCR reactions were performed using *Pfu* polymerase (Stratagene Corp., La Jolla, CA). The forward primer carried an additional 15 nucleotides from immediately upstream of the λ*R* gene. In this way, a moderately efficient Shine-Dalgarno sequence was added upstream of the start codon of L. The PCR product, referred to as SD^R-L, was digested with EcoRI and ligated into the similarly digested pRE vector. Clones in which the L gene was cloned under the control of the *pR'* promoter of the pRE vector were verified by DNA sequencing using the primer, *pR'* Seq For (Table 2.1).

Sucrose gradients

Inner and outer membranes were separated by isopycnic centrifugation on sucrose gradients essentially as described (Osborn and Munson, 1974). Briefly, 200 ml cultures of RY15194 were grown in LB containing Kan and Amp at 37°C to an A_{550} of 0.6. The culture was induced by addition of 1 mM IPTG and 40 minutes later was harvested by centrifugation at 11,000 x g at 4°C for 10 minutes. The sample was resuspended in 11 ml 0.75 M sucrose, 10 mM Tris, pH7.8, 550 µl 2 mg/ml hen egg white lysozyme was added and the sample rested on ice for 2 minutes. 23 ml of cold 1.5 mM EDTA, pH 7.5 was added and after spheroplasting was complete, the sample was sonicated using three 15 second pulses with one minute on ice between each pulse. The sample was centrifuged at 1,000 x g for 15 minutes at 4°C to remove unlysed cells. The sample was then centrifuged for 1 hour at 4°C at 100,000 x g to pellet the membranes. The pellet was resuspended in 30 ml 0.25 M sucrose, 3.3 mM Tris, pH 8.0, 1 mM EDTA, pH 7.5, and membranes were collected by centrifugation at 100,000 x g for 1

hour at 4°C. The pellet was resuspended in 6 ml 25% sucrose, 5 mM EDTA, pH 7.5 and loaded onto a 6 step gradient from 55%-30% sucrose each with 5 mM EDTA, pH 7.5. The gradient was centrifuged for 40 hours at 4°C at 26,000 rpm in an SW-28 rotor. After completion of centrifugation, fractions were collected using a density gradient fractionator (Teledyne Isco, Inc., Lincoln, NE).

Each fraction was analyzed for total protein with a Bradford assay kit as per the manufacturer's instructions (BioRad, Hercules, CA), tested for refractive index using an Abby Refractometer as per standard procedures (Bausch and Lomb, Inc., Rochester, NY) and analyzed for NADH oxidase activity according to standard protocols (Osborn and Munson, 1974). Additionally, indicated fractions containing protein were analyzed by SDS-PAGE and Western blotting using α-L (described above).

Sucrose protection of cells undergoing lysis by L

Cultures were grown overnight at 37°C in LB and 0.23 M sucrose and 10 mM Mg^{2+} with the required antibiotics. The next day, cultures were diluted 200 fold into the

same media and grown to A_{550} of 0.2, followed by induction with 1 mM IPTG. At the indicated times after induction, samples were examined and photographed using a Nikon light microscope equipped with a digital camera.

Penicillin treatment of cells infected with MS2

Cultures of 15495 were grown to A_{550} of 0.2, infected with MS2 at an MOI of 5 and ampicillin was added to a final concentration of 2 μg/ml to inhibit PBPs responsible for septation. Samples were removed at times indicated after infection and photographed as described above.

Introduction of missense mutations into L and DNA manipulations

Missense mutations were introduced into the *L* gene on pRE-L using standard procedures for QuickChange from Stratagene in conjunction with primers listed in Table 2.2. All clones were sequenced as described above using the *pR'* Seq For primer.

Table 2.2. Primers used for QuickChange mutagenesis of *L* to create single missense changes.

Primer name	Sequence
L S9C QC For	5' AAACCCGATTCCCTCAGCAATGTCAGCAAACT CCGGCATCTACT 3'
L S9C QC Rev	5' AGTAGATGCCGGAGTTTGCTGAGATTGCTGAG GGAATCGGGTTT 3'
L S15C QC For	5' CAATCGCAGCAAACTCCGGCATGTACTAATAG ACGCCGGCAATTC 3'
L S15C QC Rev	5' GAATGGCCGGCGTCTATTAGTACATGCCGGAG TTTGCTGCGATTG 3'
L S35C QC For	5' CCATCTCGAAGACAACAAAGATGTTCAACTCT TTATGTATTGATC 3'
L S35C QC Rev	5' GATCAATACATAAAGAGTTGAACATCTTTGTT GTCTTCGAGATGG 3'
L L44E For	5' ACTCTTTATGTATTGATCTTCGAGGCGATCTTT CTCTCGAAATTT 3'
L L44E Rev	5' AAATTTCGAGAGAAAGATCGCCTCAAGGATCA ATACATAAAGACT 3'
L L44R For	5' ACTCTTTATGTATTGATCTTCAGAGCGATCTTT CTCTCGAAATTT 3'
L L44R Rev	5' AAATTTCGAGAGAAAGATCGCTCTAAGGATCA ATACATAAAGAGT 3'
L A45E For	5' CTTTATGATTTGATCTTCCTCGAGATCTTTCTC TCGAAATTTACC 3'
L A45E Rev	5' GTTAAATTTCGAGAGAAAGATCTCGAGGAAG ATCAATACATAAAG 3'
L A45R For	5' CTTTATGTATTGATCTTCCTCAGAATCTTTCTC TCGAAATTTACC 3'
L A45R Rev	5' GTTAAATTTCGAGAGAAAGATTCTGAGGAAGA TCAATACATAAAG 3'
L I46E For	5' TATGTATTGATCTTCCTCGCGGAGTTTCTCTCG AAATTTACCAAT 3'
L I46E Rev	5' ATTGTTAAATTTCGAGAGAAACTCCGCGAGGA AGATCAATACATA 3'
L I46R For	5' TATGTATTGATCTTCCTCGCGAGATTTCTCTCG AAATTTACCAAT 3'
L I46R Rev	5' ATTGGTAAATTTCGAGAGAAATCTCGCGAGGA AGATCAATACATA 3'
L ILA 44-46 For	5' ACTCTTTATGTATTGATCTTCATCCTCGCGTTT CTCTCGAAATTTACCAAT 3'
L ILA 44-46 Rev	5' ATTGGTAAATTTCGAGAGAAACGCGAGGATG AAGATCAATACATAAAGAGT 3'
L S59C QC For	5' ATCTTCCTCGCGATCTTTCTCTGTAAATTTACC AATCAATTGCTT 3'
L S49C QC Rev	5' AAGCAATTGATTGGTAAATTTACAGAGAAAGA TCGCGAGGAAGAT 3'
L QC S49T For	5' ATCTTCCTCGCGATCTTTCTCACGAAATTTACC ATTCAATTGCTT 3'
L QC S49T Rev	5' AAGCAATTGATTGGTAAATTTCGTGAGAAAGA TCGCGAGGAAGAT 3'
L S58C QC For	5' TTTACCAATCAATTGCTTCTGTGTCTACTGGAA GCGGTGATCCGC 3'
L S58C QC Rev	5' GCGGATCACCGCTTCCAGTAGACACAGAAGCA ATTGATTGGTAAA 3'
L L73C QC For	5' ACAGTGACGACTTTACAGCAATGTCTTACTTA AGGGACGAATTGC 3'
L L73C QC Rev	5' GCAATTCGTCCCTTAAGTAAGACATTGCTGTA AAGTCGTCACTGT 3'

Results

Kinetics of L protein accumulation during MS2 infection

One limitation to earlier studies on the MS2 L protein was the inability to measure its relative or absolute concentration in infected cultures or in subcellular fractions derived from infected cells. To overcome this problem, we prepared a highly specific antisera against amino acids 12-26 of the L sequence. Using this reagent, the time course of L accumulation during a MS2 infection could be precisely determined for the first time. As can be seen in Figure 2.2, L can first be detected at 28 minutes after infection and culture lysis becomes evident when the levels of L reach approximately 50% of its eventual maximum.

Quantification of L expression from MS2 infections

No attempt has been made in the literature to quantify the amount of L protein produced per cell during MS2 infections. The highly specific antibodies to L and the capacity to produce a purified, quantified L standard offered a plausible solution to this. Thus, the amount of L produced during an infection of *E. coli* cells was analyzed. The amount of L protein produced just after the onset of lysis was compared to a known amount of purified His_6-L protein (from the stock of 25 μg/ml) using quantitative Western blotting. In Figure 2.3, the sample from the infected cells represents about

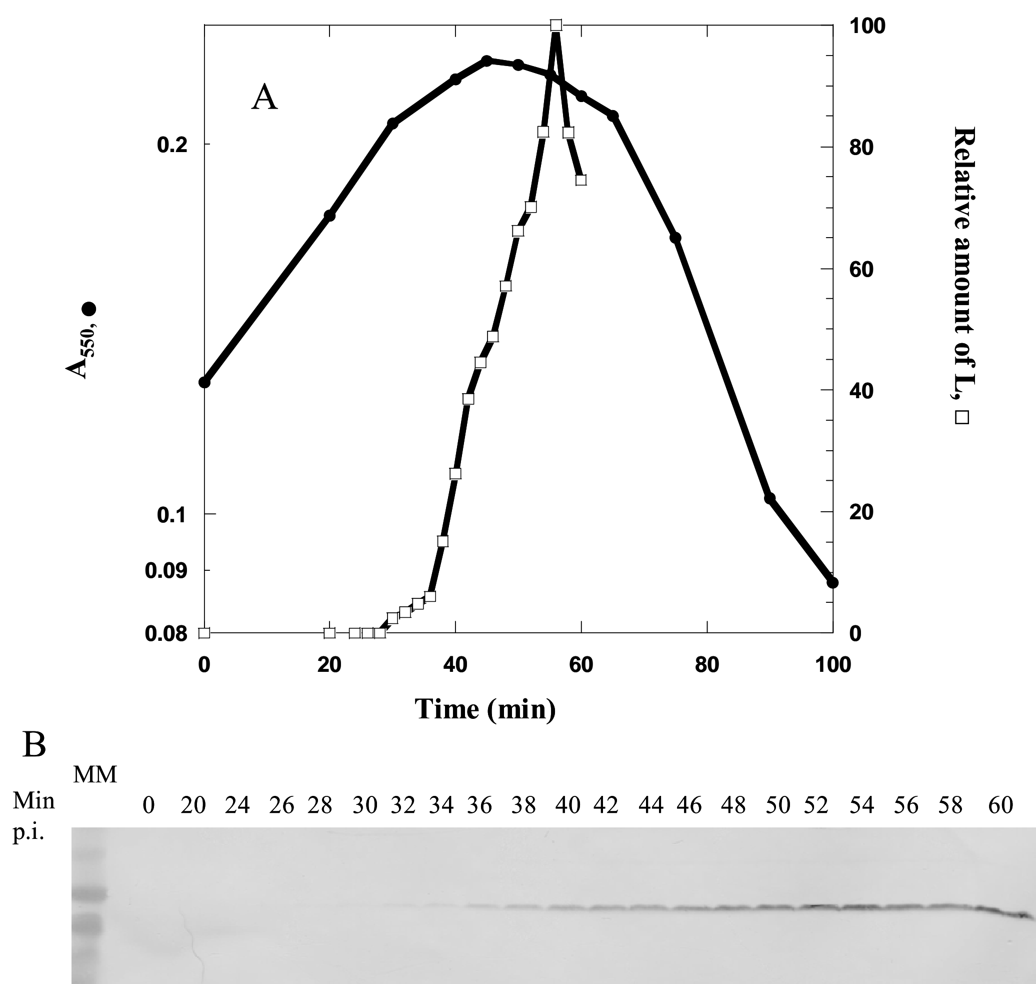

Figure 2.2. MS2 L accumulation begins at 28-30 minutes after infection at an MOI of 5. (A) A_{550} and relative amount of L produced at each time point indicated. Densitometric analysis of the Western blot shown in (B) was used to provide relative amount of L.

0.5mL of culture, infected at an A_{550} ~0.1, suggesting that there is about 250 ng of L protein per ml of culture. The His_6-L protein in lanes 1-6 runs at a higher molecular mass than wild-type L because of the addition of the oligo-histidine tag.

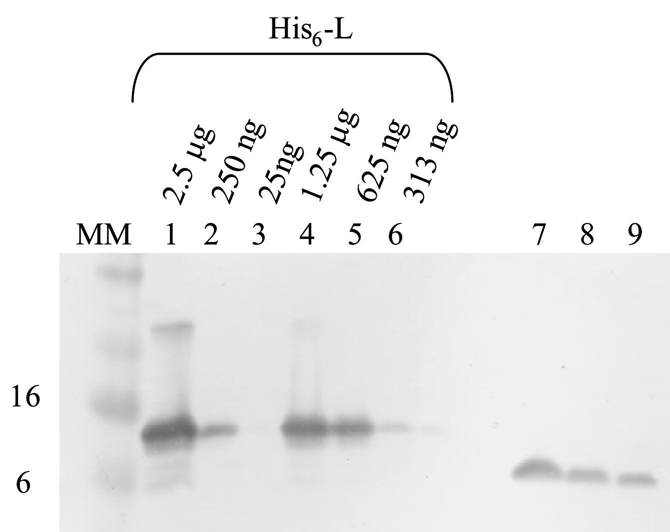

Figure 2.3. Quantification of L using purified His_6-L as a standard. The molecular mass standard is abbreviated as MM and the sizes are indicated on the left in kDa. Quantities of His_6-L are indicated above lanes 1-6. Lanes 7-9 are 5 µl, 2.5 µl and 1.25 µl of the sample collected at 60 minutes post infection of ER2738.

A dual-plasmid system for the controlled expression of L

My initial attempt to clone MS2 L with a moderately efficient Shine-Dalgarno sequence under a *lac* promoter was unsuccessful. The basal expression of L from this promoter was significantly higher than tolerable for cells, and cultures grown overnight showed signs of lysis, even without the presence of inducer. Cultures grown from these overnight samples routinely did not lyse after the addition of IPTG (data not shown), suggesting the samples had been overrun with plasmid mutants.

Thus, to facilitate our studies of L protein function, a system capable of nearly complete repression of *L* gene expression in the uninduced state and which, after induction, produced the L protein at levels comparable to those observed during an MS2

infection was sought. Based on our experience with the cloning and expression of holin genes from dsDNA phage, I decided to make use of a dual plasmid system. In this approach, the gene of interest is cloned into one plasmid under the control of the *pR'* promoter from bacteriophage λ. Since the *L* gene does not have a recognizable Shine-Dalgarno (SD) sequence upstream of the start codon, it was necessary to provide it with one to avoid the co-expression of the MS2 coat gene. To allow the translation of the *L* message, the SD sequence for the endolysin gene, *R*, from phage λ was placed upstream of the start codon of *L* (Table 2.1). The second plasmid carries the λ *Q* gene encoding the antiterminator necessary for gene expression from the *pR'* promoter. The expression of *Q* is controlled by a hybrid *lac/ara* promoter (Grundling et al., 2001) and can be almost completely repressed by the *lac* repressor in host cells with the $lacI^q$ gene. This dual plasmid system not only allows the induction of *L* gene expression by the addition of IPTG and arabinose to cultures but also permits the inductions to occur without the added complication of thermal shifts.

Using the dual plasmid system of pQ and pRE-L, *L* gene expression was sufficiently repressed to allow culture growth without loss of the pQ/pRE-L plasmids. Induction of L expression with IPTG resulted in lysis after 50-55 minutes (Figure 2.4A). The lysis time could be decreased to near 45 minutes by the addition of arabinose as well as IPTG, which results in full induction of the $P_{lac/ara}$ promoter responsible for the expression of Q. The amount of L produced from the dual plasmid system induced with IPTG is only 1.5-2 fold more than L produced during an infection by MS2, indicating the protein is not greatly overexpressed using this system (Figure 2.4B).

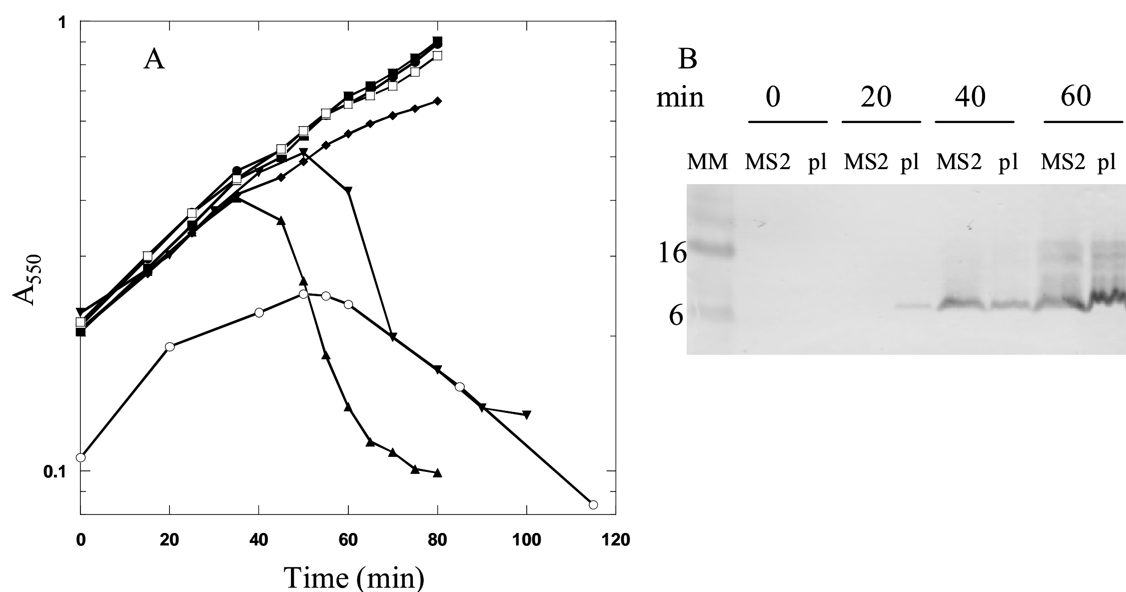

Figure 2.4. Expression of L from the dual plasmid system, pQ pRE-L, is sufficient for lysis and is comparable to L accumulation in MS2 infected cells. (A) Lysis profile from induction of the dual plasmid system with MS2 L under *pR'* promoter using 1mM IPTG or 1mM IPTG and 0.2% Arabinose (Ara) in RY15177 compared to lysis by MS2 infections of RY15177 at MOI of 5. Cultures were grown at 37°C. ●, pQ + IPTG; ■, pRE-L + IPTG; ♦, pQ pRE, + IPTG; ▼, pQ pRE-L, + IPTG; ▲, pQ pRE-L, +IPTG, +Ara; □, pQ pRE-L, uninduced; ○, MS2. (B) Plasmid-borne L is expressed at a level near to that of MS2 infected cells. MM represents the molecular mass marker, with sizes in kDa indicated on the left. Samples were prepared as described in the Materials and Methods at the indicated times after induction of the plasmids with IPTG (pl) or infection with MS2 at an MOI of 5 (MS2).

L is localized throughout the host cell envelope

In *E. coli,* specific integral membrane proteins are localized to either the inner membrane or the outer membrane, but not both. Landmark studies by Osborn and co-workers demonstrated that the inner and outer membranes of Gram-negative bacteria can be separated by isopycnic density centrifugation (Osborn and Munson, 1974). The inner

membranes are found in the low density fractions and a number of enzymes, including NADH oxidase, have been established as markers for inner membrane fractions (Bell et al., 1971; Osborn and Munson, 1974). The outer membranes are found at higher densities and are defined by the presence of the well characterized porin proteins (Bell et al., 1971; Osborn and Munson, 1974; reviewed by Osborn and Wu, 1980). Previously, Walderich et al. (1988) found that the L protein could be found in both the inner and outer membrane fractions. Additionally, using immunolabeling and electron microscopy, Walderich and co-workers claimed that L was localized to membrane adhesion sites, or Bayer's patches, where the inner and outer membranes of the cell are locally fused (Bayer, 1968; Walderich et al., 1989). In all of these experiments, the *L* gene was expressed from the *pL* promoter on a multicopy plasmid. Thus, it is likely that the L protein accumulated to levels that are higher than those seen during a MS2 infection. Since the overproduction of proteins often results in their aggregation, denaturation, and mislocalization, these findings must be viewed with caution. For this reason, I examined the distribution of L in cells infected with MS2 and in cells expressing the *L* gene from the dual plasmid system where the *L* gene is expressed at physiological levels. Surprisingly, in both cases, L was found in the inner, intermediate, and outer membrane fractions (Figure 2.5A -D) as defined by the protocol developed by Osborn and Munson (1974).

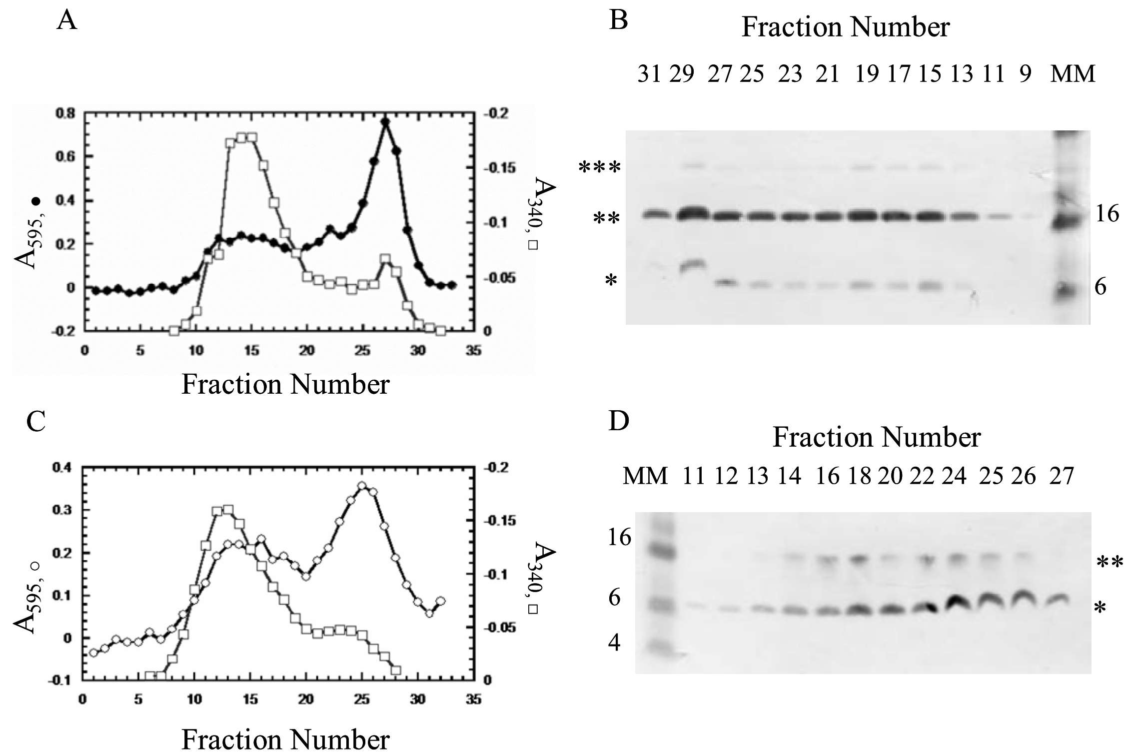

Figure 2.5. Isopycnic sucrose gradients performed on MS2-infected cells or cultures expressing L from a plasmid result in detection of L protein in all fractions. (A) Fractions of the sucrose gradient of MS2-infected cells were tested for total protein using a Bradford assay, ●, and assayed for NADH oxidase activity, □. (B) Western blot of indicated fractions of MS2-infected cells using anti-L antibody. MM designates the molecular mass marker (in kDa), while one, two or three asterisks specify monomer, dimer and trimer species of L, respectively. (C) Fractions of the sucrose gradient from L plasmid induction were tested for total protein using a Bradford assay, ○, and assayed for NADH oxidase activity, □. (D) Western blot of indicated fractions from plasmid-borne L expression using anti-L antibody. One or two asterisks specify monomer and dimer species of L, respectively. MM represents the molecular mass marker (in kDa).

Lytic function of MS2 L protein homologs

The identical approach was taken to clone the *L* genes of two other Leviviruses, AP205 and KU1 (Fig. 2.1). Both of these phage require an F pilus for adsorption, though AP205 will not form plaques on *E. coli*. The L protein produced by KU1 shares significant sequence similarity with the MS2 L protein. Both L proteins have a similar overall appearance as they have a positively charged N-terminus followed by a mostly uncharged and hydrophobic C-terminal domain. Interestingly, the *L* gene of KU1 does not overlap the coat gene in the KU1 genome, and thus is not evolutionarily constrained by the coat gene sequence. However, L^{KUI} maintains the highly charged N-terminal sequences seen in all L proteins. KU1 and MS2 L proteins share a hydrophobic, leucine-enriched domain from residues 38-52 of the MS2 sequence, LYVL/WIFG/ALAIF/VLSK/DFT. This sequence includes the fundamental XLS triad used to align the *Leviviridae* L proteins. By contrast, the L protein with the least similarity to MS2 L is that produced by the *Acinetobacter*-specific phage AP205. In this case, the similarity is limited to the overall charge distribution and the XLS triad.

Host lysis was clearly evident after induction of pRE-L^{KU1} (Fig. 2.6). However, expression of the AP205 *L* gene in *E. coli* resulted in the cessation of growth rather than overt lysis reflected by a decrease in culture A_{550} (Figure 2.6). Such behavior might be due to a general toxicity of the AP205 L protein and, thus, is unrelated to its lytic function in *Acinetobacter*. This would not be surprising given the fact that *Acinetobacter* and *Escherichia* are very distantly related and that the similarity between the L proteins from MS2 and AP205 are only superficial (Figure 2.1).

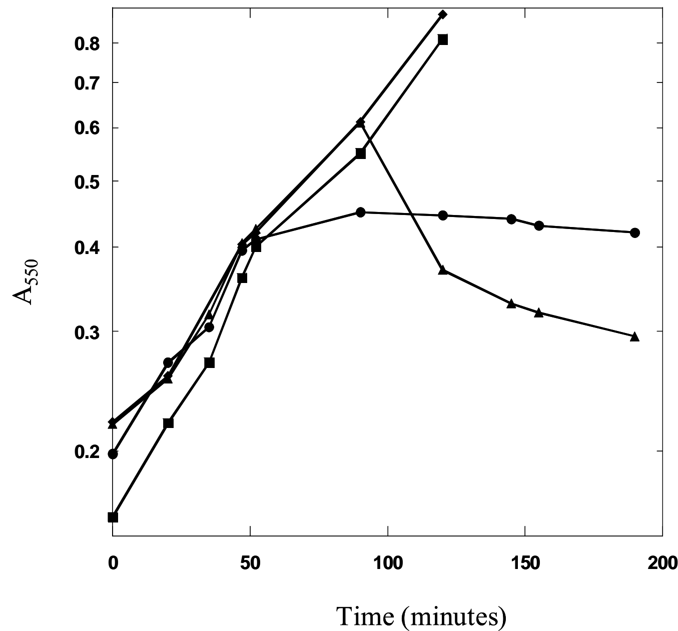

Figure 2.6. Lysis profiles from induction of other *L* genes from AP205 and KU1. Cultures of XL1 Blue were grown at 37°C and induced by the addition of IPTG and arabinose to a final concentration of 1 mM and 0.2%, respectively. ●, pQ pRE-L^{AP205}, induced; ■, pQ pRE- L^{AP205}, uninduced; ▲, pQ pRE-L^{KU1}, induced; ▼, pQ pRE- L^{KU1}, no induction; ♦,pQ pRE, no induction.

L-mediated envelope defects are located randomly on the host cell surface

In order to determine if the L protein causes weakening of the cell envelope at discrete sites, cells harboring the dual plasmid L system were grown in standard LB media supplemented with 0.23 M sucrose and 10 mM $MgCl_2$. Under these conditions, cells are osmotically stabilized allowing preservation of morphologies present immediately before membrane rupture and overt cell lysis. As can be seen in Panel A of Figure 2.7, expression of the MS2 *L* gene results in membrane protrusions that can be located anywhere along the cell periphery. In some cells, the lesions appear to be at the

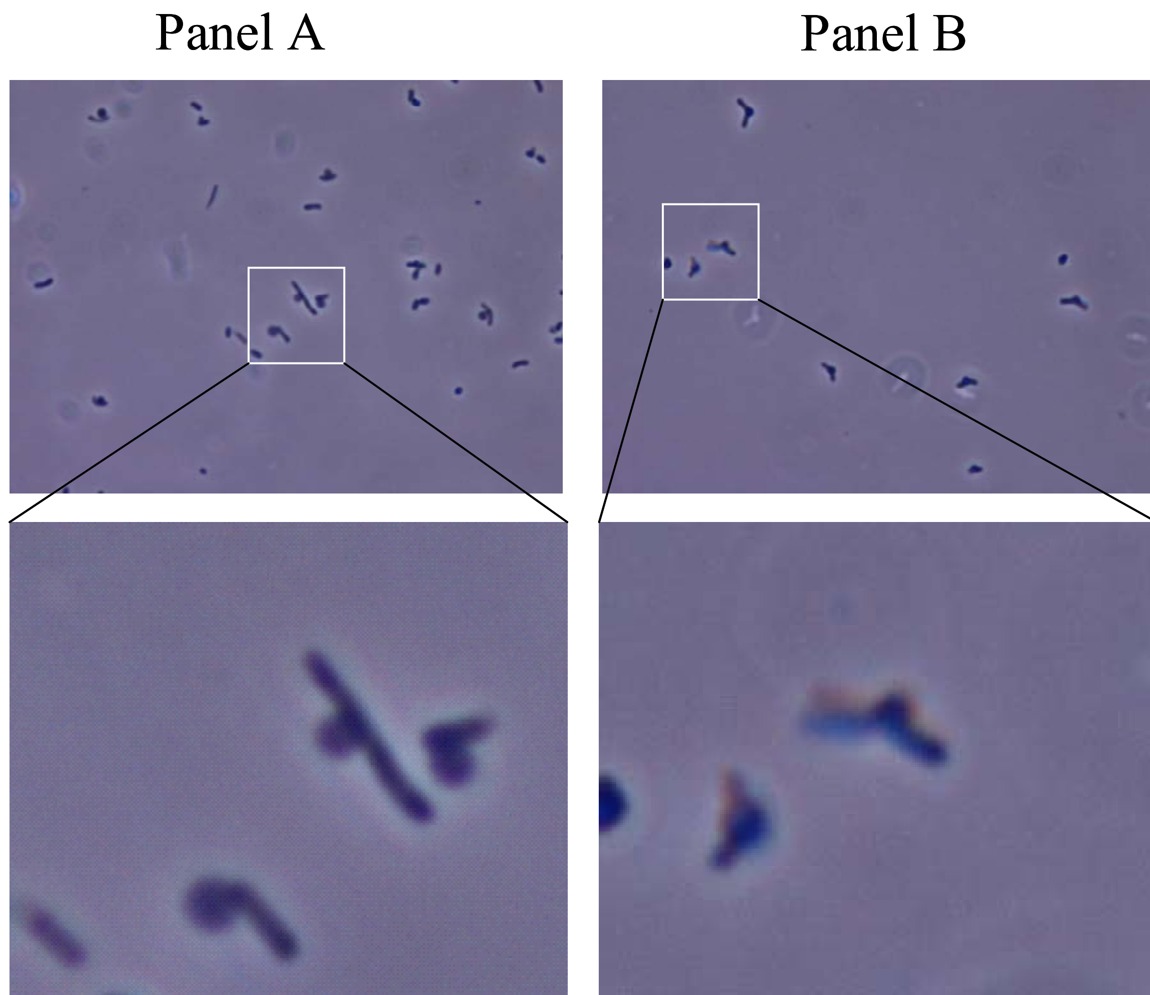

Figure 2.7. The morphologies of L-lysing cells are different from those seen with A_2 when cells are protected from lysis by sucrose and Mg^{2+}. Cultures were induced with 1 mM IPTG. Panel A: RY15177 pQ pRE-L induced for 45-60 minutes. Panel B: RY15177 pZE12-A_2 induced for 30-45 minutes.

midpoint (septum), while in others they are clearly present at various other sites along the cell, including the poles. This L-mediated lysis phenotype is markedly different from the septally localized protrusions that characterize A_2-mediated lysis (shown in

Figure 2.7 Panel B) and E-mediated lysis and suggests that L causes host lysis by a fundamentally different mechanism.

Septation is not required for MS2 L-mediated lysis

The addition of low concentrations of penicillin and related β-lactams to cultures of *E. coli* results in the inhibition of septation; cells remain capable of elongation of the murein sacculus, but cannot initiate and complete the septation event required for cell division (Satta et al., 1980). If the L protein of MS2 caused host lysis by interfering with septation, its lytic function might be blocked by the treatment of cells with sublytic concentrations of ampicillin that block septation but allow filamentation. As can be seen in Figure 2.8A, MS2 is fully capable of lysing *E. coli* cells treated with 2 μg/ml Amp

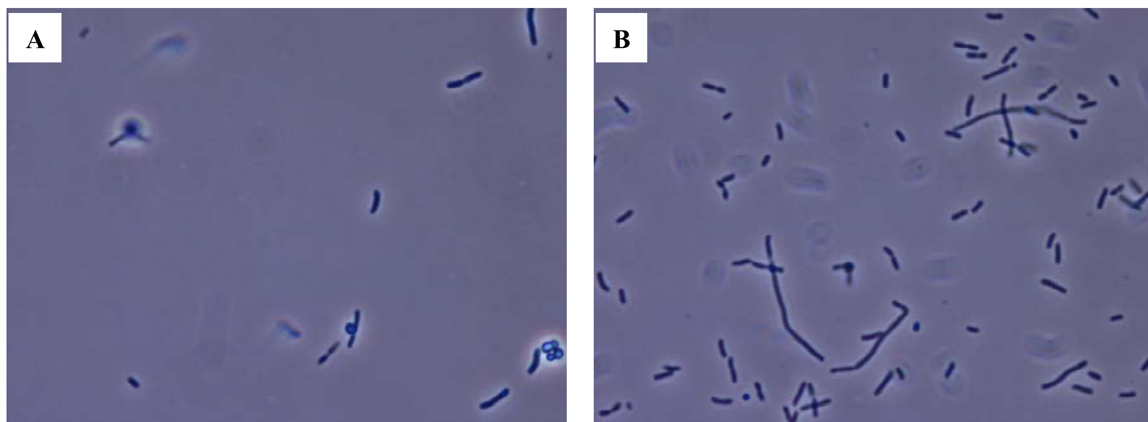

Figure 2.8. Images of RY15495 treated with 2 μg/mL Amp to prevent septation and infected with either MS2 or Qβ at an MOI of 10. (A) MS2-infected cells. (B) Qβ-infected cells.

and cells still show the membrane protrusions that typify L-mediated lysis. Cells treated with 2 μg/ml Amp and infected with Qβ do not lyse or show septally localized membrane protrusions (Figure 2.8B). Since lysis does not occur in these cells, there are more cells in the field than the MS2-infected cells.

Mutational analysis of L

Initial efforts to define the lytic domain(s) of L employed N-terminal deletions of the L protein. This study suggested that the C-terminal half of the protein was both necessary and sufficient for host lysis (Berkhout et al., 1985). However, the interpretation of these results is open to question. First, the analysis of the lytic domain of L was performed using fusion proteins. To create the truncations, various segments at the 5'-end of the lysis gene were deleted and fused to the coat protein, or constructed so that the truncated *L* gene was under the control of the ribosome binding site of the *rep* gene. The resulting coat-L fusion proteins were not subjected to any subcellular fractionation to show the fusion protein was properly localized. Second, the expression of the full-length L and truncated proteins was performed using expression from the *pL* promoter. The levels of expression from any of these constructs were never compared to the expression of L during MS2 infections, though it is likely that this system results in overexpression of the cloned L proteins along with the other intact MS2 proteins encoded on the plasmid. Third, the induction of expression from the *pL* promoter required a thermal shift to 42°C, but the cultures were never returned to 37°C, even though this would have also allowed for expression from the *pL* promoter. Fourth, the

authors reported that the construct in which the C-terminal 32 residues were fused to the N-terminal 8 amino acids of the coat protein was lytic, in a manner nearly equivalent to full-length L on the plasmid. However, the lytic effect of the truncated protein encoding the C-terminal 32 residues could not be reproduced using the dual plasmid system described above (data not shown).

To define specific regions and residues required for lysis mediated by L, a different approach was taken. Numerous conserved residues, based on comparisons of *Leviviridae* L proteins, were mutated to different amino acids. Most of the mutations had no effect on lysis kinetics, though one of the mutants (H24F) showed a 20-25 minute delay in the onset of lysis (Figure 2.9A, B & C). The accumulation of the L^{H24F} protein was not analyzed and delayed accumulation may account for delayed lysis times. Although cysteine scanning mutagenesis was initiated for studying the localization of L, the mutagenesis led to the discovery of an essential residue, L^{S49C}. This mutant is not lytic, though it does appear to slow the growth of cells about sixty minutes after

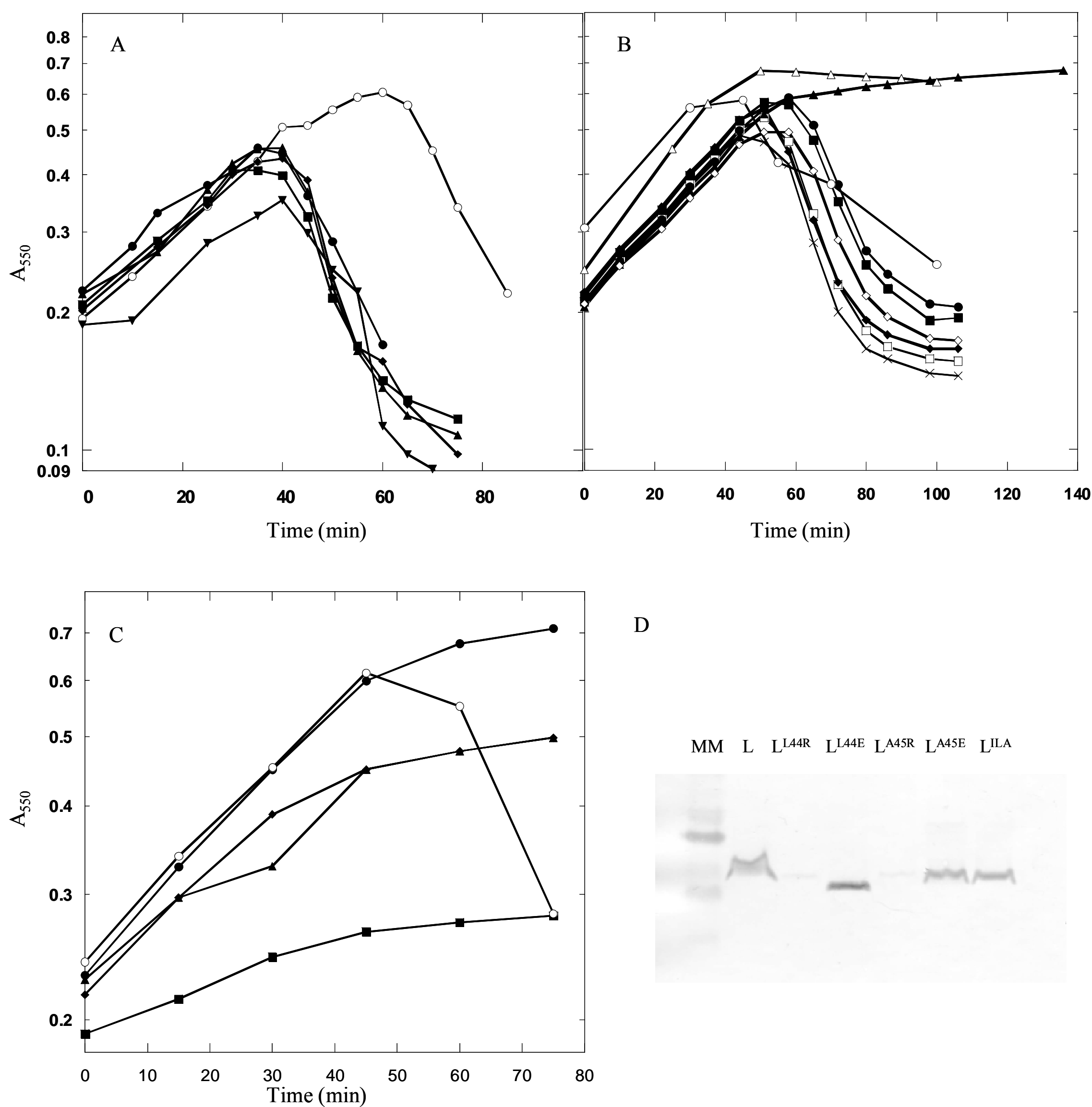

Figure 2.9. Lysis profiles and Western blots of various missense mutants of L. (A) Lysis profiles of missense mutants of L at positions H24, C29 and R34. Cultures were grown to A550 of 0.2 and induced with IPTG at a final concentration of 1mM. ●, L^{C29S}; ■, L^{H24A}; ○, L^{H24F}; ♦, L^{R34E}; ▼, L^{R34K}; ▲, L^{R34A}. (B) Lysis profiles of various cysteine scanning and non-lytic mutants. x, L; □, L^{C29S};●, L^{S9C}; ■, L^{S15C}; ♦, L^{S35C}; ▲, L^{S49C}; Δ, L^{S49T}; ◊, L^{S58C}; ○, L^{L73C}. (C) Lysis profiles of mutants from residues 44 to 46 of L. ●, L^{L44R}; ■,L^{L44E}; ♦, L^{A45R}; ▲, L^{A45E}; ○, $L^{ILA44-46}$. (D) Western blot of accumulation of L proteins in (C). Samples were harvested as described in the Materials and Methods 75 minutes after induction.

induction. Ser49 is located in the most conserved domain of the coliphage L proteins, a region that is non-charged and hydrophobic (refer to Figure 2.1). The incorporation of either a threonine or cysteine at this position prevents L function, which is somewhat unexpected since neither substitution is charged or bulky. This suggests that serine at position 49 of L may be essential for interacting with the target to elicit lysis. The conserved serine residue is flanked by upstream residues that are highly conserved throughout L proteins of coliphage, which became of particular interest after the characterization of the lysis defect of Ser49. A series of mutants were produced where three of the five residues preceding Ser49 were altered. The introduction of positive or negative charges into positions 44-46 prevented proper functioning of the protein. As can be seen in Figure 2.9D, several of the charge mutants failed to accumulate L protein, which would explain the non-lytic phenotype. A scramble of the residues that maintained the overall charge, but changed the order of the amino acids, maintained a lysis time near that of wild-type L and accumulated the L protein (Figure 2.9D).

Discussion

Translation of the *L* gene message is coupled to the translation of the coat protein from the ssRNA genome of MS2. Normally, the start codon for L is present in a stem-loop preventing its recognition by the translational machinery. However, ribosomes completing the translation of coat disrupt this stem-loop allowing initiation to occur at the L start codon. By this mechanism, the ratio of coat to L protein produced during an infection is thought to be 5-10% (Beremond and Blumenthal, 1979). I have found that the L protein can first be detected approximately 28 minutes after infection and eventually accumulates to a level of approximately 100,000 molecules per infected cell. This number is not significantly different from that predicted by Beremond and Blumenthal (1979).

Although the genome of MS2 is roughly the size of the plasmids routinely used in molecular genetics, the fact that it is a molecule of ssRNA precludes the genetic manipulation of the *L* gene in this context. Although dsDNA versions of the MS2 genome have been constructed, even these are not well suited for a genetic analysis of *L* since it overlaps the coat protein and replicase genes for MS2. Thus, in order to study the effect of the L protein on the physiology of the host cell, it was necessary to develop a system that allowed expression of the *L* gene at levels that were comparable to what is seen in an MS2 infection. This goal was achieved using a dual plasmid system where the *L* gene was placed under the control of the λ *pR'* promoter on one plasmid while a second plasmid carried *Q*, encoding the anti-terminator for *pR'*, under the control of a hybrid *lac-ara* promoter. Upon induction, this system brought about cell lysis with

kinetics similar to that seen in a MS2 infection. Moreover, the level of L protein in an induced culture is roughly equal to that in an infected culture at the time of lysis. Unlike the thermally inducible system based on the *pL* promoter (Renaut et al., 1981), this system will allow investigation of L function under conditions that allow *L* gene expression to more accurately reflect what occurs in infected cells.

Osmotic stabilization of cells expressing the *L* gene revealed that envelope defects occur all along the cell periphery including the poles. This lies in contrast to what is seen with cells exposed to penicillin, which develop large membrane protrusions at mid-cell (Schwarz and Asmus, 1969). It is thought that these lesions occur at the septum because this site has the highest localized activity of the PBP (penicillin-binding proteins) as the cell attempts to build the septum, a physical barrier composed of murein between its two daughter cells (Schwarz and Asmus, 1969; Tomasz, 1979, 1982). E- and A_2-mediated lysis also show membrane protrusions localized to the septum. Previous studies have characterized Qβ-infected, osmotically stabilized cells and shown that the membrane protrusions form spherical cells or spheroplasts (Ozaki and Valentine, 1973), which are also evident in Figure 2.6, Panel B. This is in stark contrast to the morphologies of L-lysing cells. These cells appear to have membrane protrusions or become emptied ghosts, resulting in a much more soiled field during observation. Septation requires large amounts of precursor to assemble into the growing septum and E and A_2 are known to inhibit enzymes (MraY and MurA, respectively) involved in de novo synthesis of peptidoglycan. This is likely why the disturbance in the envelope of cells on the brink of lysing from E or A_2 expression is localized to the septum. Thus, not

only does L not block the incorporation of diaminopimilic acid into peptidoglycan, L-mediated lysis does not seem to resemble at the microscopic level lysis brought about by chemical or protein inhibitors of peptidoglycan synthesis. To discriminate between important residues in L, a mutational analysis was employed. A series of cysteine mutants produced were still capable of lysis, but one particular position, Ser49, was discovered as essential for L-mediated lysis. Mutation of this amino acid to cysteine resulted in an accumulation of the protein, but failed to cause obvious clearing of the culture. This result suggests that the serine residue plays a critical and required role in the lytic function of L. Preliminary evidence from a set of mutations introduced into a conserved domain of the coliphage L proteins (position 44-46 of MS2 L) indicated that the amino acids are not significant to the overall function of the L protein, but that the important characteristic of the region is the net neutral charge and overall hydrophobicity.

CHAPTER III

MISSENSE MUTATIONS IN PCNB PREVENT ENTRY OF ssDNA AND ssRNA GENOMES FROM F-SPECIFIC BACTERIOPHAGES

Introduction

Three classes of phages with small single-stranded RNA or DNA genomes and icosahedral morphologies, accomplish lysis of their hosts without expressing a muralytic enzyme: the *Microviridae* (single-stranded circular DNA), represented by ϕX174 (Sertic and Bulgakov, 1935; Sinsheimer, 1959); and *Leviviridae* and *Alloleviviridae* (single-stranded linear RNA), with MS2 and Qβ as the prototypic phages, respectively (Zinder and Lyons, 1967). Early studies on the mechanism of host lysis by small phages led to the suggestion that a phage-encoded protein acted as an inhibitor of a step in peptidoglycan synthesis (Ozaki and Valentine, 1973). This view was consistent with the observation that conditional mutants in cell wall biosynthesis would undergo lysis under nonpermissive conditions and that lysis under these conditions, or as a result of infections with these small phage, was morphologically indistinguishable from penicillin-induced lysis. The effects of penicillin on cells were observed more than fifty years ago as having several fundamental characteristics, including the necessity for cells to be growing, cellular content release into the medium and lysis occurring at pH 7, but not pH 5 (Goodell et al., 1976; Hahn and Ciak, 1957). Hahn and co-workers visualized penicillin-treated cells grown in sucrose and showed membrane protrusions, which they referred to as “rabbit ears”, at the center or poles of the cells (Hahn and Ciak, 1957). E-

mediated lysis had the same characteristics, showing a necessity for growing cells and a bleb occurring at mid-cell of the lysing cell (Bradley, 1968; Bradley et al., 1969; Roof and Young, 1993). Despite these early insights, several groups investigating the mechanism of action of E and L, the lysis proteins of bacteriophages ϕX174 and MS2, respectively, made claims that these proteins acted by stimulating a cellular autolytic system or by forming pores or tunnels spanning the entire cell envelope (Holtje and van Duin, 1984; Lubitz et al., 1984; Witte et al., 1990; Witte et al., 1992). Autolysis was hypothesized after the observation that radiolabeled DAP (diaminopimilic acid) incorporation not only stopped prior to the initiation of lysis by E, but began to decrease (Lubitz ad Plapp, 1980). This showed that the murein layer was being degraded, which the authors suggested was the result of E-induced unregulated activity of the cell's peptidoglycan-degrading enzymes. Walderich and co-workers estimated the length of peptidoglycan strands during L expression at pH 7 and pH 5 (Walderich et al., 1988). The length of the strands was shortened about 18%, which they attributed to an activation of murein glycosidases present in the cell (Walderich et al., 1988). Alternatively, the transmembrane tunnel model was hypothesized for E-mediated lysis after electron microscopy of E-lysed cells showed holes spanning the cell envelope (Witte et al., 1992). It should be noted, however, that the 91 amino acid E protein was localized to the inner membrane in sucrose gradients (Roof and Young, 1993). It was proposed that the cellular contents leaked out of these envelope-spanning holes located at the pole or mid-cell and eventually led to lysis, leading to the proposal of the transmembrane tunnel model. In any case, the autolysis and transmembrane tunnel

models were the result of studies based on physiology and morphology of E- and L-mediated lysis.

A different picture, consistent with the original proposal of Valentine and Ozeki, emerged when a genetic approach was taken. By selecting for cells that survived expression of the cloned E gene from ϕX174, Bernhardt et al. (2000) found that recessive mutations in *slyD* blocked E-mediated lysis. Later, it was found that the E protein was unstable and did not accumulate in the *slyD* hosts, suggesting that SlyD was not the target of E but was required for its stability (Bernhardt et al., 2001). By plating ϕX174 on *slyD* hosts, alleles of E (Epos) whose products were independent of SlyD were identified (Bernhardt et al., 2001a). Selecting for Epos-resistant cells resulted in isolation of dominant mutations in the gene *mraY*, a conserved, essential gene in the pathway for synthesis of murein precursors. Subsequently, it was demonstrated that the expression of E blocked the incorporation of DAP into SDS-insoluble material and resulted in the accumulation of UDP-MurNAc-oligopeptides, which include the substrate for MraY. Thus, both biochemical and genetic evidence indicated that the E protein brings about cell lysis by acting as a specific inhibitor of MraY (Bernhardt et al., 2001a). Shortly thereafter, the same genetic and biochemical strategies were used to demonstrate that A_2, the lysis protein of Qβ, inhibited MurA, the enzyme catalyzing the first committed step in peptidoglycan synthesis (Bernhardt et al., 2001b). Although the mechanistic details of how these phage proteins actually effect inhibition of their specific targets remain an area of active investigation, at least the underlying molecular

strategy employed by the *Microviridae* and the *Alloleviviridae* to affect lysis, was solved.

At the strategic level, the remaining mystery is thus the mechanism for host lysis used by MS2 and other members of the *Leviviridae*. L, the lysis protein of MS2, is encoded by a gene that spans the 3' end of the *coat* gene and 5' end of the *rep* gene (Atkins et al., 1979; Beremond and Blumenthal, 1979; Model et al., 1979). L expression does not inhibit the incorporation of DAP into SDS-insoluble cell wall material (Holtje and van Duin, 1984; T. G. Bernhardt dissertation, 2001). Thus L does not function analogously to E and A_2, which inhibit early cytoplasmic steps in peptidoglycan synthesis. Formally, one can say that L either interferes with a late but essential step in murein maturation or brings about cell lysis by a mechanism distinct from cell wall synthesis. L-mediated lysis of *E. coli* has been variously ascribed to activation of an uncharacterized autolytic system, the formation of adhesion sites between the inner and outer membranes and an ill-defined pathway involving membrane-derived oligosaccharides (Holtje and van Duin, 1984; Holtje et al., 1988; Walderich et al., 1988). Since the targets of the lysis proteins of ϕX174 and Qβ were identified using a genetic approach, I was optimistic the target of L could be similarly identified. Here I report a genetic strategy implemented to that end.

Materials and methods

Bacterial strains, bacteriophages, plasmids and culture growth

BMC1 (RY15495 *tonA*::Tn10 *pcnB*^{D90A}), BMC2 (RY15495 *tonA*::Tn10 *pcnB*^{R78C}), BMC3 (RY15495 *tonA*::Tn10 *pcnB*^{G74S}), BMC4 (RY15495 *tonA*::Tn10 *pcnB*^{G75E}), and BMC5 (RY15495 *tonA*::Tn10 *pcnB*^{R78C}) were used where indicated or strains were as described in Chapter II. Bacteriophage GA was a generous gift from Dr. van Duin, University of Leiden. GA and other phages were prepared as described in Chapter II. Plasmids are described below or were described in Chapter II. Conditions for growth were as described in Chapter II.

General bacteriological methods

P1 transductions and matings between Hfr and F' males and female recipients were performed as described by Miller (1992). Phage sensitivities were determined by cross-streaking on LB agar plates containing the appropriate antibiotics. Plasmids were cured by the growth of cultures in LB without antibiotic selection. In some cases, novobiocin was added to facilitate plasmid loss (Hooper et al., 1984). The level of MS2 accumulation in infected cultures was determined as previously described except that cultures were grown to an A_{550} ~0.4-0.5 and then diluted to an A_{550} ~0.1 before infection at a multiplicity indicated in the legends (Bernhardt et al., 2001b). MS2 titers were determined by plating serial dilutions on lawns of RY15177. Titers of other

bacteriophage were determined by spot titering 5 µl of serial ten-fold dilutions of phage on indicator lawns (RY15177) with H-top agar and incubating overnight at 37°C.

RNA and DNA manipulations and DNA sequencing

All methods and materials are described below or were as described in Chapter II.

pCA24N-*pcnB* was obtained from Kitagawa et al. (2005). Using this plasmid, the PcnB protein is fused to a His tag located at the N-terminus and GFP at the C-terminus. Expression of this fusion can be induced from a *T5/lac* promoter with the addition of 1 mM IPTG. The plasmid was digested with NotI and ligated to remove the GFP fused at the C-terminus of the His_6-*pcnB* construct according to the manufacturer's instructions (New England BioLabs, Ipswich, MA). Primers used for QuickChange are listed in Table 3.1 and used as described in Chapter II. Clones were sequenced using the primers pCA-F and pCA-R listed in Table 3.1 also (Kitagawa et al., 2005).

Table 3.1. Primers used for QuickChange or sequencing of *pcnB*.

Primer name	**Sequence**
R78C QC For	5' GGCTGGTTGGCGGCGGCGTGTGCGACCTGTTACTTGGCAAAAA 3'
R78C QC Rev	5' TTTTTGCCAAGTAACAGGTCGCACACGCCGCCGCCAACCAGCC 3'
D90N QC For	5' GCAAAAAGCCGAAAGATTTTAACGTAACCACTAACGCCACGCCT 3'
D90N QC Rev	5' AGGCGTGGCGTTAGTGGTTACGTTAAAATCTTTCGGCTTTTTGC 3'
PcnB QC D90A For	5' GGCAAAAAGCCGAAAGATTTTGCCGTAACCACTAACGCCACGCCT 3'
PcnB QC D90A Rev	5' AGGCGTGGCGTTAGTGGTTACGGCAAAATCTTTCGGCTTTTTGCC 3'
pCA-F	5' GGGCGTATCACGAGGCCCTTTCGTCTTCACC 3'
pCA-R	5' TTTGCATCACCTTCACCCTCTCCACTGACAG 3'

Isolation of BMC1-5 mutants

RY15495 was mutagenized with EMS for thirty minutes as described by Miller (1992). This level of exposure led to 50% killing and increased the frequency of rifampicin-resistant cells from approximately 10^{-8} to 10^{-4}. The mutagenized cells were allowed to recover by overnight growth in LB supplemented with Kan and Amp. The next day, the overnight culture was diluted 1/100 in LB with antibiotics, grown to an A_{550} of 0.2 and induced for L gene expression by the addition of IPTG and Ara. After two hours, the induced cells were collected by centrifugation, washed three times to remove the inducers and allowed to recover by overnight growth in LB with antibiotics. This sequence of dilution, growth and induction was repeated and the survivors from the second induction were recovered by plating on LB agar containing Kan and Amp. Individual colonies were gridded onto MacConkey lactose agar containing 1 mM IPTG, 0.2% arabinase, and antibiotics. Many of the surviving colonies grew poorly on this medium or were Lac^- and these were discarded. Initially, almost 4000 survivors were screened that were not subjected to chemical mutagenesis, but none of the survivors passed these initial screens. Clones that exhibited good growth and were strongly Lac^+ were cross-streaked against λΔSR and λ20. Cells that were sensitive to both phages presumably contained defective pQ plasmids and were not analyzed further. Positive candidates (λΔSR- resistant and λ20-sensitive) were tested for their sensitivity to MS2 and a second male-specific phage, M13. Clones that showed complete or partial resistance to MS2 but remained fully sensitive to M13 were designated as *fnd*. In two independent experiments, a total of 3300 survivors were screened for the MS2-resistant

phenotype, resulting in the isolation of 5 (2 from the first screen and 3 from the second) mutants, BMC1-5 (B. McIntosh clones 1-5).

SDS-PAGE and Western blotting

L protein was assessed as described in Chapter II.

Poly(A) polymerase purification and assays

RY15177 was transformed with pCA24N-*pcnB* carrying one of the mutated residues. For each allele of *pcnB,* 200ml of these cells was grown to A_{550} ~0.4, induced with IPTG for one hour and harvested at 4°C for 15 minutes at 11,000 x g. The pellets were resuspended in 7 ml 0.1 M Tris, pH 8.0 and disrupted by passage through a French press. The lysate was subjected to a second centrifugation to remove unlysed cells and cell debris at 4°C (1,000 x g for 10 minutes). Urea was added to the supernatant to a final concentration of 1 M and 5ml was loaded onto a TALON column (ClonTech Laboratories, Inc., Mountain View, CA) and monitored by absorbance at A_{280}. The column was washed with 0.1 M Tris, pH 8.0 until the trace returned to baseline. The column was eluted using a 0 M to 0.5 M gradient of imidazole in 0.1 M Tris, pH 8.0.

To assay pyrophosphate release by the purified His_6-PcnB, 50 µl reactions were prepared containing 33.25 ml purified protein, 2.5 mM $MnCl_2$, 0.25 mM ATP, 1 µg carrier yeast tRNA (Sigma Aldrich, St. Louis, MO), 1 unit of Yeast inorganic pyrophosphatase (New England Biolabs, Ipswich, MA) and 1X PAP buffer (Ambion Inc., Austin, TX). The reaction was incubated at 37°C for 20 minutes. To 10 µl, 1 µl of

100% TCA was added and samples were centrifuged at 18,000 x g for 10 minutes at room temperature. The supernatant was assayed for the presence of phosphate using a Malachite Green/Ammonium molybdate assay (Lanzetta et al., 1979).

Electroporation of MS2 RNA

Electrocompetant cells of each strain indicated were prepared and electroporated as described previously (Sambrook et al., 1989). Cells were electroporated with 5 µg carrier yeast tRNA (Sigma Aldrich, St. Louis, MO.) and 0.05 µg MS2 RNA (described above). 10 µl aliquots were removed, diluted to 100 µl with LB and combined with 500 µl of an indicator strain (RY15177) and plated in H-top agar on LB-agar plates.

Real time PCR

E. coli RNA from 1 ml of cells was purified using a QIAGEN (Valencia, CA) RNeasy purification kit according to the manufacturer's instructions for use as a negative control in the real time PCR. 100 µl of CsCl-purified MS2 phage at a titer of

2 x 10^{13} pfu/ml was prepared using the QIAGEN RNeasy purification kit and used as a positive control for the real time experiments. Concentration of A_{260} was measured using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). Cultures were grown at 37°C to A_{550} ~0.4-0.5, diluted to 0.1 and infected with MS2 at MOI (multiplicity of infection) of 20. At specified times, 1 ml samples of culture were removed and 5 μl of toluene was added to each tube. Samples were kept on ice until processed using the QIAGEN RNeasy purification kit. A_{260} was measured for each sample. Real-time PCR was performed essentially as described by O'Connell et al. (2006), except that the final concentration in each well was 200 ng of total RNA. The primers used were as described by O'Connell et al. (2006) and were MS2 Forward (5' GCTCTGAGAGCGGCTCTATTG), MS2 Reverse (5' CGTTATAGCGGACCGCGT) and MS2 probe (5' CCGAGACCAATGTGCGCCGTG). The probe labeled with the fluorescent moieties FAM at the 5' end and TAMRA at the 3' end was purchased along with TaqMan Universal PCR Master Mix from Applied Biosystems (Foster City, CA).

Results

Selection of BMC1-5 mutants

For the purpose of identifying the target of *L*, a system was established in which mutants that survived induction of a plasmid-borne *L* gene could be selected. Using the two-plasmid system described in Chapter II, survivors of *L* induction were selected by successive two rounds of *L* induction, with an overnight non-selective growth period between the two inductions. A number of screens were undertaken to identify host mutants that conferred resistance to both plasmid-borne expression of L, as well as L from MS2 phage. Following plating for colony forming units, individual clones were screened by the induction of the two-plasmid system on plates. Colonies that showed no growth were presumed to be persisters that had failed to be induced in liquid culture, yet remained sensitive to L, and were discarded. Meanwhile, colonies that were Lac^- were assumed to be host mutants that prevented induction of L using the two-plasmid system. 3300 positive clones were then subjected to a round of screening to test the functionality of each of the two plasmids. After several rounds of selections and screens, twenty-four clones were tested for resistance to the Levivirus, MS2. Each mutant was screened for sensitivity to the filamentous phage M13 (to show the F pilus was intact) and to MS2, which provided an independent source of L. Two clones remained sensitive to M13, while being resistant to MS2, and were designated BMC1 and BMC2 (see below). A second independent selection/screen identified an additional three mutants (BMC3-5).

Genetic mapping of BMC1-5 mutants

Hfr mapping localized the mutations to an early region of the *E. coli* genome between *leuA* (2 min) and *ade* (12 min). P1 mapping showed a co-transduction frequency of about 84% and 81% with a *tonA*::Tn10 allele located at 3.2 min for BMC1 and BMC2, respectively. Surprisingly, the *pcnB* gene, located at 3.5 min and encoding a poly(A) polymerase of *E. coli*, proved to be the site of the mutations. In each mutant, there was a single base change resulting in a missense change to PcnB (Figure 3.1). One of the three mutants from the second independent selection/screen was a duplicate of one isolated in the first experiment, suggesting that this mutational system is not far from saturation. These mutations are located in known catalytic residues or conserved amino acids within a known catalytic domain (Raynal and Carpousis, 1998).

Figure 3.1. Sequence of residues 71-93 of PcnB with the mutations of BMC1-4 indicated above. BMC1 has an aspargine at position 90, BMC2 has a cysteine at position 78, while BMC3 has a serine at position 74 and BMC4 has an aspartate at position 75. BMC2 was isolated twice, as BMC5, also.

To ensure the plasmids in the mutants were functional, plasmids were isolated from each and transformed into RY15177. In all cases, induction of *L* resulted in normal lysis of the isogenic parental strain (RY15177), indicating there were no mutations

affecting the plasmids (data not shown). The plasmids were cured from the mutant strains using treatment with novobiocin by the method described by Hooper et al. (1984).

The BMC1-5 alleles of pcnB *are dominant with respect to sensitivity to MS2*

We obtained pCA24N-*pcnB* from the ASKA clone collection (Kitagawa et al., 2005) and tested the lysis phenotype of MS2 in the presence of multiple, extrachromosomal copies of *pcnB*. pCA24N-*pcnB* was transformed into HfrH strains with either wild-type *pcnB*, a *pcnB* null allele (*pcnB*::Tn*10*), BMC1 or BMC2. As shown in Figure 3.2A, MS2 is able to cause lysis of the *pcnB*$^+$ strain as well *pcnB*::Tn*10*. On the other hand, the mutants are resistant to lysis by the phage, indicating that the mutations are dominant.

The mutant alleles of pcnB *remain sensitive to L-mediated lysis*

BMC1 and BMC2 alleles of *pcnB* were tested for dominance over of the plasmid-borne L, which were expected to be dominant (similar to the result obtained from the phage infections). Surprisingly, in the presence of plasmid-borne *pcnB*, the mutants were sensitive to L (Figure 3.2B). *pcnB* was originally identified as a locus required for maintaining plasmid copy number of ColE1 plasmids (Liu and Parkinson, 1989). The copy number of ColE1 and other plasmids is controlled by small RNAs transcribed from the origin region. In *pcnB*$^+$ cells, this antisense RNA is modified with a poly(A) tail by PcnB, signaling its degradation. However, in *pcnB*$^-$ strains, this antisense

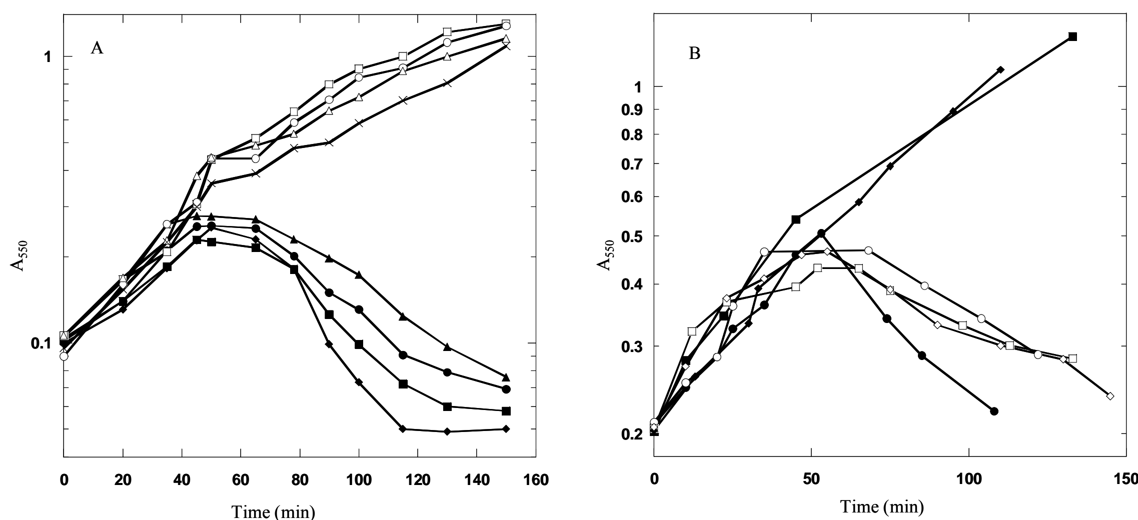

Figure 3.2. Lysis profiles of *pcnB* alleles are different for MS2 infected cells compared to plasmid-borne *L* in the presence of PcnB supplied in trans. (A) Infection of *pcnB* alleles with MS2 at MOI 5 at time 0. ●, *pcnB*$^{+}$, vector; ■, *pcnB*$^{+}$, pCA24N-*pcnB*; ♦, *pcnB*$^{-}$, vector; ▲, *pcnB*$^{-}$, pCA24N-*pcnB*; □, BMC1, vector; ○, BMC1, pCA24N-*pcnB*; Δ, BMC2, vector; x, BMC2, pCA24N-*pcnB*. (B) Induction of plasmid-borne *L* using IPTG (final concentration of 1mM) at time 0. ●, *pcnB*$^{+}$, vector; ○, pcnB^{+}, pCA24N-*pcnB*; ■, BMC1, vector; □, BMC1, pCA24N-*pcnB*; ♦, BMC2, vector; ◊, BMC2, pCA24N-*pcnB*;

RNA is neither modified nor degraded, resulting in severely reduced replication of the plasmid (Liu & Parkinson, 1989). Plasmid DNA isolated and analyzed by gel electrophoresis confirmed that the mutant alleles of *pcnB* exhibited a reduced plasmid copy number phenotype. When PcnB was provided in trans on a plasmid, the copy number of a ColE1 plasmid was restored (Figure 3.3A). Additionally, purified PcnB was assayed for pyrophosphate release. As shown in Figure 3.3B, the mutants were reduced in the ability to release pyrophosphate, as was a previously characterized catalytically inactive mutant, D90A, compared to wild-type activity (Raynal and Carpousis, 1999). The activity of BMC2 was reduced to about 40%, whereas BCM2 and PcnB^{D90A} had

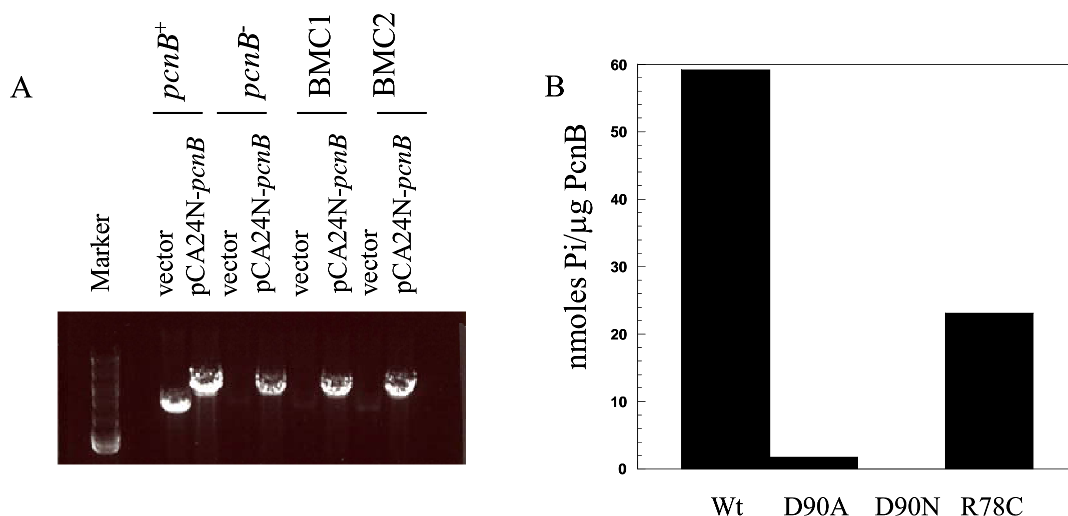

Figure 3.3. The mutations in PcnB result in dramatically reduced poly(A) polymerase activity. (A) Plasmid DNA isolated from the indicated strains was analyzed on a 0.7% agarose gel. (B) Poly(A) polymerase activity was measured in a coupled assay using inorganic pyrophosphatase. The phosphate released was measured a standard colorimetric assay described in Materials and Methods.

essentially no activity. It was concluded that the mutations in PcnB affected a different step in the phage life cycle other than lysis mediated by L, since restoration of the copy number of the L plasmid in the mutants resulted in lysis. PcnB had not previously been characterized for its involvement in any phage life cycle. This is expected since the knockout of *pcnB* maintained the same phenotype as the wild-type. This suggests that MS2 is wholly able to infect, replicate in and lyse cells with either the presence of the wild-type protein or cells that are null for PcnB. According to this perspective, the presence of the catalytically inactive PcnB protein was detrimental to the life cycle of MS2, though not the lysis step. This leaves several steps in the infection cycle, including binding, injection of RNA into the cell, and replication of the progeny phage.

The alleles of *pcnB* were tested for a decreased ability to adsorb phage, but no difference was found (data not shown).

The appearance of the L protein and progeny phage is reduced/delayed in MS2-infected BMC1 and BMC2

To test if the *pcnB* mutants were capable of accumulating L protein, hosts carrying the indicated alleles of *pcnB* were infected with MS2 at MOI (multiplicity of infection) of 1 and samples were collected and analyzed. As shown in Figure 3.4, the accumulation of the L protein is significantly reduced and/or delayed in the mutant hosts.

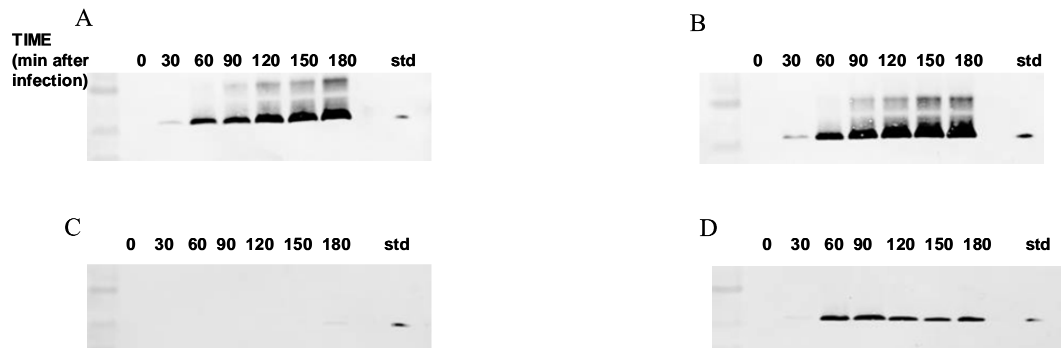

Figure 3.4. Accumulation of L protein was significantly reduced in the mutant alleles of *pcnB*. Cultures of the indicated alleles were infected at an MOI of 5. Samples were collected at the indicated times and TCA precipitated. Samples, normalized for total protein, were analyzed by Western blot with anti-L antibodies. A standard amount of L was loaded into the last lane of each gel (std).

To demonstrate whether and when functional progeny phage were produced in the mutants, hosts carrying the indicated alleles of *pcnB* were infected with MS2 and

total phage production was assayed (Figure 3.5). Over the course of infection, the appearance of progeny phage was greatly delayed in the mutants. These results could be

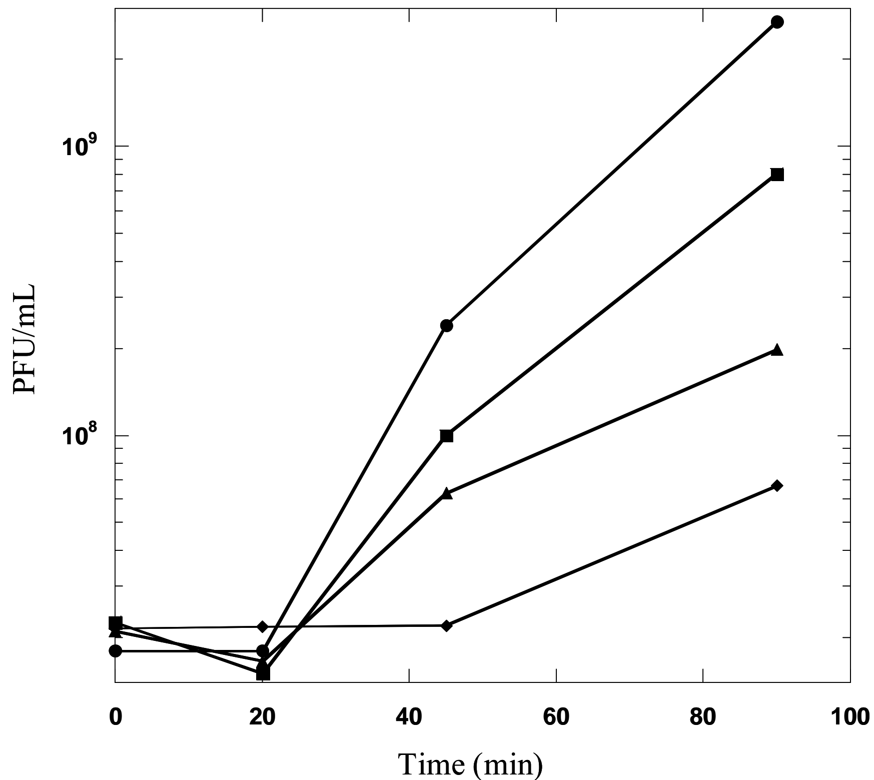

Figure 3.5. PcnB mutants accumulate MS2 with delayed kinetics compared to *pcnB*$^+$ and *pcnB*::Tn10. Infections of strains with each allele were performed at an MOI of 1. 1 ml samples were removed at the indicated times and 1% chloroform added to prevent further growth. The samples were then diluted with LB to 4 ml total, and subjected to French Press. Samples were titered for total pfu/mL on lawns of RY15177. ●, *pcnB*$^+$; ■, *pcnB*::Tn10; ♦, BMC1; ▲, BMC2.

explained in several ways. First, the mutants might allow normal injection of the RNA into the cell, but prevent replication of the phage. In this way, there would only be a small amount of L produced from the few genomes replicated in each cell, which would

be insufficient to result in lysis. Alternatively, the injection process could be affected in the mutants and result in an inability to uniformly infect all of the cells. If this was the case, some of the cells might be infected normally and accumulate a adequate amount of L to result in lysis, but the fraction of cells in which this occurs is not sufficient to be seen in the lysis curves.

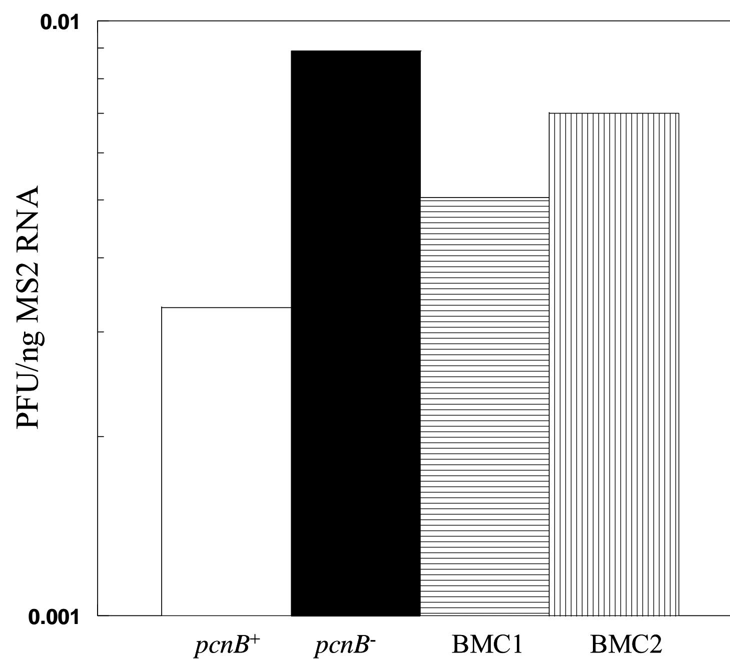

Figure 3.6. Electroporation of MS2 RNA into PcnB mutants results in normal plaque formation. 5 µg of carrier tRNA and 0.05 µg of MS2 RNA were electroporated into 40 µl aliquots of each indicated strain. Mixtures were diluted into an indicator strain, RY15177, and plated on LB plates using H-top. Plates were grown overnight at 37°C and plaques were counted.

The replication of the MS2 genome is delayed in MS2-infected BMC1 and BMC2 hosts

In an attempt to identify the step in the MS2 infection cycle affected by the mutant *pcnB* alleles, real time PCR was performed to quantify the amount of MS2

molecules over the course of an MS2 infection. A delay was seen for the BMC2 mutant, while the overall quantity of molecules or genomes does not significantly increase in the BMC1 mutant (Figure 3.6). This result was consistent with the delayed or impaired L protein and phage production.

The cytoplasm of BMC1 and BMC2 is permissive for MS2 replication

To test for the ability of the cytoplasm of BMC1 and BMC2 to allow for MS2 replication and lysis, MS2 RNA was electroporated into the cytoplasm directly. By mixing these cells with a sensitive indicator strain before plating, the number of infectious centers was assayed and normalized to each strain's transformation efficiency Electroporation of MS2 RNA into host cells bypasses early steps in the infection process (phage adsorption, RNA ejection from the capsid, RNA entry into the cytosol). As shown in Figure 3.7, the ability of BMC1 and BMC2 to form infectious centers was not significantly altered compared with the wild-type or knockout of *pcnB*. This suggests that the cytoplasm of the mutant *pcnB* hosts is normal for supporting MS2 replication and subsequent lysis. Furthermore, this suggests that the impairment of the mutant *pcnB* alleles on MS2 is restricted to the injection process. Since PcnB is a cytoplasmically localized protein, these results suggest that the step of injection that is being impaired is the actual entry of the RNA into the cell, rather than its fate from the release of the capsid and migration to the surface of the cell.

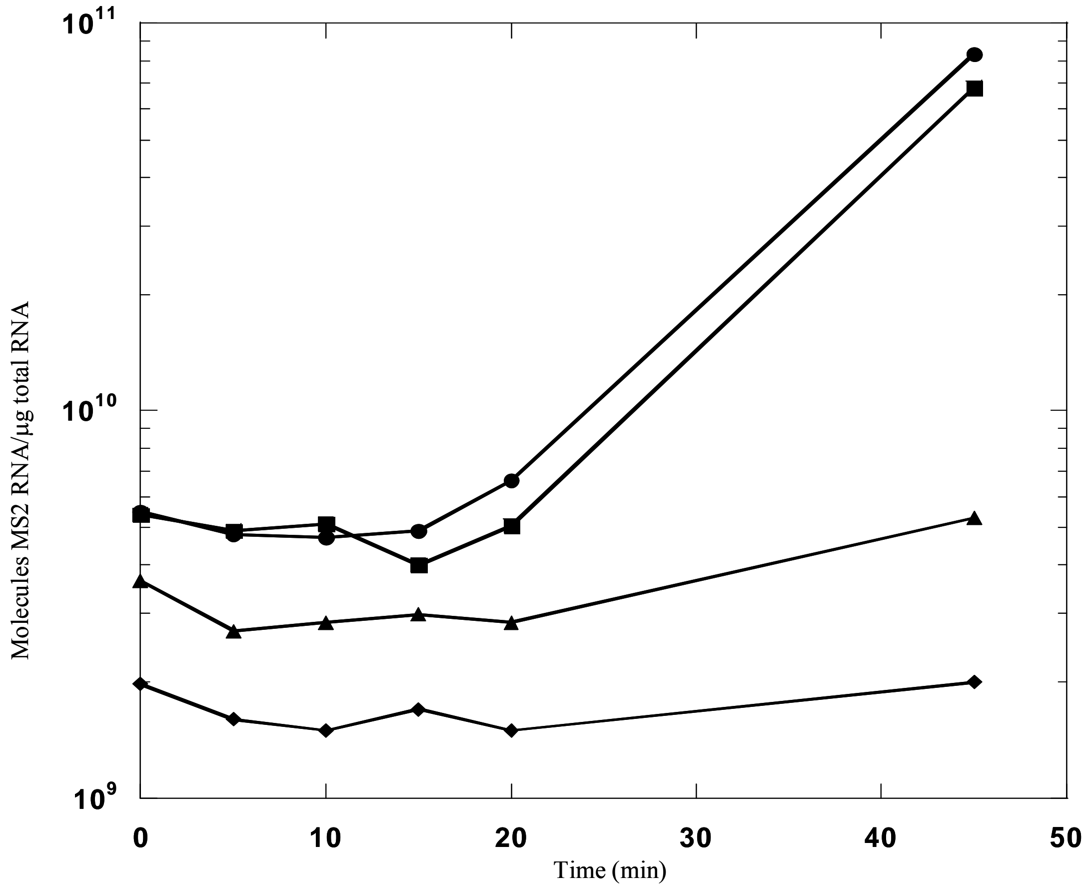

Figure 3.7. Real time PCR indicates the *pcnB* alleles are deficient in production of MS2 RNA molecules. Using an MOI of 20, 1 ml samples were removed at indicated times and 5 μl toluene added to each sample. RNA was isolated using RNeasy kit and concentrations were measured using a NanoDrop spectrometer and 500 ng of total RNA added to real time PCR. ●, *pcnB*$^{+}$; ■, *pcnB*::Tn10; ♦, BMC1; ▲, BMC2.

The mutant alleles of pcnB *affect the plating efficiency of numerous F-specific phage*

The ability to complete the life cycle and form plaques of GA, a ssRNA bacteriophage related to MS2, was also tested on the *pcnB* mutant strains. GA had a similar, noticeable reduction in plating efficiency, as did Qβ, the prototypic type III ssRNA bacteriophage. The use of the filamentous ssDNA phage, M13, in the original

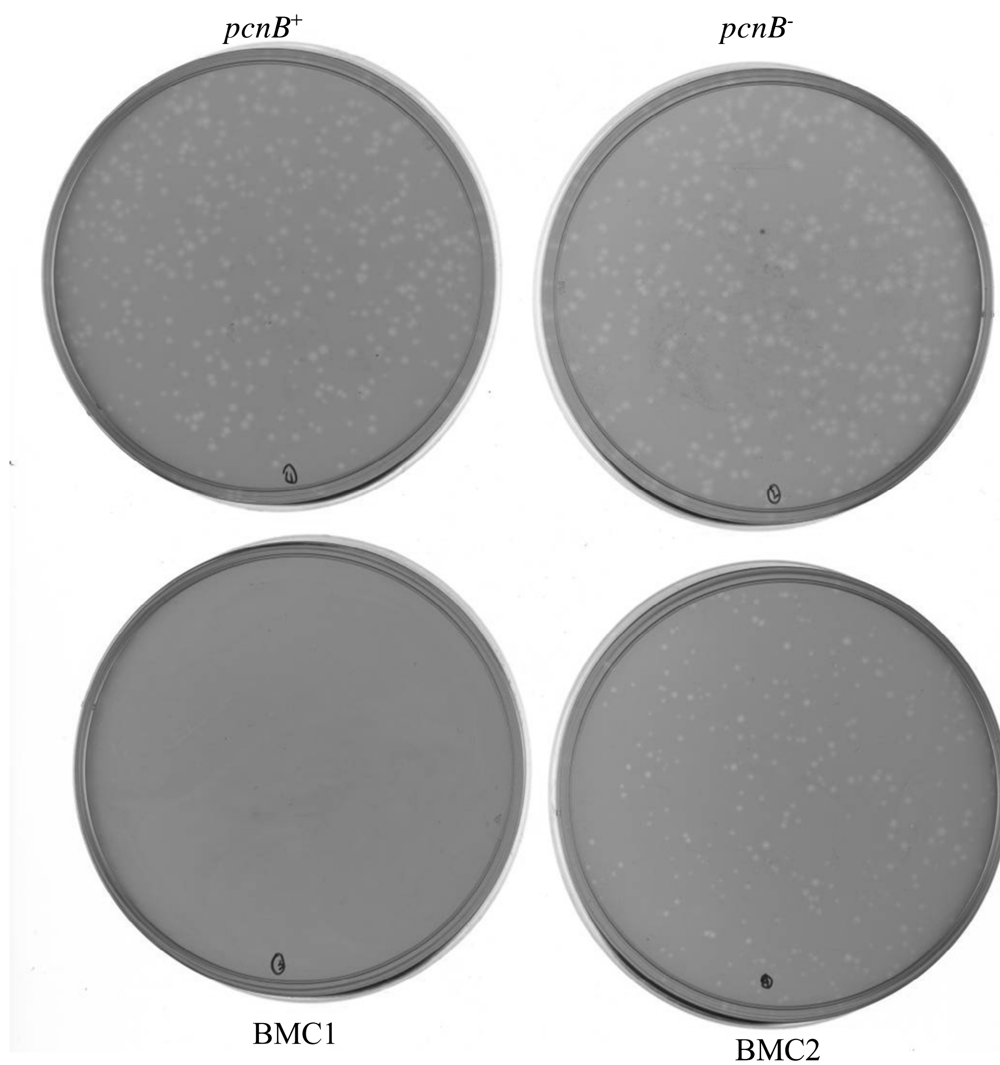

Figure 3.8. Plaque morphology of M13 plated on strains carrying indicated alleles of *pcnB*. 100 µl of M13 phage (~100 pfu) were mixed with 500 µl of each culture before plating in H-top on LB plates.

screen was not quantitative, but plating of serial dilutions revealed M13 displayed an altered plating efficiency (Figure 3.8). Thus, the plating phenotype exhibited by the mutant *pcnB* alleles is general for F-specific bacteriophages. The plating efficiency of M13 on BMC2, which retained about 40% of it poly(A) polymerase activity, is not significantly decreased, but the plaques formed by M13 are significantly more opaque. On the other hand, the efficiency of plating of M13 on the *pcnB* allele with no catalytic activity (BMC1) was not determined since the plaques were not easily perceptible. The mutants were also tested for their capacity to transfer F DNA to a recipient under standard mating conditions. The transfer of a selectable marker was reduced by several orders of magnitude in the presence of the mutated *pcnB* alleles, with the BMC1 mutants being the most severely affected. This is taken to mean that the ability of these strains to permit the early stages of infection, including the transfer of nucleic acids, is diminished. The BMC2 mutant, which retains about 40% of its poly(A) polymerase activity, is the less severe of the two mutants characterized here. This is shown by its greater capacity to support phage infection compared to the inactive BMC1 mutant.

Discusssion

The original goal of this study was to uncover the molecular target of the lysis protein, L. However, the mutants that were isolated as survivors of *L* induction were found to be missense alleles of *pcnB*, the major poly(A) polymerase of *E. coli.* Moreover, these mutants were found to be fully sensitive to *L*-mediated lysis and, in fact, completely permissive for MS2 replication and morphogenesis. It is important to note that undoubtedly many *pcnB* knockout alleles were also selected as survivors, because, like the BMC1-5 alleles, the lethal plasmid number would be reduced to one or less per cell (Liu and Parkinson, 1989). However, these were eliminated from further evaluation because they retained full sensitivity to MS2, which does not require *pcnB*. The specific missense changes in *pcnB* can give rise not only to reduce plasmid copy number, thus sparing the cell from *L* lethality, but also to severely reduce the ability of MS2 to plate on the mutant strains. It is the latter phenomenon that makes these particular *pcnB* mutants significant, because they establish a new genetic requirement for levivirus and filamentous phage growth. No new host requirement for the life cycle of these paradigm phages have been identified for more than 20 years.

What can be deduced about the role of PcnB in the levivirus phage infection cycle? The data presented above indicate that PcnB is involved in MS2 RNA entry into the pilus or cytoplasm. It is unclear whether the effect of the PcnB in the lifecycle of ssRNA and ssDNA phage is direct or indirect. Since PcnB is a cytoplasmically localized protein, a direct effect would only be achieved on the RNA entry in the cytoplasm. This could explain why the mutants can replicate in and lyse host cells, but are deficient in the

early steps of RNA phage infection. On the other hand, the presence of the inactive protein could have an indirect effect on the F pilus, though this is much less likely. The pilus is still capable of adsorbing phage, which suggests that PcnB is not involved in expression or stability of the F pilus. The exact mechanism through which the ssRNA and ssDNA genomes are transported down the pilus to the cell surface and enter into the cytoplasm remains a mystery, but it appears that PcnB may have a role in these steps of phage infection.

CHAPTER IV

BIOCHEMICAL CHARACTERIZATION OF THE MS2 L PROTEIN

Introduction

As part of their life cycle, bacteriophages must have a mechanism that allows for timely release of progeny from their bacterial hosts. To fail in this process means a failure of the infection, as progeny remain trapped within the cell. Bacteriophages have evolved two mechanisms for this last step, either extruding the progeny phage without killing the host or by causing host lysis. ssDNA filamentous phage, such as M13 and Ff, secrete completed progeny without killing their host. All other bacteriophages, with ssDNA, ssRNA and dsDNA genomes, encode one or more proteins that bring about lysis by interfering with the integrity of the peptidoglycan cell wall. dsDNA phages encode one or more proteins that actively degrade the murein layer, whereas ssDNA and ssRNA phages do not encode a muralytic enzyme (Eigner et al., 1963; Robertson, 1975). These small single-stranded genome phages were long known to each encode a single protein for lysis (Coleman et al., 1983; Heinrich et al., 1982; Karnik and Billeter, 1983; Winter and Gold, 1983; Young and Young, 1982), but the mechanism through which these lysis proteins functioned was the subject of wide debate.

For many years, there were two different hypotheses to explain the mechanism of lysis mediated used by the small bacteriophages. According to one scheme, the lysis proteins from these phage activated an autolytic system of *E. coli*. Lubitz and co-workers first proposed this model after following [^{3}H]-diaminopimilic acid (DAP)

incorporation into the murein layer (Lubitz and Plapp, 1980). DAP is a component of the pentapeptide side chain and is also a precursor in lysine synthesis. Thus, incorporation of DAP into peptidoglycan can be monitored in a *lysA* mutant grown in minimal medium supplemented with lysine. This mutant is incapable of utilizing DAP for lysine synthesis, so the radiolabeled DAP will only be incorporated into the cell wall. The radiolabeled amino acid in the side chain of the peptidoglycan can be detected in the insoluble fraction after boiling cells in sodium dodecyl sulfate (SDS) followed by a brief centrifugation, since all of the precursors are soluble. Based on this premise, the expression of E was induced in a *lysA* strain and radiolabeled DAP incorporation was measured (Lubitz and Plapp, 1980). Initially after induction of *E* expression, the incorporation of DAP occurred normally. After about 10-15 minutes, DAP incorporation was inhibited, even though lysis did not begin for at least another 10 minutes. The level of radiolabeled substrate in the cell wall did not remain static after E prevented further incorporation at 10-15 minutes after induction, rather, the amount of radiolabel decreased until the onset of lysis (Lubitz and Plapp, 1980). Lubitz and co-workers noticed this phenomenon and proposed that autolytic enzymes, which are the cell's murein hydrolyzing enzymes, were responsible for degrading the peptidoglycan (Lubitz and Plapp, 1980). Lubtiz and co-workers went on to show that E-mediated lysis was impaired in hosts with mutations in autolytic enzymes (Lubitz et al., 1984). However, the authors made no attempt to quantify the expression levels of E during their experiments, nor compare the expression of E from their plasmid-based system to that of ϕX174. Later, using electron microscopy, Lubitz and co-workers reported that the E

protein of the ssDNA phage, ϕX174, formed "transmembrane tunnels" that spanned the cellular envelope. These structures were proposed to be the trigger for induction of autolysis, or, alternatively, even the portal for release of phage (Witte et al., 1992; Witte et al., 1990). Incredibly, an entire industry has been established based on bacterial ghosts (caused by transmembrane tunnels produced by E) as a potential drug delivery system (Jalava et al., 2003; Kudela et al., 2007; Paukner et al., 2003). Using the same approach, other groups claimed that the L protein of the ssRNA phage, MS2, accumulated at membrane adhesion sites (Walderich et al., 1988), or Bayer patches (Bayer, 1968), where the inner and outer membranes came into contact. These Bayer patches were shown to contain L using immunolabeling and EM and were suggested to be the trigger for activation of autolytic enzymes (Walderich and Holtje, 1989).

A second hypothesis had its origins in the work of Ozaki and Valentine (1973). These authors found that the incorporation of [^{3}H]-DAP into peptidoglycan was significantly inhibited about 30-40 minutes after infection by the ssRNA phage Qβ while the synthesis of other macromoleucles was relatively unaffected. These authors noted that in this respect, lysis by Qβ was similar to lysis caused by penicillin and suggested that "attention should be given to viral proteins not as lysozymes but as inhibitors of essential steps in cell wall mucopeptide biosynthesis" (Ozaki and Valentine, 1973).

Roughly thirty years after Valentine's original prediction, the cellular targets of E and A_2 were identified as the enzymes MraY and MurA, respectively, which are both involved in cytoplasmic steps in peptidoglycan biosynthesis (Bernhardt et al., 2001a, 2001b). The protein targets of E and A_2 were identified using simple, yet elegant, genetic selections and screens (Bernhardt et al., 2000, 2001b). As recounted in Chapter III, I attempted to identify the target of L, the lysis protein of phage MS2, using a similar, genetic approach. These studies identified the *pcnB* gene as being a potential target of L based on selection for resistance to expression of the L protein. However, further analysis revealed that the PcnB protein is involved in the process of MS2 infection rather than in host cell lysis. For this reason, I initiated a biochemical analysis of the L protein. These studies have shown that L is not a conventional integral membrane protein, but rather is localized to the periplasm and self associates to form oligomers.

Materials and methods

Bacterial strains, bacteriophages, plasmids and culture growth

The bacterial strains, plasmids, bacteriophages used in this work were described in Chapter II or were CS109 (W1485 F^- *rpoS rph*) (Denome et al., 1999), CS315-1 (CS109 *dacA*::Res512-1 *pbpG*::Res501-1 *dacB*::Res516-2), CS802-2 (CS109 *ampC*::Res459-2 *dacC*::Res520-1 *pbpG*::Res501-1 *ampH*::Res480-2 *dacA*::Res512-1 *dacB*::Res516-2 *mrcB*::Res461-3 *dacD*::Res536-2), CS804-1 (CS109 Δ*mrcA*::Res1-862 *ampC*::Res459-2 *dacC*::Res520-1 *pbpG*::Res501-1 *ampH*::Res480-2 *dacA*::Res512-1 *dacB*::Res516-2 *dacD*::Kan532-6) (Denome et al., 1999). Conditions were as described in Chapter II.

Standard DNA manipulations, PCR and sequencing

Plasmid DNA isolation, PCR, DNA transformation and sequencing were performed as previously described (Xu et al., 2004). Primers were purchased from Integrated DNA Technologies, Coralville, IA. Single-base changes were introduced using the QuickChange kit according to standard protocols with commercially available primers (Stratagene Corp, La Jolla, CA). Ligations were performed according the the manufacturer's instructions (Roche Molecular Biochemicals, Pleasanton, CA). All other

enzymes were purchased from New England Biolabs (Ipswich, MA), except *Pfu* (Stratagene Corp., La Jolla, CA). Automated fluorescent sequencing was performed at the Laboratory for Plant Genome Technology at the Texas Agricultural Experiment Station.

SDS-PAGE and Western blotting

SDS-PAGE and Western blotting was performed essentially as described previously (Bernhardt et al., 2002). For detection of L, anti-L was used, which was synthesized against the peptide 5'-TPASTNRRRPFHEDYP-3' in rabbit. For detection of the cmyc tag, cmyc-specific antibodies were used (Covance, Princeton, NJ). Detection of the His tag was performed using α-His (Sigma Aldrich, St. Louis, MO). For detection of biotinylated proteins, streptavidin-conjugated horseradish peroxidase was used according to the manufacturer's instructions (Amersham Biosciences, Piscataway, NJ). Colormetric development of Western blots was performed as described in Chapter II, while chemiluminescent development was performed according to the manufacturer's instructions (Pierce Biotechnology, Inc., Rockford, IL).

Construction of R^{S141C}-cmyc

The addition of an exposed cysteine residue to R-cmyc was performed using the primers lbd R S141C For (5'-

CGAGCATAAGGCTGACTGCCTGATTGCAAAATTCAAAGAAGCG) and lbd R S141C Rev (5'-CGCTTCTTTGAATTTTGCAATCAGGCAGTCAGCCTTATGCTCG) as described above. To verify the presence of the cysteine at position 141, sequencing was performed as described above using the primer pZE12 For (5'-GTGAGCGGATAACAAATTGACA).

Addition of epitope tags to the 5' end of the L *gene*

Primers are listed in Table 4.1.

Table 4.1. Primers used for addition of epitope tags to *L*.

Primer	Sequence
Long cmyc L For	5' TTCAAGCCGGAGTAGAAGATGGAGCAGAAACTGATCTCTGAAGAAGATCTGGAAACCCGATTCCCTCAGCAA 3'
Long cmyc L Rev	5' TTGCTGAGGGAATCGGGTTTCCAGATCTTCTTCAGAGATCAGTTTCTGCTCCATCTTCTACTCCGGCTTGAA 3'
Short cmycL For	5' TTCAAACCGGAGTAGAAG 3'
Short cmyc L Rev	5' TTGCTGAGGGAATCGGGT 3'
BamHI pETDUET L For	5' ATATATATGGATCCGGAAACCCGATTCCCTCAGCAA 3'
HindIII pETDUET L Rev	5' ATATATATAAGCTTGGATGCTTTGTGAGCAATTCG 3'

Cmyc (5' EQKLISEEDL) was added to the N-terminus of L using a modified version of a standard QuickChange method (Stratagene Corp., La Jolla, CA). Briefly, two separate reactions were set up according to the manufacturer's instructions, except that each reaction only contained one of the long cmyc L primers (Forward or Reverse). These reaction mixtures were then combined and a second round of PCR was performed using the respective short cmycL primers and subsequent products treated as per standard protocols (Stratagene, La Jolla, CA). Clones with verified by automated fluorescent sequencing using the *pR'* Seq For primer (Table 2.1). The addition of the His tag was accomplished by cloning the L gene into BamHI/HindIII digested the pETDUET vector (Novagen, Gibbstown, NJ). This resulted in the fusion of the residues MGSHHHHHHSQDP to the N-terminus of L beginning at the second amino acid of L (Figure 4.1). The DNA fragment containing the His_6-L fusion and the upstream Shine-Dalgarno sequence was excised using XbaI and NotI, treated with the Klenow fragment of DNA polymerase and cloned into the pRE vector digested with SmaI. Clones with His_6-L in the correct orientation were identified by DNA sequencing using the *pR'* Seq For primer (Table 2.1).

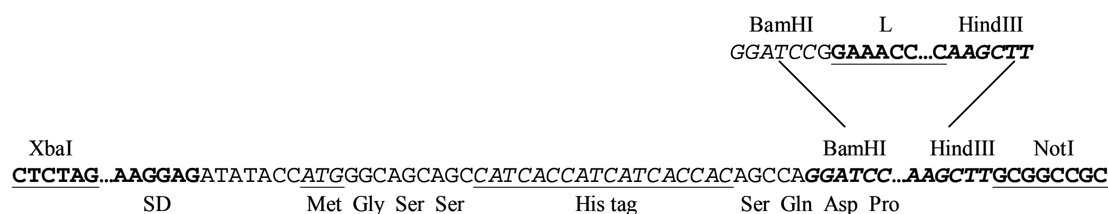

Figure 4.1. Schematic of the construction of His_6-L. Initially, *L* was cloned into pETDUET (Novagen, Gibbstown, NJ) using BamHI and HindIII (italics). One additional nucleotide was added in front of the L sequence to complete the codon for the proline residue. The Shine-Dalgarno (SD) sequence is in bold, while the start codon and His tag are underlined and italicized. XbaI and NotI sites are underlined and were used to remove His_6-L from pETDUET for cloning into pRE.

Purification of His_6-L

Six liters of RY15177 carrying pQ and either pRE-His_6-L or pRE- His_6-L^{S49C} were grown to an A_{550} ~0.6 and induced with 1 mM IPTG. After 30 minutes of induction, cultures were chilled on ice and harvested by centrifugation at 11,000 x g for 15 minutes. The cell pellets were resuspended in 40 ml of 25 mM Tris, pH 8.0, 150 mM NaCl, 1 mM PMSF and disrupted by passage through a French pressure cell (Spectronic Instruments, Rochester, NY) at 16,000 psi (pounds per square inch). The supernatants

obtained by a ten minute centrifugation at 1,000 x g were then subjected to centrifugation at 100,000 x g for 1 hour at 4°C to collect a total membrane fraction. The membrane pellets were extracted overnight at 4°C in 25 mM Tris, pH 8.0, 150 mM NaCl and 1% Empigen BB (Sigma Aldrich, St. Louis, MO) and subjected to a second centrifugation at 100,000 x g for 1 hour at 4°C. The supernatant from this centrifugation was purified by immobilized metal affinity chromatography on a 5 ml TALON (ClonTech, Mountain View, CA) column. Bound proteins were eluted with a 0 to 0.5 M imidazole gradient in 25 mM Tris, pH 8.0, 150 mM NaCl, 1% Empigen BB. The L containing fractions appeared at the beginning of the gradient.

L topology prediction

Topology prediction was analyzed using the following servers: TatP, http://www.cbs.dtu.dk/services/TatP-1.0; TMPred, http://www.ch.embnet.org/cgi-bin/TMPRED; PHDhtm, http://cubic.bioc.columbia.edu; TopPred, http://bioweb.pasteur.fr/seqanal/tmp/toppred; TMHMM, http://www.cbs.dtu.dk/cgi-bin/nph; HMMTOP, http://www.enzim.hu/hmmtop/servfer/hmmtop.cgi; and Dense Alignment Surface (DAS), http://www.sbc.cu.se/~miklos/DAS/tmdas.cgi.

Chemical cross-linking of L

300 ml cultures of RY15177 transformed with the indicated plasmids were grown to A_{550} ~0.4 and induced for 30-35 minutes with 1 mM IPTG. The culture was harvested at 11,000 x g for 15 minutes at 4°C, resuspended in 3.5 ml PBS (phosphate buffered saline), pH 7.2, 1 mM PMSF and disrupted by passage through a French pressure cell as described above. The membranes were collected on a cushion of 55% sucrose, 5 mM EDTA, pH 7.5 overlaid with 20% sucrose, 5 mM EDTA, pH 7.5 by centrifugation at 26,000 rpm at 4°C for 3 hours in an SW-28 rotor. Membranes were recovered by puncturing the tube, extracting the membrane cushion with an 18-guage needle and syringe. The samples were subjected to chemical crosslinking with the amino-directed homobifunctional reagent dithiobis (succinimidyl propionate) (DSP) as previously described (Grundling et al., 2000). Briefly, 60 µl of membrane sample prepared above was removed and treated with 6 µl of dimethylsulfoxide as a negative control. 440 µl of membrane sample was treated with freshly prepared 100 mM DSP in dimethylsulfoxide at a final concentration of 16 mM. Immediately upon addition, a 60 µl sample was removed and quenched with the addition of glycine to a final concentration of 100 mM, representing the 1 minute sample. At the other times indicated, 60 µl samples were withdrawn and quenched with glycine. Samples were

mixed with 2X sample loading buffer devoid of reducing agents (Sambrook et al., 1989) and analyzed by SDS-PAGE and Western blotting using α-His as described above.

Alternatively, samples were crosslinked with the heterobifunctional reagent, 4-maleimidobenzophenone (MBP) (Invitrogen, Carlsbad, CA). A 25 ml culture of RY15194 carrying the desired plasmids was grown to A_{550} of 0.2, induced with 1 mM IPTG for 35 minutes, harvested by centrifugation and washed once with PBS, pH 7.2. The cell pellet was resuspended in 1 ml PBS, pH 7.2, 2 mM EDTA, pH 8.0 and 1 mM PMSF and disrupted by passage through a French pressure cell. Large debris was removed by a 5 minute centrifugation at 1,000 x g at 4°C. 100 μl of the supernatant was pipetted into a sterile Petri dish (15 mm diameter per well, 24 wells per dish) and 1 μl of 100 mM MBP prepared in dimethylformamide, or 1 μl of dimethylformamide for the mock treatment, was added. The mixture was incubated at room temperature for 30 minutes in subdued light after which 1 μl 1 M DTT was added to quench unreacted MBP. The samples were placed on ice and irradiated with 366 nm light using a hand held lamp for 5, 10 or 20 minutes. Samples were mixed with 2X sample loading buffer (Sambrook et al., 1989) and analyzed by SDS-PAGE and Western blotting using α-L.

To demonstrate that the L protein in the crude cell lysates was modified with MBP, duplicate samples were precipitated with TCA (trichloroacetic acid), washed once

with acetone and allowed to air dry. The pellet was resuspended in 1 ml of 0.5 M Tris, pH 7.0, 1% SDS, 1 mM EDTA and 10 μl 60 mM PEG-OPSS (methoxy- polyethylene glycol-bis(ortho-pyridyldisulfide)) (Creative Chemlabs, Winston-Salem, NC) was added and incubated at room temperature for 30 minutes. 1.4 ml cold acetone was added and the sample incubated at -20°C for 10 minutes. Precipitated protein was collected by centrifugation at 18,000 x g for 15 minutes at 4°C and the pellet briefly air dried before resuspension in non-reducing sample loading buffer for analysis by SDS-PAGE and Western blotting using α-L.

Biotinylation of proteins that interact with L

In an alternative approach to identify proteins that interact with L, host membrane proteins were reacted with a sulfhydryl-modifying biotin labeling reagent, (Maleimide-polyethylene oxide-Biotin) (Pierce, Rockford, IL). A 20 mM stock solution of Maleimide-PEO$_2$-Biotin was prepared in dimethylformamide. 150 μl of the membrane cushion (described above) of the indicated strain was incubated with 1.76 μl 20 mM Maleimide-PEO$_2$-Biotin for 2 hours or overnight on ice, or mock treated with dimethylformamide. 1 M DTT to quench the unreacted reagent was added to a final concentration of 1 mM and the proteins were then immunoprecipitated.

Before immunoprecipitation, a 10 µl aliquot of the 150 µl of Maleimide-PEO_2-Biotin labeled membrane sample was removed, 2X sample loading buffer was added. This sample was analyzed using streptavidin-conjugated horseradish peroxidase as described above, representing a sample that was not subjected to immunoprecipitation. To the remaining sample that was to be immunoprecipitated, Empigen BB was added to a final concentration of 1% to solubilize the membranes. Samples were then brought up to a final volume of 0.5 ml with lysis buffer (0.1 M Na_2PO_4, 0.15 M NaCl, pH 7.2, 1% NP-40), with NP-40 purchased from USB (Cleveland, OH). 5 µl of α-L was added to the sample and incubated on ice for 1 hour. Meanwhile, *S. aureus* (Sigma Aldrich, St. Louis, MO) were prepared as previously described by washing twice in lysis buffer (Harlow and Lane, 1999). 50 µl of *S. aureus* were added to the sample-α-L mixture, incubated on ice for 30 minutes and centrifuged at 10,000 x g for 1 minute at 4^oC. The supernatant was removed by aspiration and the pellet was washed four times with lysis buffer. Samples were resuspended using 2X sample loading buffer and analyzed as described above using α-L antibodies or streptavidin-conjugated horseradish peroxidase.

Subcellular localization of L by MTSES accessibility

MTSES (sodium (2-sulfonatoethyl) methanethiosulfonate) (Anatrace, Maumee, OH) protection was done essentially as described (Guan and Kaback, 2007). Cultures of

cells carrying the desired plasmids were grown to A_{550} ~0.6 and induced with IPTG for 35 minutes. At that time, separate 500 μl and 2 ml aliquots were withdrawn from each culture and harvested by centrifugation. Both samples were washed once with 0.1 M KH_2PO_4 buffer, pH 7.0, and resuspended to their original volume in the same buffer. The 2 ml sample was lysed by passage through a French pressure cell and a 500 μl aliquot was removed for MTSES modification. EDTA was added to both the intact and broken cell samples to a final concentration of 2 mM. Each sample was subdivided into four 130 μl aliquots. 13 μl 1 M MTSES was added to two of these aliquots while 13 μl of water was added to the other two. After 30 minutes at room temperature, the samples subjected were precipitated with a mixture of MeOH and $CHCl_3$ as described in Chapter II. The samples were each resuspended in 100 μl of buffer containing 10 M urea, 1% SDS, 1 mM EDTA. 50 μl of an MTSES-labeled and an unlabeled sample was then reacted with 10 μl of 1.2 mM PEG-Mal (methoxy-Polyethylene glycol-maleimide) (Creative Chemlabs, Winston-Salem, NC) for 30 minutes at room temperature. Samples were precipitated using 1 ml cold 100% EtOH, chilled at -20°C for at least one hour and centrifuged at 13,000 x g for 15 minutes. Samples were allowed to air dry and were then resuspended in 15 μl sample loading buffer (Sambrook et al., 1989) and analyzed by SDS-PAGE and Western blotting.

Results

L is predicted to be an inner membrane protein with a single transmembrane domain

Since L fractionates with membranes, the sequence of L was used to predict the topology of the protein using multiple web-available programs. According to all of the transmembrane domain prediction programs, L contains a single TMD with an N-terminus inside, C-terminus outside topology (Figure 4.2). Von Heinje (1995) described

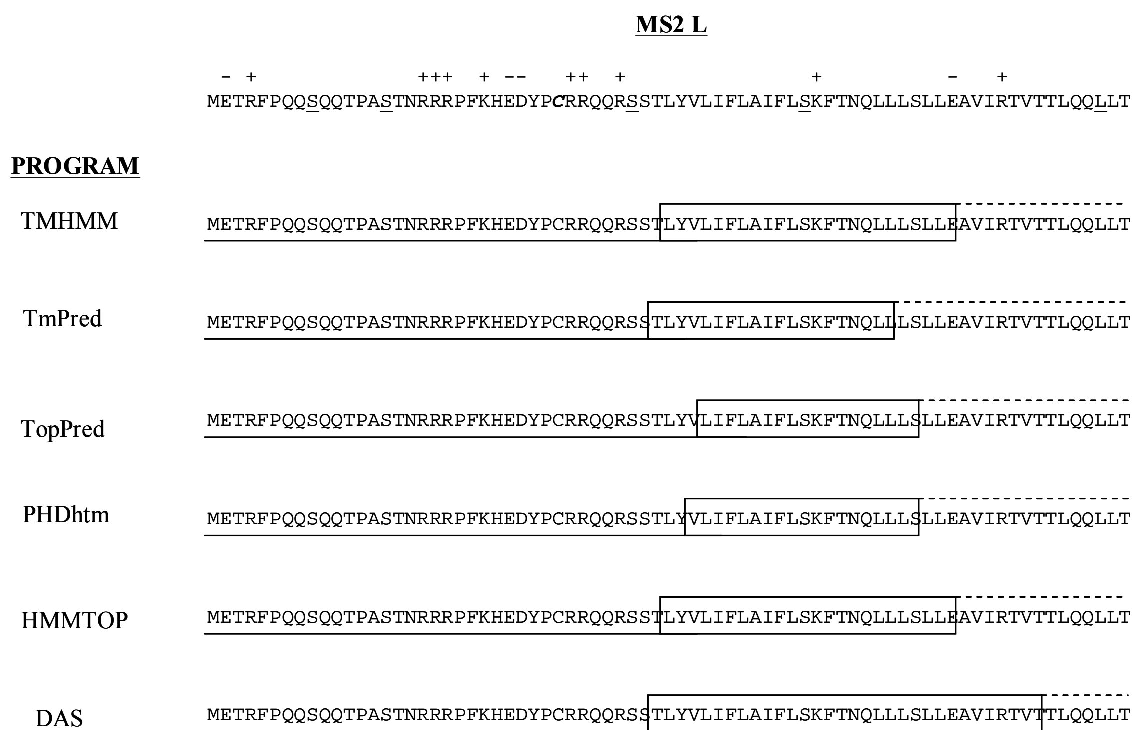

Figure 4.2. Sequence of MS2 L and putative topologies based web-based protein topology prediction programs. Residues that were used for cysteine-scanning mutagenesis are underlined in the first sequence while the native cysteine at position 29 is italicized. For each program, residues in cytoplasm, solid line below; transmembrane domain, boxed; residues in periplasm, hashed line above.

a set of characteristics of most membrane proteins, which included the ‘positive-inside’ rule. This suggests that the topology of a membrane protein will be dependent on the positively charged domains being present in the cytoplasm of the cell (von Hiejne, 1995). Thus, the predicted topologies of L are consistent with this, since the N-terminus with a net positive charge would be in the cytoplasm. The TMD was most often predicted to include residues 36-60, with the threonine at position 36 being the most N-proximal predicted residue and threonine at position 67 as the last predicted residue by any of the programs. As such, the TMD would include the majority of the C-terminus, leaving only 15-17 residues in the periplasm. The domain spans the most hydrophobic regions of the protein and includes the essential serine at position 49 in the middle of the predicted TMD. As seen in Figure 2.1, a Lys residue at position 51 of MS2 L is conserved in all coliphage L proteins and is predicted to be present in all of the transmembrane domains, without a compensating acidic residue. Charged residues are not normally found in a TMD unless it is neutralized by a nearby residue of the opposite charge (Elofsson and von Heijne, 2007; von Heijne, 1995). Nevertheless, charged residues are not unheard of in transmembrane domains, though they are lumenal-facing residues involved in functioning of a channel (Bass et al., 2002; Sahin-Toth et al., 1994). Cysteine-scanning mutagenesis, described in Chapter II, was used to introduce cysteines at various positions of the L protein. According to these L topology models, this collection of mutants would include alleles with single Cys resiudes in all three compartments: in the cytoplasm (Ser to Cys 9 and the native Cys 29), the membrane

(Ser 49 to Cys and possibly the Cys replacements at positions Ser 35 and Ser 58) and periplasm (Leu 73 to Cys). As noted above, all of these except L^{S49C} lytically functional.

A cmyc-tagged allele of L is functional, but has an altered cellular localization

Derivatives of L with N-terminal oligo-histidine or cmyc tags were not only lytically active, but both brought about host lysis before the wild type (Figure 4.3A). Surprisingly, unlike wild type L or His_6-L approximately 20% of the total cmyc-L protein was present in the soluble fraction (Fig. 4.3B-D). Since it is highly unlikely that the addition of the cmyc-tag to the N-terminus of an integral membrane protein would convert it into a soluble protein, we decided to re-examine the distribution of L within the cell.

L is periplasmic and membrane associated, but is not an integral membrane protein

MTSES, a cysteine-specific modification reagent, has been utilized for mapping the topology of membrane proteins in both eukaryotic and prokaryotic cells (Frillingos et al., 1998; Holmgren, et al., 1996; Kaback et al., 2007; Pascual et al., 1995; Sun et al., 1996; Yagur-Kroll and Amster-Choder, 2005). This reagent is small enough to penetrate the outer membrane of Gram-negative organisms but its negative charge makes it impermeable to the inner membrane. Thus, while it can react with sulfhydryl groups that are exposed to the periplasm, it will not react with those that are embedded in a membrane or that are cytoplasmically disposed. To determine the topology of L, we first subjected the protein to cysteine-scanning mutagenesis. Next, whole cells

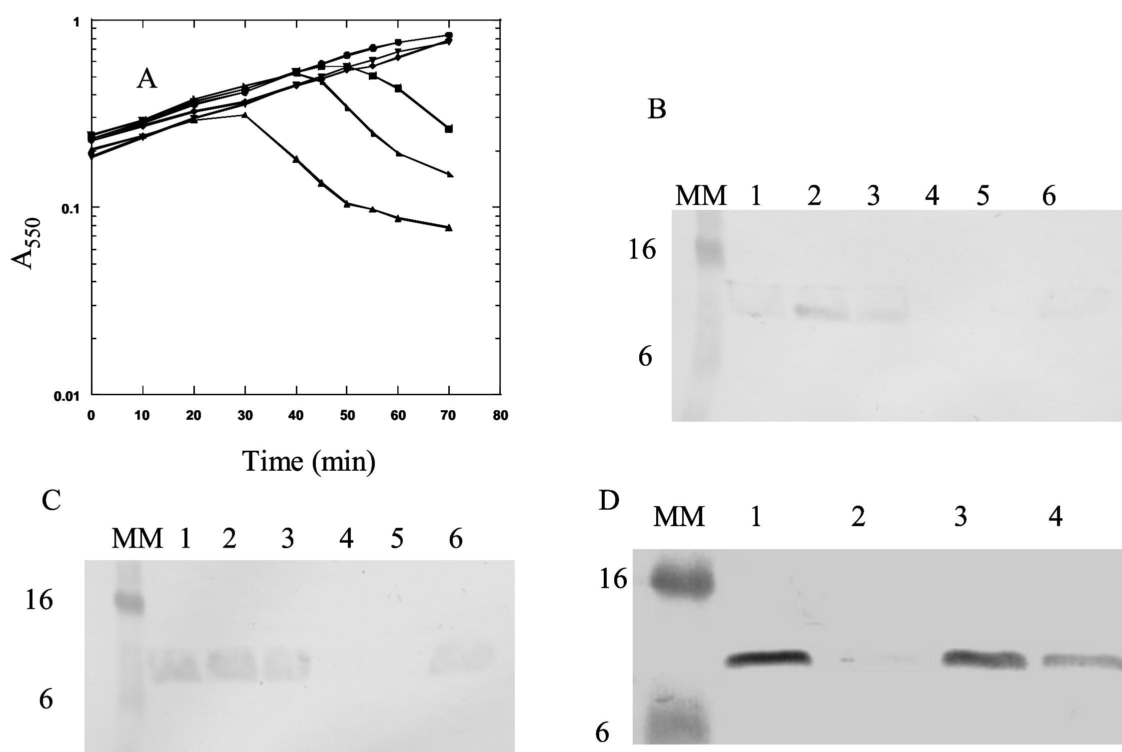

Figure 4.3. Subcellular fractionation of epitope-tagged L. In all panels, MM is size marker and kDa are to the left. (A) Lysis profile of epitope-tagged clones of L compared to wild-type L. Cultures were induced at A_{550} 0.2 with 1mM IPTG (final concentration). ●, pQ pRE-L, uninduced; ■, pQ pRE, + IPTG; ♦, pQ pRE-His_6-L, uninduced; ▲,pQ pRE-His_6-L, + IPTG; ▼, pQ pRE-cmyc-L, uninduced; ►, pQ pRE-cmyc-L, + IPTG. (B) His_6-L fractionates entirely with the membrane fraction. Lane 1, whole cells; lane 2, total protein after French press; lane 3, supernatant after low speed centrifugation; lane 4, unlysed cells after low speed centrifugation; lane 5, supernatant after ultracentrifugation; lane 6, ultracentrifugation pellet. (C) L fractionates with the membrane. Lanes as above. (D) Cmyc-L fractionates as a soluble and membrane protein. Lane 1, total protein after disruption by French press; lane 2, undisrupted cells and cell debris after low speed centrifugation; lane 3, soluble cmyc-L protein after ultracentrifugation; lane 4, detergent- extracted, membrane-bound protein after ultracentrifugation. The pellet after ultracentrifugation was extracted overnight at 4°C in 1% Triton X-100.

expressing the desired *L* alleles using the dual plasmid system described earlier were treated with MTSES to block cysteines external to the cytoplasmic membrane. After

quenching the reaction with free cysteine, the cellular proteins were delipidated and denatured by a chloroform-methanol extraction. The residue was dissolved in a buffer containing SDS and urea and was then treated with PEG-maleimide which, in principle, will modify any available cysteine not blocked by reaction with MTSES. Although MTSES labeling is not associated with a change in the molecular mass of the protein, mPEG-Mal increases the apparent size by 10kDa for each residue modified, as well as increasing the ability of the protein to transfer to a nitrocellulose membrane. As seen in Figure 4.4, cysteine residues at positions 9, 29, 35, 49 and 73 were all accessible to

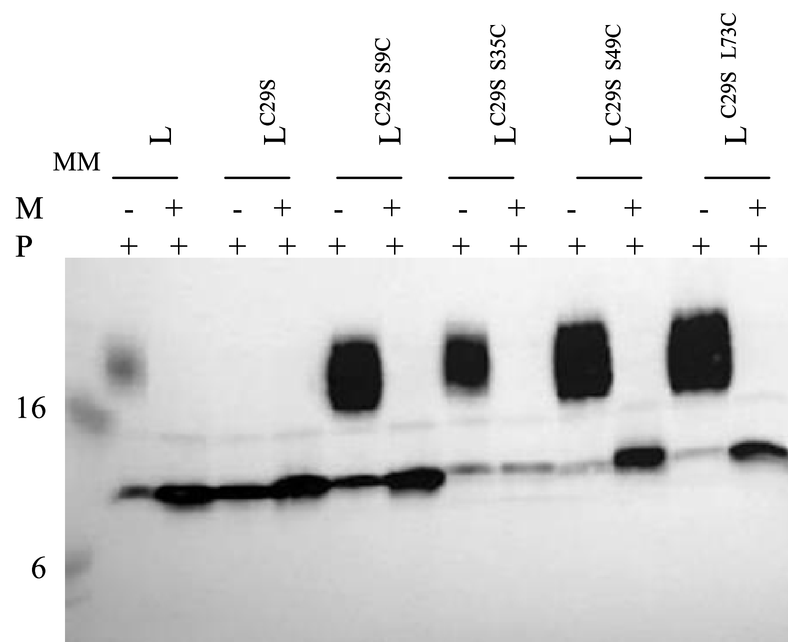

Figure 4.4. The entire L polypeptide chain is accessible to externally added MTSES. MTSES labeling of the single cysteine residue indicated protects the residue from modification by PEG-Mal. Samples were collected 35 minutes after induction with 1 mM IPTG and half of the sample was reacted with PEG-Mal only while the other half of the sample was subjected to MTSES labeling followed by PEG-Mal as described in the Materials and Methods. Samples were subjected to Western blotting using anti-L antibody. MM, molecular mass standard (kDa).

modification by external MTSES. Without pretreatment with MTSES, all of the cysteine-containing L derivatives were efficiently modified by PEG-mal.

Although MTSES has been shown to be impermeant to liposomal bilayers (Holmgren et al., 1996), it had not previously been shown that this reagent is unable to penetrate the inner membrane of *E. coli*. To establish this property, we examined the MTSES accessibility of a soluble cytoplasmic protein, the λ endolysin, R. R contains a single cysteine residue at position 120. However, the crystal structure of R indicates that this residue is buried and, thus, should not be accessible to cysteine modification when R is in its native conformation (Evrard et al., 1997). Consequently, we constructed the R^{S141C}*-cmyc* allele to provide a cysteine that is predicted to be accessible in the native protein. The R^{S141C}-cmyc protein has two cysteines, one that is accessible to MTSES and one that is not. As expected, when whole cells expressing the R^{S141C}*-cmyc* gene were treated with MTSES neither cysteine was protected from subsequent modification by PEG-maleimide (Figure 4.5). By contrast, in disrupted cells, MTSES blocked one of the cysteines, presumably the exposed cysteine at position 141. This clearly demonstrates that MTSES/PEG-maleimide can be used to determine the subcellular localization of proteins as well as their membrane topology.

The soluble cmyc-L protein is located in the cytoplasm

Since it was not clear from the membrane experiments whether the soluble cmyc-L protein was present in the cytoplasm or periplasm, cells expressing the *cmyc-L* gene were converted to spheroplasts by EDTA-lysozyme treatment. The periplasmic fraction

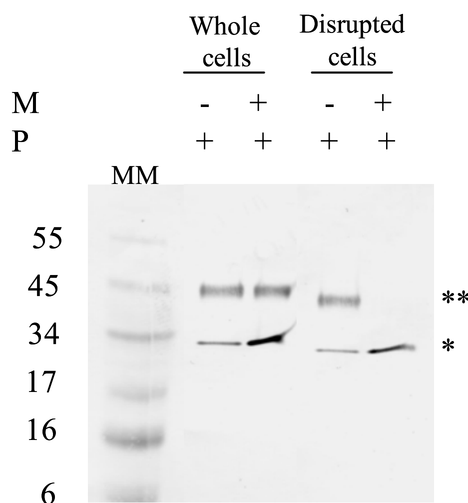

Figure 4.5. MTSES is membrane-impermeable and cannot modify cysteine residues located in the cytoplasm of *E. coli* cells. MM, molecular mass standard, sizes in kDa on left. R^{S141C}-cmyc is 17 kDa without modification by PEG-Mal, each PEG-Mal is 10 kDa. Samples were subjected to Western blot using anti-cmyc antibody. M, MTSES; P, PEG-Mal. Asterisk, single PEG-Mal modification; Double asterisk, two PEG-Mal modifications.

was separated from the spheroplasts by centrifugation well in advance of the onset of lysis. The spheroplasts present in the pellet were disrupted and the cytoplasm was separated from the cell membranes by centrifugation. The soluble fraction of cmyc-L is found in the cytoplasm and not in the periplasm (Figure 4.6).

L-mediated lysis does not require any specific PBP

It is widely held that the lysis of bacteria requires disruption of their peptidoglycan cell wall. The only proteins that are involved in cell wall synthesis that function in the periplasm are the penicillin-binding proteins (PBPs) (refer to Figure 1.5). Several of the 12 known PBPs are responsible for catalyzing the polymerization, or

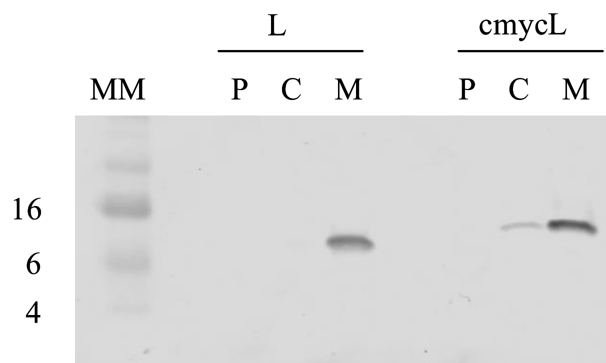

Figure 4.6. Subcellular fractionation of L and cmyc-L. Subcellular fractionation using spheroplasting followed by French press to disrupt spheroplasts. Samples were analyzed by Western blotting with anti-L antibody. MM, molecular mass standard (kDa) indicated on left. P, periplasmic fraction; C, cytoplasmic fraction; M, membrane fraction.

transglycosylation, reaction whereby the monomeric units of precursor molecules are covalently attached to the growing strand of cell wall (Denome et al., 2002). Meanwhile, others catalyze the cross-linking, or transpeptidation reactions that link the strands together to provide rigidity to the peptidoglycan. Two high-molecular weight PBPs, PBP1a and PBP1b, are capable of performing both reactions and at least one of these PBPs is essential for viability (Goffin and Ghuysen, 1998; Young, 2001). Since MTSES accessibility indicates that L is present on the periplasmic side of the cytoplasmic membrane, we tested the dependence of L-mediated lysis on the presence of specific penicillin-binding proteins (PBP) involved in the polymerization and crosslinking of the growing peptidoglycan chains. L was expressed in cells in which 3 to 8 of the 12 known PBPs were deleted and also an isogenic, wild-type parental strain. As shown in Figure 4.7, L is able to lyse *E. coli* cells that are deleted for as many as 8

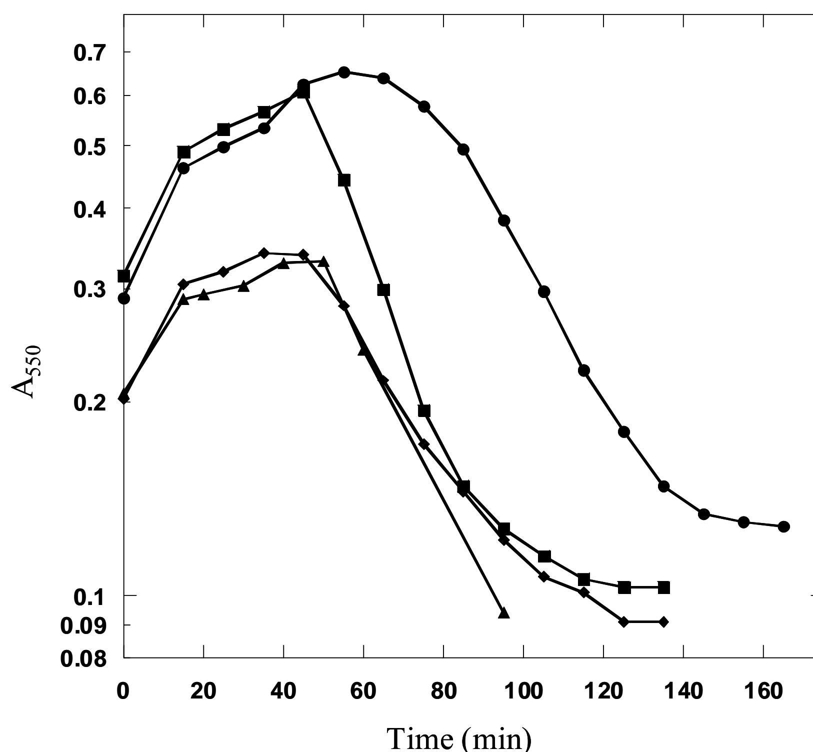

Figure 4.7. PBP1b, PBP4, PBP5, PBP6, PBPG, DacA, AmpH and AmpC are non-essential for L-mediated lysis. Each strain is lysogenized with λΔSR Cam^R and carries pRE-L. Cultures grown to A_{550} ~0.2-0.3 at 30°C, then were subjected to a thermal shift at 0 min, with 15 minutes at 42°C, followed by incubation at 37°C.●, CS109; ■, CS315-1; ♦, CS802-2; ▲, CS804-1.

PBPs. Thus, the lytic function of L does not derive from its interaction with any one member of the PBP family or with any of the combinations tested.

Chemical cross-linking of L did not reveal interactions with host proteins

Using a biochemical approach, I attempted to identify host proteins that directly interacted with L by chemical cross-linking. The first cross-linking reagent used, MBP

(4-Maleimidobenzophenone), is a heterobifunctional reagent. After reaction of the maleimide moiety with the sulfhydryl group of a cysteine, photoactivation leads to reaction of the carbonyl group of the benzophenone with any C-H bond within 10Å. Since MBP specifically modifies cysteine residues, the L proteins produced during cysteine scanning mutagenesis were employed (refer to Figure 4.2). By using the different Cys alleles of L, this would move the modification reagent along the length of the L protein to maximize the chances of cross-linking L to a cellular protein. An MBP-dependent increase in the molecular mass of the L protein was expected if the allele was cross-linked to a host protein after photoactivation. The detection of the different alleles of the L protein using L-specific antibodies was possible, but no novel shift was obtained with the addition of MBP followed by irradiation to activate the secondary reaction with carbonyl groups in close proximity (Figure 4.8A). Since the primary reaction of MBP requires modification of cysteines, it is possible that the failure to detect a cross-linked host protein was the result of a failure to modify the cysteine on L. Thus, a control was required to show that the cysteine residues on the various L proteins were modified and thereby inaccessible to modification by another cysteine-specific reagent, PEG-OPSS. Samples were reacted with MBP, which does not alter the molecular mass, followed by treatment with PEG-OPSS, which would result in an increase of 10kDa and increase reactivity with the nitrocellulose membrane. As shown in Figure 4.8B, the samples labeled with MBP were not accessible to modification (and associated molecular mass shift), indicating that in each case, MBP modified the cysteine residue.

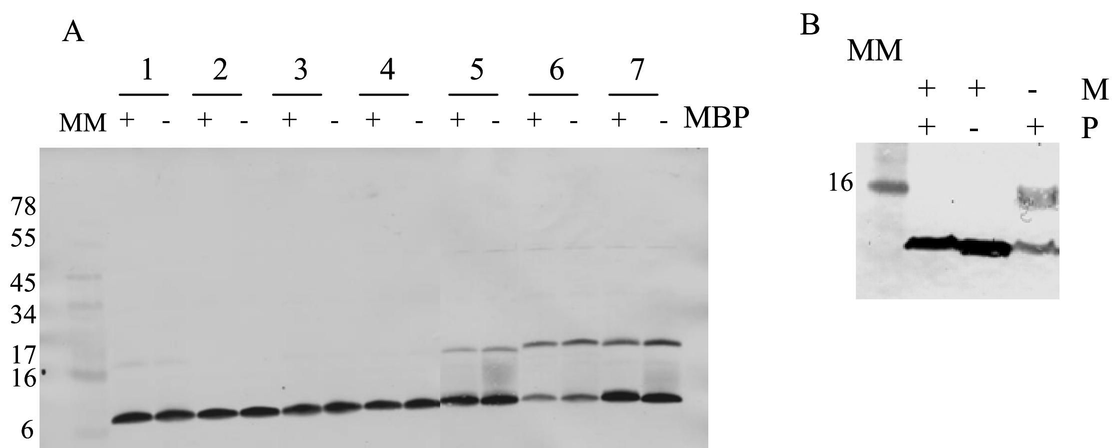

Figure 4.8. Labeling of L with MBP (4-Maleimidobenzophenone). MM, molecular mass standard. Samples were subjected to Western blot with anti-L antibody. (A) MBP labeling of L and subsequent photoactivation does not cause the appearance of a band indicative of an association with another protein. +, addition of MBP; -, no MBP. 1, L; 2, L^{C29S}; 3, $L^{C29S\ S9C}$; 4, $L^{C29S\ S15C}$; 5, $L^{C29S\ S35C}$; 6, $L^{C29S\ S49C}$; 7, $L^{C29S\ S58C}$. (B) MBP modification blocks PEGylation by PEG-OPSS. M, MBP; P, PEG-OPSS.

Membranes containing His_6-L were reacted with DSP, an amino-directed homobifunctional reagent, with the expectation that L would be cross-linked to any nearby proteins and that these cross-links would be sensitive to reducing agents. The His tagged version of L was used here since the antigenic sequence used to produce the L-specific antibodies contains Lysine residues, which are modified by DSP. This reduces the reactivity with the antibodies and occurs with increasing frequency over the time course of the experiment (data not shown). As can be seen in Figure 4.9, L forms homo-oligomers, with the formation of at least an octomer visible (indicated by asterisks). The cross-links are reversible with the addition of the reducing reagent, β-mercaptoethanol.

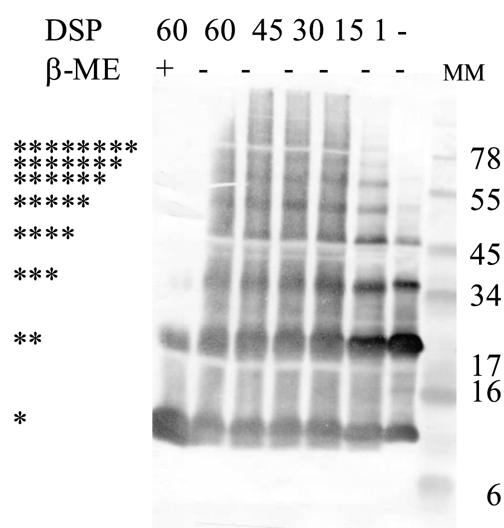

Figure 4.9. Reaction with DSP indicates that L forms homo-oligomers. Samples were detected using α-His antibody for Western blotting. MM, molecular mass (kDa) indicated in right and length of treatment in minutes at top. DSP, dithiobis(succinimidyl propionate); β-ME, reducing reagent β-mercaptoethanol. Asterisks indicate the oliogmeric state of L, with one asterisk for the monomer, two asterisks for the dimer, and so on.

Identifying interacting proteins by co-immunoprecipitation

Another biochemical approach to identify host proteins that interact with L was used. In this system, membranes from cells expressing L^{C29S} were reacted with Maleimide-PEO_2-Biotin. In this case, all cellular proteins with a modifiable cysteine were labeled with a biotin moiety. The mixture was then immunoprecipitated with L-specific antibodies. Host proteins that co-immunoprecipitated with the L protein would be subsequently detected with streptavidin-conjugated antibodies. Figure 4.10 shows that no additional protein species co-immunoprecipitated with L. Although there are a number of biotin-labeled proteins present in the co-immunoprecipitated samples, these species are also present in the control lanes as well.

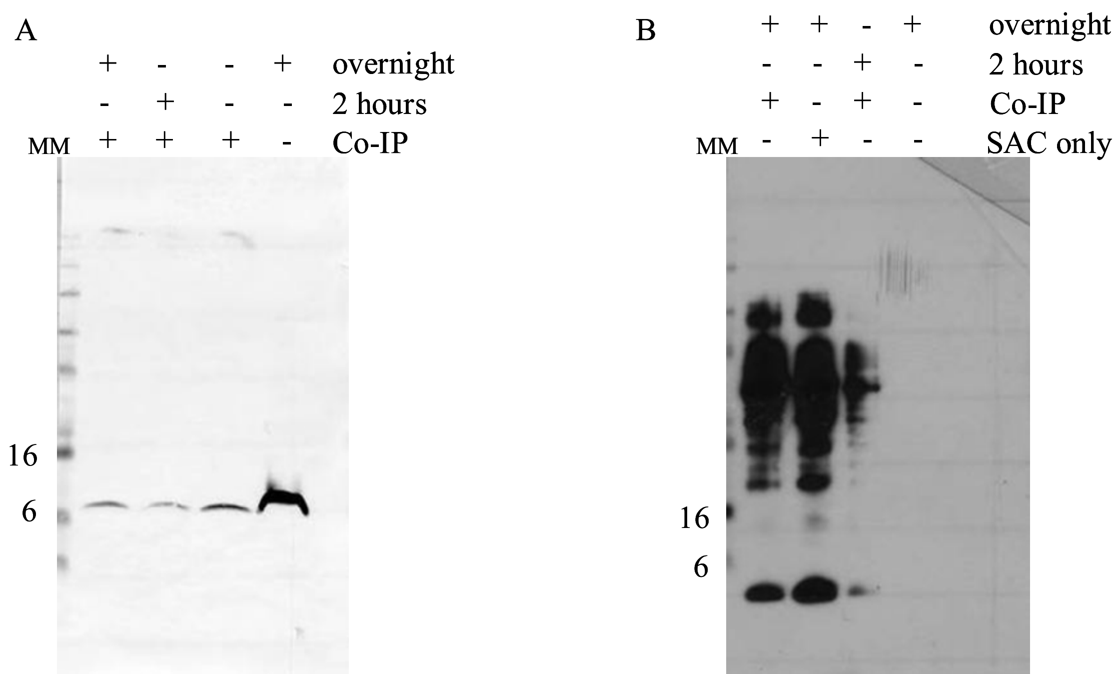

Figure 4.10. Maleimide-PEO_2-Biotin modification does not indicate a host protein that co-immunoprecipitates with L. (A) Western blot using α-L with colorometric detection after co-immunoprecipitation with α-L. Overnight, MBP treatment overnight before quenching; 2 hours, MBP treatment for only 2 hours; Co-IP, co-immunoprecipitation performed with α-L. (B) Western blot using streptavidin-conjugated horseradish peroxidase after co-immunoprecipitation and detected with chemiluminescence. Lanes as in (A), with addition of SAC only, no incubation with α-L before addition of SAC.

Purification of oligohistidine-tagged L

In order to physically characterize the L protein, the His_6-L and His_6-L^{S49C} derivatives were purified by IMAC in buffer with the detergent, Empigen BB at a final concentration of 1%. The L-containing fractions had an UV spectrum consistent with protein, but not with potential UV-absorbing contaminants such as nucleic acid (Figure 4.11A). Using an extinction coefficient calculated from the amino acid sequence of L

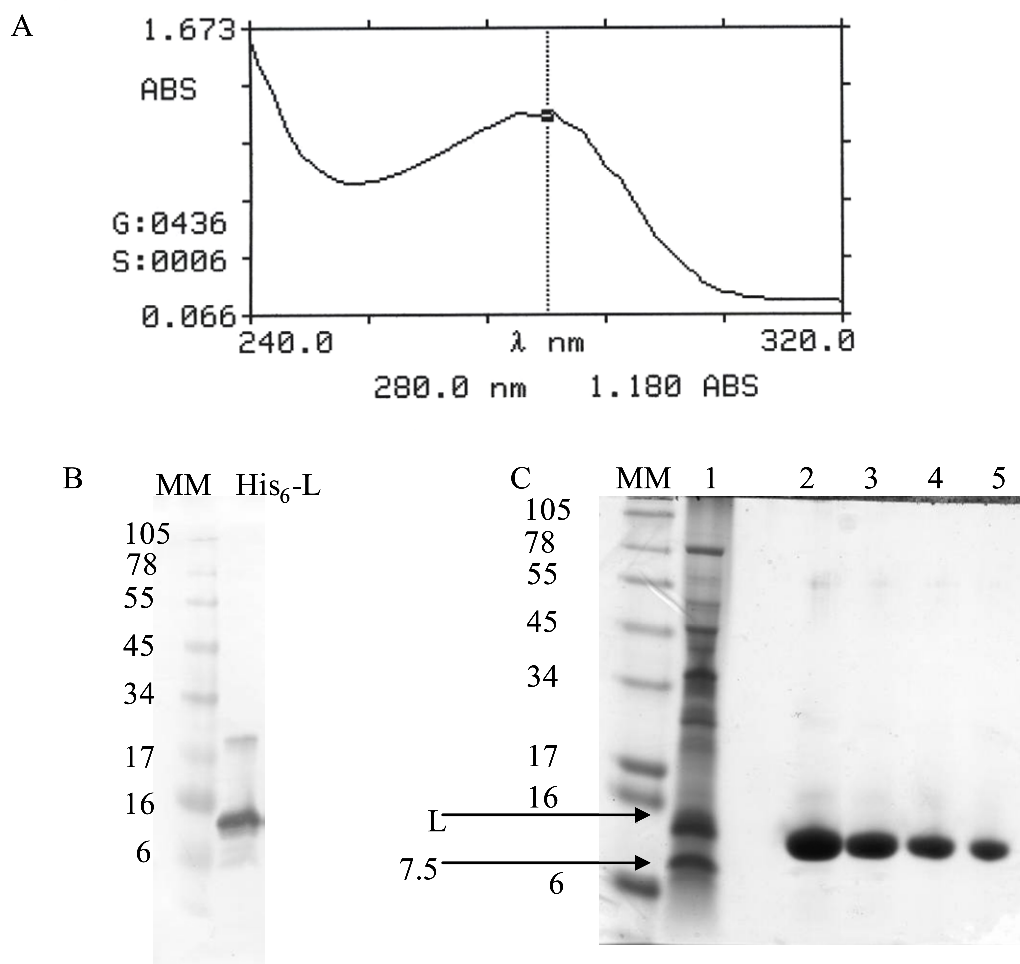

Figure 4.11. Purified His_6-L protein absorbance spectrum, Western blot with α-L and Coomassie-stained gel for comparison to a known protein standard. MM, molecular mass standard. (A) Absorbance spectrum of His_6-L from A_{240}-A_{320}. (B) Western blot of His_6-L (1X) using L-specific antibodies. (C) Coomassie stained gel of 20X purified protein sample in comparison to HEW lysozyme standard., sizes in kDa on left; 1, 20X concentrated purified sample; 2, 10μg HEW lysozyme; 3, 5μg; 4, 2.5μg; 5, 1.25μg. Arrows are labeled to indicate the His_6-L species and an unknown *E. coli* protein at 7.5kDa.

(Pace et al., 1995), these fractions should contain L protein at the nominal concentration of 3.5 mg/ml. However, subjecting 10 µl (approximately 35 µg of L protein by A_{280}) of this fraction to SDS-PAGE and staining with Coomassie blue did not reveal the presence of any protein (or combination of proteins) that could account for the absorbance at 280 nm (not shown). When the L-containing fractions were concentrated 20-fold and then examined by SDS-PAGE, a number of Coomassie blue staining bands became apparent, indicating the purified samples are not pure (Figure 4.11C). As expected, a major band recognized by anti-L antibodies migrated at 10 kDa, which is characteristic for L (Figure 11B & C). A second major band migrating at 7.5 kDa was also observed and its staining intensity suggests that it was present in a 1:1 molar ratio with L. The concentration of L in the eluted fractions was estimated by comparing the intensity of the Coomassie blue stained bands observed after SDS-PAGE with a lysozyme standard (Figure 4.11C). By this method, the yield of L was approximately 100 µg from 6 L of culture. Based on the estimation of ~10^5 molecules of L per cell using quantitative Western blotting (refer to Figure 2.2), the purification yielded only ~100 molecules per cell, or approximately 0.1% of the total His_6-L protein.

Discussion

From its earliest characterization, the MS2 lysis protein, L, has been described as a membrane protein (Beremond and Blumenthal, 1979; van Duin and Tseareva, 2006; Walderich et al., 1988, 1989). In fact, all six of the topology prediction algorithms we have used categorize L as a single-pass integral inner membrane protein with an N-in, C-out topology (Figure 4.2). The consensus prediction is that residues 1-37 of L reside in the cytoplasm, residues 36-60 represent a transmembrane domain (TMD), and residues 61-75 are in the periplasm. However, our findings indicate that it is very unlikely that L is an integral membrane protein. First, we found that adding a cmyc tag to the N-terminus of L resulted in about 20% of the protein appearing in the soluble cytoplasmic fraction (Figure 4.6). Second, the MTSES-accessibility of cysteine residues placed along the length of the L polypeptide suggests that L is periplasmic (Figure 4.4). Moreover, even a cysteine at position 49, in the middle of the predicted TMD, is accessible to MTSES. These results suggest that L is translocated completely across the inner membrane. To account for its fractionation with the membrane, the L protein can be viewed as strongly interacting with either the outer leaflet of the inner membrane, the inner leaflet of the outer membrane, or some other structure which sediments at 100,000 x g. The finding that L is localized to the periplasm is consistent with the observation that it does not block DAP incorporation into peptidoglycan and, thus, cannot inhibit any of the cytoplasmic steps in cell wall biosynthesis (Holtje and van Duin, 1984; T. G. Bernhardt dissertation, 2001). Presumably, L interferes with some step in cell wall assembly or processing that occurs in the periplasm.

Several attempts were made to identify host proteins that interact with L. DSP and maleimidyl-benzopheonone were used in an attempt to crosslink L to its putative target. The former reagent is an amine-specific homobifunctional crosslinker while the latter is cysteine-specific heterobifunctional photo-crosslinker. In neither case, however, was there a clear indication that L was associated with a cellular protein (Figures 4.8 and 4.9). However, DSP crosslinking did demonstrate that L formed oligomers.

In a second approach, L was immunoprecipitated from detergent-solubilized cell lysates and then treated the immunoprecipitates with biotinyl-maleimide to attach biotin groups to any cellular proteins present with free cysteines. These samples were then analyzed by SDS-PAGE and Western blotting with avidin-horseradish peroxidase. This approach also failed to identify a potential cellular target for L (Figure 4.10). Finally, since L is found in the periplasm, we reasoned that its function might involve an interaction with a specific PBP. However, deleting various combinations 8 of the 12 of the PBPs had no discernable effect on the time course of L-mediated lysis (Figure 4.7).

It is certainly provocative that a 7.5kDa protein copurifies with oligohistidine-tagged L using IMAC (Figure 4.11C). Although we do not currently know the identity of this protein, one likely candidate that would fit with the lytic function of L is Lpp, Braun's lipoprotein. Lpp is the major *E. coli* outer membrane lipoprotein and approximately one-third of the Lpp molecules are crosslinked to peptidoglycan (Braun, 1975). It has been shown that the mislocalization of Lpp to the inner membrane is lethal and results in cell lysis (Suzuki et al., 2002). It is tempting to speculate that L might interact with Lpp causing its retention in the inner membrane resulting in lysis of the

cell. Lpp is the most prevalent outer membrane protein of *Escherichia coli*, with nearly 10^6 molecules per cell (Braun, 1975). It is not yet clear whether the presence of Braun's lipoprotein in the purified samples is due to co-purification or merely an artifact contaminating the samples.

CHAPTER V

DISCUSSION AND CONCLUSIONS

Max Delbruck considered bacteriophage a simple model system, and since the ssRNA *Leviviridae* are the simplest phage, it would stand to reason that the processes involved in the life cycle of ssRNA phage would be the most straightforward to characterize. Though many stages of the life cycle of a prototypic Levivirus, MS2, have been identified and described, other steps have remained mysterious for more than forty years after their initial identification. The preceding chapters describe the characterization of specific steps and proteins involved in the MS2 life cycle, from the early stages of infection to the very last – the lysis event mediated by the L protein.

The life cycle of MS2 begins with adsorption to the pilus of a host cell, but the subsequent steps whereby the genome travels down the pilus, crosses the cell envelope and enters the cytoplasm are relatively less well understood. The results presented in Chapter III identify and characterize the involvement of PcnB, a poly(A) polymerase, in these steps. While attempting to isolate mutants resistant to lysis by L using a series of genetic selections and screens, a set of mutants arose that were unexpected. As a result of expression of L from a ColE1-based plasmid, followed by screening using MS2 phage, missense mutations were uncovered in the *pcnB* gene. These mutations dramatically reduced or eliminated catalysis by PcnB, which is known to control the copy number of plasmids with ColE1 origins. Yet, surprisingly, the presence of these catalytically inactive mutants of PcnB was also detrimental to the ability of MS2 to infect the cell, through a step after the initial binding of phage to the pilus. Cells

expressing these non-catalytic versions of PcnB remained capable of replicating progeny phage and allowed for lysis to occur, so long as the RNA enters efficiently. It is likely that the effect of these mutations had not previously been identified because the insertionally inactivated mutant has the same phenotype as wild-type *E. coli*.

The work presented in Chapter II and IV was aimed at characterizing the lysis brought about by L. It has been known for nearly a quarter century that L does not inhibit the incorporation of radiolabeled precursor into the murein sacculus; this result has remained as the most significant, yet least understood, characteristic of L-mediated lysis. Chapter II includes quantification of L protein during MS2 infections and a detailed microscopic analysis of the phenotypic differences between lysis mediated by L and that of chemical or protein antibiotics, such as A_2. The quantification of L suggests that L is synthesized at levels of about 10^5 molecules per cell and this correlates with the levels of protein roughly estimated using radiolabeling and comparison to coat protein accumulation by Beremond and Blumenthal (1979). Unlike penicillin and A_2, L appears to have no preference for causing membrane perturbations at the septum, which suggests that L-mediated lysis is achieved through a fundamentally different mechanism.

Also in Chapter II, a dual plasmid system, pQ/pRE-L, was described that was developed based on the system used for the controlled expression of cloned dsDNA lysis genes. This system enabled the expression of L at close to physiological levels and clearly maintained relevant characteristics of L protein expressed from the phage. Additionally, the pQ/pRE-L system provided the capability to study single missense mutants of L, which is otherwise not possible in the context of the ssRNA genome. For

the first time, a non-lytic mutant of L has been identified, L^{S49C}. A region near Ser49, L44-I46, has been highly conserved throughout the coliphage L proteins of the *Leviviridae* family. The important character of this region has been shown to be the overall hydrophobicity and nearly neutral charge.

Perhaps most significantly, L has been shown to be accessible to membrane-impermeant chemical reagents (Chapter IV). This remarkable result indicates that the long-standing assumption in the literature that L is an integral inner membrane protein with a single transmembrane domain is incorrect. Also, the addition of a small epitope tag to the N-terminus of the L protein altered the subcellular localization, which is unprecedented for inner proteins. Nonetheless, L has now been shown not to span the membrane bilayer. The accessibility of many residues along the length of L to modification by external MTSES demonstrated L behaves like a periplasmically localized protein. Consistent with these results, isopycnic sucrose gradient analysis showed that L was distributed across all of the envelope fractions. These results indicate that L, rather than being an integral membrane protein, is instead intimately associated with the periplasmic face of the inner or outer membrane or the cell wall, without spanning the bilayer. Additionally, L protein was purified using an oligohistidine tag and co-purifies with an unknown *E. coli* protein of 7.5 kDa.

Future work on lysis by L

Export of L to the periplasm

Based on the MTSES protection results that indicate L is localized to the periplasm, the important question arises as to how L gets to this location in the cell. There are a number of pathways that are possible, including the TAT pathway, Sec secretion system and YidC-assisted transport. Preliminary data indicated that the Sec system is not essential to lysis mediated by L. Addition of azide, a known inhibitor of the SecA protein, does not alter the kinetics of lysis (data not shown). In order to test dependence of L export on other known secretion systems, experiments will be performed using a TatC deletion strain available from the EGCS or *secE* and *yidC* depletion strains graciously provided by Dr. R. Dalbey (Ohio State University). In each strain, the localization of L protein will be analyzed by the MTSES protection experiment described above.

Identification of the target of L

The mechanism of lysis induced by L remains unknown. L must compromise the integrity of the murein sacculus in order to allow release of progeny, as is the requirement for all lytic bacteriophage. dsDNA phages encode multiple proteins for lysis, with the essential endolysin being used to actively degrade the cell wall. The lysis proteins, E and A_2, of two prototypic ssDNA and ssRNA phages, ϕX174 and Qβ, respectively, were shown to behave as protein antibiotics. These proteins bring about

lysis by inhibiting the catalysis of two different enzymes involved in de novo synthesis of peptidoglycan and in doing so prevent incorporation of radiolabeled precursor into the SDS-insoluble murein sacculus. In a different mechanism, L does not inhibit incorporation of this radiolabeled precursor. This leads to one of two potential models that will be tested in the near future. In both cases, the models must account for the ability of L to allow for incorporation, or covalent attachment, of the monomeric unit of peptidoglycan (N-acetyl-glucosamine-β-1-4-N-acetylmuramic acid-pentapeptide) into the cell wall, yet also must be compatible with L as a membrane-associated protein. The first model would suggest that L could allow for the covalent attachment of the precursor to the murein sacculus, but would interact with the strands to prevent proper cross-linking. This would affect the cell wall similarly to penicillin, in that the cell wall would not be sufficiently cross-linked and lysis would eventually ensue. In this case, it should be possible to isolate [^{3}H-DAP]-labeled individual monomeric units of peptidoglycan at a detectably higher rate than in cells without L expression. The detection of monomeric, dimeric, and trimeric species of murein has previously been shown (Waxman et al, 1980; Li et al., 2003).

The second model stems from the fact that a small, 7.5 kDa protein appears to co-purify with His_6-L. If this protein is Lpp, then it is possible that L binds to the premature form of Lpp, or Braun's lipoprotein, in the inner membrane. Normally, mature Lpp is inserted into the outer membrane by the presence of three fatty acid chains attached at the N-terminal cysteine and about one-third is covalently crosslinked at its C-terminal lysine to the peptidoglycan (Braun, 1975). The modification of the cysteine

residue is essential for its cleavage and release from the inner membrane (Hussain et al., 1980; Tokunaga et al., 1984). Isopycnic sucrose gradients performed on cells that fail to properly process the premature form with the addition of the fatty acid chains demonstrated that Lpp did not appear in the fractions of the outer membrane (Suzuki et al., 2002). Instead, Lpp was present in a single, broad peak that represented both of the membranes. The cumulative effect of this failure was lysis, which was proposed to be the result of the covalent attachment of the premature Lpp present in the inner membrane to peptidoglycan (Suzuki et al., 2002). This leads to the second model for L-mediated lysis by which L protein binds premature Lpp to prevent its modification and subsequent release from the inner membrane, though still allowing for covalent linkage of Lpp to the peptidoglycan. Studies are currently being performed to identify the 7.5 kDa protein that co-purifies with oligohistidine-tagged L and to show that the band is absent from purified non-lytic protein (L^{S49C}).

REFERENCES

Achtman, M., Willets, N., Clark, A.J. (1971). Beginning a genetic analysis of conjugational transfer determined by the F factor in *Escherichia coli* by isolation and characterization of transfer-deficient mutants. J. Bacteriol. 106: 529-38.

Adhin, M.R., and van Duin, J. (1990). Scanning model for translational reinitiation in eubacteria. J. Mol. Biol. 213: 811-818.

Armstrong, J., Perham, R.N., Walker, J.E. (1981). Domain structure of bacteriophage fd adsorption protein. FEBS Lett. 135: 167-172.

Atkins, J.F., Steitz, J.A., Anderson, C.W., Model, P. (1979). Binding of mammalian ribosomes to MS2 phage RNA reveals an overlapping gene encoding a lysis function. Cell. 18: 247-256.

Barrell, B.G., Air, G.M., Hutchison, C.A. (1976). Overlapping genes in bacteriophage ϕX174. Nature. 264: 34-41.

Bass, R.B., Strop, P., Barclay, M., Rees, D. (2002). Crystal structure of *Escherichia coli* MscS, a voltage-modulated mechanosensitive channel. Science. 298: 1582-1587.

Bayer, M.E. (1968). Areas of adhesion between wall and membrane of *Escherichia coli*. J. Gen. Microbiol. 53: 395-404.

Bell, R.M., Mavis, R.D., Osborn, M.J., Vagelos, P.R. (1971). Enzymes of phospholipid metabolism: Localization in the cytoplasmic and outer membrane of the cell envelope of *Escherichia coli* and *Salmonella typhimurium*. Biochim. Biophys. Acta. 249: 628-635.

Benzer, S. (1955). Fine structure of a genetic region in bacteriophage. Proc Natl Acad Sci USA. 41: 344-354.

Beremand, M.N., and Blumenthal, T. (1979). Overlapping genes in RNA phage: A new protein implicated in lysis. Cell. 18: 257-266.

Berkhout, B., de Smit, M.H., Spanjaard, R.A., Blom, T., van Duin, J. (1985). The amino terminal half of the MS2-coded lysis protein is dispensable for function: Implications for our understanding of coding region overlaps. EMBO J. 4: 3315-3320.

Berkhout, B., and van Duin, J. (1985). Mechanism of translational coupling between coat protein and replicase genes of RNA bacteriophage MS2. Nucl. Acids Res. 13: 6955-6967.

Berkhout, B., Schmidt, B.F., van Strien, A., van Bloom, J., van Westrenen J., van Duin, J. (1987). Lysis gene of bacteriophage MS2 is activated by translation termination of the overlapping coat gene. J. Mol. Biol. 195: 517-524.

Bernardi,A., and Spahr, P.F. (1972). Nucleotide sequence at the binding site for coat protein on RNA of bacteriophage R17. Proc. Natl. Acad. Sci. 69: 3033-3037.

Bernhardt, T.G. (2001). Breaking free: Small phages inhibit murein synthesis to lyse their host. Ph.D. Thesis, Texas A & M University, College Station, TX.

Bernhardt, T.G, Roof, W.D., Young, R. (2000). Genetic evidence that the bacteriophage phiX174 lysis protein inhibits cell wall synthesis. Proc. Natl. Acad. Sci. USA. 97: 4297-4302.

Bernhardt, T.G., Wang, I.N., Struck, D.K., Young, R (2001a). The lysis protein E of phi X174 is a specific inhibitor of the MraY-catalyzed step in peptidoglycan synthesis. J. Biol. Chem. 276: 6093-6097.

Bernhardt, T.G, Struck, D.K., Young, R. (2001b). A protein antibiotic in the phage Qbeta virion: Diversity in lysis targets. Science. 292: 2326-2329.

Bernhardt, T.G., Roof, W.D., Young, R. (2002). The *Escherichia coli* FKBP-type PPIase SlyD is required for the stabilization of the E lysis protein of bacteriophage phiX174. Mol Microbiol. 45: 99-108.

Blumenthal, T., Landers, T.A., Weber, K. (1972). Bacteriophage Qβ replicase contains the protein biosynthesis elongation factors EF-Tu and EF-Ts. Proc. Natl. Acad. Sci. USA 69: 1313-1317.

Blumenthal, T., and Carmichael, G.G. (1979). RNA replicase: Function and structure of Qβ replicase. Annu. Rev. Biochem. 48: 525-548.

Boni, I.V., Isaeva, D.M., Musychenko, M.L., Tzavera, N.V. (1991) Ribosome-messenger recognition: mRNA target sites for ribosomal protein S1. Nucl. Acids Res. 19: 155-162.

Bradley, D.E. (1968). Spheroplast formation in cells of *Escherichia coli* infected with a ϕX174 type bacteriophage. J. Gen. Virol. 3: 141-142.

Bradley, D.E., Dewar, C.A., Robertson, D. (1969). Structural changes in *Escherichia coli* infected with a ϕX174 type bacteriophage. J. Gen. Virol. 5: 113-121.

Braun, V. (1975). Covalent lipoprotein from the outer membrane of *Escherichia coli*. Biochim. Biophys. Acta. 415: 335-377.

Brinton C.C., Jr. (1965). The structure, function, synthesis and genetic control of bacterial pili and a molecular model for DNA and RNA transport in gram negative bacteria. Trans. N.Y. Acad. Sci. 27: 1003-1054.

Brinton, C.C. (1971). The properties of the sex pili, the viral nature of "conjugal" genetic transfer systems, and some possible approaches to the control of bacterial drug resistances. Crit. Rev. Microbiol. 1: 105-159.

Brown, D., and Gold, L. (1996). RNA replication by Q beta replicase: A working model. Proc. Natl. Acad. Sci. USA. 93: 11558-11562.

Brüssow, H., and Hendrix, R.W. (2002). Phage genomics: Small is beautiful. Cell. 108: 13-16.

Caputo, G.A., London, E. (2004). Position and ionization state of Asp in the core of membrane-inserted a helices control both the equilibrium between transmembrane and nontransmembrane helix topography and transmembrane helix positioning. Biochemistry. 43: 8794-8806.

Chang, Y.F., Ma, D.P., Young, R., Struck, D.K. (1993). Cloning, sequencing and expression of a *Pasteurella haemolytica* A1 gene encoding a PurK-like protein. DNA Seq. 3: 357-367.

Coleman, J., Inouye, M., Atkins, J. (1983). Bacteriophage MS2 lysis protein does not require coat protein to mediate cell lysis. J. Bacteriol. 153: 1098-1100.

Crick, F.H.C., Barnett, L., Brenner, S., Watts-Tobin, R.J. (1961). General nature of the genetic code for proteins. Nature. 192: 1227-1232.

Curtiss, L.K., and Krueger, R.G. (1974). Localization of coliphage MS2 A-protein. J. Virol. 14: 503-508.

de Fernandez, M.T., Eoyang, L., August, J.T. (1968). Factor fraction required for the synthesis of bacteriophage Qβ RNA. Nature. 219: 588-590.

de Smit, M.H., van Duin, J. (1990). Secondary structure of the ribosome binding site determines translational efficiency: A quantitative analysis. Proc. Natl. Acad. Sci. USA. 87: 7668-7672.

Demerec, M. and Fano, U. (1945). Bacteriophage-resistant mutants in *Escherichia coli*. Genetics. 30: 119-136.

Denome, S.A., Elf, P.K., Henderson, T.A., Nelson, D.E., Young, K.D., (1999). *Escherichia coli* mutants lacking all possible combinations of eight penicillin binding proteins: Viability, characteristics and implications for peptidoglycan synthesis. J. Bacteriol. 181: 3981-3993.

Devos, R., Contreras, R., van Emmelo, J., Fiers, W. (1979). Construction and characterization of a plasmid containing a nearly full-size DNA copy of bacteriophage MS2 RNA. J. Mol. Biol. 128: 595-619.

Drake, J. (1993). Rates of spontaneous mutation rates among RNA viruses. Proc. Natl. Acad. Sci. USA. 90: 4171-4175.

Eigner, J., Stouthmaer, A.H., van der Sluys, I., Cohen, J.A. (1963). A study of the 70S component of bacteriophage ϕX174. J. Mol. Biol. 6: 61-84.

Elofsson, A., von Heijne, G. (2007). Membrane protein structure: Prediction versus reality. Annu. Rev. Biochem. 76: 125-140.

Engelberg, H., and Soudry, E. (1971). RNA bacteriophage release: Requirement for host-controlled protein synthesis. J. Virol. 8: 257-264.

Evrard, C., Fastrez, J., Declercq, J.P. (1997). Crystal structure of the lysozyme from bacteriophage lambda and its relationship with V and C-type lysozymes. J. Mol. Biol. 276: 151-164.

Fiers, W., Contreras, R., Duerinick, F., Haegeman, G., Iserentant, D., Merregaert, J., Min Jou, W., Molemans, F., Raemaekers, A., Van den Berghe, A., Volckaert, G., Ysebaert, M. (1976). Complete nucleotide sequence of bacteriophage MS2 RNA: Primary and secondary structure of the replicase gene. Nature. 260: 500-507.

Frillingos, S., Sahin-Toth, M., Wu, J., Kaback, H.R. (1998). Cys-scanning mutagenesis: A novel approach to structure function relationships in polytopic membrane proteins. FASEB J. 12: 1281-1299.

Frost, L.S., Finlay, B.B., Opgenorth, A., Paranchych, W., Lee, J.S. (1985). Characterization of sequence analysis of pilin from F-like plasmids. J. Bacteriol. 164: 1238-47.

Furuse, K. (1987). Distribution of coliphages in the environment: General considerations. In Phage Ecology, S.M. Goyal, ed. (New York, NY: Wiley), pp87-124.

Goelz, S., and Steitz, J.A. (1977). *Escherichia coli* ribosomal protein S1 recognizes two sites in bacteriophage Qβ RNA. J. Biol. Chem. 252: 5177-5179.

Goessens, W.H., Driessen, A.J., Wilschut, J., van Duin, J. (1988). A synthetic peptide corresponding to the C-terminal 25 residues of phage MS2 coded lysis protein dissipates the proton motive force in *Escherichia coli* membrane vesicles by generating hydrophilic pores. EMBO J. 7: 867-873.

Goffin, C., and Ghuysen, J-M. (1998). Multimodular penicillin-binding proteins: An enigmatic family of orthologs and paralogs. Microbiol. Mol. Biol. Rev. 62: 1079-1093.

Goodell, E.W., Lopez, R., Tomasz, A. (1976). Supression of lytic effect of β-lactams on *Escherichia coli* and other bacteria. Proc. Natl. Acad. Sci. USA. 73: 3293-3297.

Gray, C.W., Brown, R.S., Marvin, D.A. (1981). Adsorption complex of filamentous fd virus. J. Mol. Biol. 146: 621-627.

Groeneveld, H., Thimon, K., van Duin, J. (1995). Translational control of maturation-protein synthesis in phage MS2: A role for the kinetics of RNA folding? RNA 1: 79-88.

Grundling, A., Blasi, U., Young, R. (2000). Genetic and biochemical analysis of dimer and oligomers interactions of the λ S holin. J. Bacteriol. 182: 6082-6090.

Grundling, A., Manson, M.D., Young, R. (2001). Holins kill without warning. Proc. Natl Acad Sci. 98: 9348-9352.

Guan, L., and Kaback, H.R. (2007). Site-directed alkylation of cysteine to test solvent accessability of membrane proteins. Nature Protocols. 2: 2012-2017.

Gussin, G.N. (1966). Three complementation groups in bacteriophage R17. J. Mol. Biol. 21: 435.

Hahn, F.E. and Ciak, J. (1957). Penicillin-induced lysis of *Escherichia coli*. Science. 125: 119-120.

Harlow, E. and Lane, D. (1999). Immunoprecipitation. In Using Antibodies: A Laboratory Manual. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), pp. 221-266

Harrington, L.C., and Rogerson, A.C. (1990). The F pilus of *Escherichia coli* appears to support stable DNA transfer in the absence of wall-to-wall contact between cells. J. Bacteriol. 172: 7263-7264.

Harris, R.L., Hombs, V., Silverman, P.M. (2001). Evidence that F-plasmid proteins TraV, TraK and TraB assemble into an envelope-spanning structure in *Escherichia coli*. Mol. Microbiol. 42: 757-766.

Harris, R.L., and Silverman, P.M. (2002). Roles of internal cysteines in the function, localization and reactivity of the TraV outer membrane lipoprotein encoded by the F plasmid. J. Bacteriol. 184: 3126-3129.

Heisenberg, M. (1966). Formation of defective bacteriophage particles by *fr* amber mutants. J. Mol. Biol. 17: 136-144.

Henrich, B., Lubitz, W., and Plapp, R. (1982). Lysis of *Escherichia coli* by induction of cloned ϕX174 genes. Mol. Gen. Genet. 185: 493-497.

Herrmann, R., Schubert, D., Rudolph, U. (1968). Self-assembly of protein subunits from bacteriophage fr. Biochem. Biophys. Res. Comm. 30: 576-581.

Hershey, A.D. and Rotman, R. (1949). Genetic recombination between host-range and plaque-type mutants of bacteriophage in single bacterial cells. Genetics. 34: 44-71.

Holmgren, M., Liu, Y., Xu, Y., Yellen, G. (1996). On the use of thiol-modifying agents to determine channel topology. Neuropharmacology. 35: 797-804.

Holtje, J.V. (1998). Growth of the stress-bearing and shape-maintaining murein sacculus of *Escherichia coli*. Microbiol. Mol. Biol. Rev. 62: 181-203.

Holtje, J.V., Fielder, W., Rotering, H., Walderich, B., van Duin, J. (1988). Lysis induction of *Escherichia coli* by the cloned lysis protein of the phage MS2 depends on the presence of osmoregulatory membrane-derived oligosaccharides. J. Biol. Chem. 263: 3539-3541.

Hori, K., Eoyang, L., Banerjee, A.K., August, J.T. (1967). Replication of RNA viruses. V. Template activity of synthetic riboploymers in the Qβ RNA polymerase reaction. Proc. Natl. Acad. Sci. USA. 57: 1790-1797.

Housman, D. (1971). The role of methionine in the initiation of protein synthesis. Ph.D. Thesis, Brandeis University, Waltham, MA.

Hung, P.P., Ling, C.M., Overby, L.R. (1969). Self-assembly of Qβ and MS2 phage particles: Possible function of initiation complexes. Science. 166: 1638-1640.

Hung, P.P., and Overby, L.R. (1969). The reconstitution of infective bacteriophage Qβ. Biochemistry. 8: 820-828.

Hussain, M., Ichihara, S., Mizushima, S. (1980). Accumulation of glyceride-containing precursor of the outer membrane lipoprotein in the cytoplasmic membrane of *Escherichia coli* treated with globomycin. J. Biol. Chem. 255: 3707-3712.

Ikeda, M., Wachi, M., Jung, H.K., Ishino, F., Matsuhashi, M., The *Escherichia coli mraY* gene encoding UDP-N-acetylmuramoyl-pentapeptide: Undecaprenyl-phosphate phospho-N-acetylmuramoyl-pentapeptide transferase. J. Bacteriol. 173: 1021-1026.

Ippen-Ihler, K.A., and Minkley, E.G., Jr. (1986). The conjugation system of F, the fertility factor of *Escherichia coli*. Ann. Rev. Genet. 20: 593-624.

Jalava, K., Eko., F.O., Riedmann, E., Lubitz, W. (2003). Bacterial ghosts as carrier and targeting systems for mucosal antigen delivery. Expert. Rev. Vaccines. 2: 45-51.

Kaback, H.R., Dunten, R., Frillingos, S., Venkatesan, P., Kwaw, I., Zhang, W., Ermolova, N. (2007). Site-directed alkylation and the alternating access model for LacY. Proc. Natl. Acad. Sci. USA 104: 491-494.

Kaerner, H.C. (1970). Sequential steps in the *in vivo* assembly of the RNA bacteriophage fr. J. Mol. Biol. 53: 515-529.

Kamen, R. (1970). Characterization of the subunits of Qβ replicase. Nature. 228: 527-533.

Karnik, S., Billeter, M. (1983). The lysis function of RNA bacteriophage Qβ is mediated by the maturation (A2) protein. EMBO J. 2: 1521-1526.

Karring, H., Mathu, S.G., van Duin, J., Clark, B.F., Kraal, B., Knudsen, C.R. (2004). Qβ-phage resistance by deletion of the coiled-coil motif in Elongation Factor Ts. J. Biol. Chem. 279: 1878-1884.

Kastelein, R.A., Remaut, E., Fiers, W., van Duin, J. (1982). Lysis gene expression of RNA phage MS2 depends on a frameshift during translation of the overlapping coat protein gene. Nature. 295: 35-41.

Kitagawa, M., Ara, T., Arifuzzaman, M., Ioka-Nakamichi, T., Inamoto, E., Toyonaga, H., Mori, H. (2005). A complete set of ORF clones of *Escherichia coli* ASKA library (a complete set of *E. coli* K-12 ORF archive): Unique resources for biological research. DNA Res. 12: 291-299.

Klovins, J., Tsareva, N.A., de Smit, M.H., Berzins, V., van Duin, J. (1997). Rapid evolution of translational control mechanisms in RNA genomes. J. Mol. Biol. 265: 372-384.

Klovins, J., Berzins, V., van Duin, J. (1998). A long-range interaction in Qβ RNA that bridges the thousand nucleotides between the M-site and the 3' end is required for replication. RNA 4: 948-957.

Knolle, P. (1969). Incorporation of amino acids into phage-like particles in cell-free systems of *E. coli*. Biochim. Biophys. Acta. 190: 496-503.

Knolle, P. (1972). Fate of R17 maturation protein after infection. Abstr. 8th Annu. FEBS Meeting, Amsterdam.

Kondo, M., Gallerani, R., Weissmann, C. (1970). Subunit structure of Qβ replicase. Nature. 228: 525-527.

Kozak, M., and Nathans, D. (1971). Fate of maturation protein during infection by coliphage MS2. Nature New Biol. 234: 209.

Krahn, P.M., O'Callaghan, R.J., Paranchych, W. (1972). Stages in phage R17 infection. VI. Injection of A protein and RNA in the host cell. Virology. 47: 628-637.

Kudela, P., Paukner, S., Mayr, U. B., Cholujova, D., Kohl, G., Schwarczova, Z., Bizik, J., Sedlak, J., Lubitz, W. (2007). Effective transfer to melanoma cells using bacterial ghosts. Cancer Lett. [Epub ahead of print.]

Lanzetta P.A., Alvarez, L.J., Reinach, P.S., Candia, O. (1979) An improved assay for nanomole amounts of inorganic phosphate. Anal. Biochem. 100: 95-97.

Lawley, T.D., Klimke, W.A., Gubbins, M.J., Frost, L.S. (2003). F factor conjugation is a true type IV secretion system. FEMS Microbiol. Lett. 224: 1-15.

Li, S.Y., Holtje, J.V., Young, K.D. (2003). Comparison of high-performance liquid chromatography and fluorophore-assisted carbohydrate electrophoresis methods for analyzing peptidoglycan composition of *Escherichia coli*. Anal. Biochem. 326: 1-12.

Licis, N., van Duin, J., Balklava, Z., Berzins, V. (1998). Long-range translational coupling in single-stranded RNA bacteriophages: An evolutionary analysis. Nucl. Acids Res. 26: 3242-3246.

Liu, J.D., and Parkinson, J.S. (1989). Genetics and sequence ananlysis of the *pcnB* locus, an *Escherichia coli* gene involved in plasmid copy number control. J. Bacteriol. 171: 1254-1261.

Lodish, H.F., Cooper, S., Zinder, N.D. (1964). Host-dependent mutants of bacteriophage f2. IV. On the biosynthesis of a viral RNA polymerase. Virology. 24: 60.

Lodish, H.F., Horiuchi, K., Zinder, N.D. (1965) Mutants of bacteriophage f2. V. On the production of noninfectious phage particles. Virology. 27: 139.

Lodish, H.F., and Zinder, N.D. (1966). Mutants of bacteriophage f2. VII. Control mechanisms for phage-specific synthesis. J. Mol. Biol. 19: 333.

Loeb, T. (1960). Isolation of a bacteriophage specific for the F^+ and Hfr mating types of *Escherichia coli* K12. Science. 131: 932-933.

Loeb, T., and Zinder, N.D. (1961). A bacteriophage containing RNA. Proc. Nac. Acad. Sci. 47: 282.

Lubitz, W. and Plapp, R. (1980). Murein degradation in *Escherichia coli* infected with bacteriophage ϕX174. Curr. Microbiol. 4: 301-304.

Lupker, J.H., Verschoor, G.J., de Rooij, F.W., Rorsch, A., Bosch, L. (1974). An *Escherichia coli* mutant with an altered elongation factor Tu. Proc. Natl. Acad. Sci. USA. 70: 460-463.

Luria, S.E. and Delbrück, M. (1943). Mutations of bacteria from virus sensitivity to virus resistance. Genetics. 28: 491-511.

Manchak, J., Anthony, K.G., Frost, L.S. (2002). Mutational analysis of F-pilin reveals domains for pilus assembly, phage infection and DNA transfer. Molec. Microbiol. 43: 195-205.

Markert, A., and Zillig, W. (1965). Studies on the lysis of *Escherichia coli* C by bacteriophage fX174. Virology. 25: 88-97.

Marquardt, J.L, Siegele, D.A., Kolter, R., Walsh, C.T. (1992). Cloning and sequencing of *Escherichia coli murZ* and purification of its product, a UDP-N-acetylglucosamine enolpyruvyl transferase. J. Bacteriol. 174: 5748-5752.

Miranda, G., Schuppli, D., Barrera, I., Hausherr, C., Sogo, J.M., Weber, H. (1997). Recognition of bacteriophage Qβ plus strand RNA as a template for Qβ replicase: Role of RNA interactions mediated by ribosomal proteins S1 and Host Factor. J. Mol. Biol. 267: 1089-1103.

Min-Jou, W., Haegeman, G., Ysebaert, M., Fiers, W. (1972). Nucleotide sequence of the gene coding for the bacteriophage MS2 coat protein. Nature. 237: 82.

Model, P., Webster, R.E, Zinder, N.D. (1979). Characterization of Op3, a lysis-defective mutant of bacteriophage f2. Cell. 18: 235-246.

Model, P., and Russel, M. (1988). Filamentous bacteriophage. In The Bacteriophages, Volume 2, R. Calendar, ed. (New York, NY: Plenum Press), pp 375-456.

Muffler, A., Fischer, D., Hengge-Aronis, R. (1996). The RNA-binding protein HF-I, known as a host factor for phage Qβ RNA replication, is essential for *rpoS* translation in *Escherichia coli*. Genes Dev. 10: 1143-1151.

Nishihara, T., Haruna, I., Watanabe, I., Nozu, Y., Okada, Y. (1969). Comparison of coat proteins from three groups of RNA phages. Virology. 37: 153.

Okazi, M., and Valentine, R.C. (1973). Inhibition of bacterial cell wall mucopeptide synthesis: A new function of RNA bacteriophage Qβ. Biochem. Biophys. Acta. 304: 707-714.

Osborn, M.J., and Munson, R. (1974). Separation of the inner (cytoplasmic) and outer membranes of Gram-negative bacteria. Methods Enzymol. 31: 642-653.

Osborn, M.J., and Wu. H.C. (1980). Proteins of the outer membrane of gram-negative bacteria. Annu Rev Microbiol. 34: 369-422.

Ou, J.T. (1973). Inhibition of formation of *Escherichia coli* mating pairs by f1 and MS2 bacteriophages as determined with a Coulter counter. J. Bacteriol. 114: 1108-1115.

Ou, J.T., and Anderson, T.F. (1972). Effect of Zn^{2+} on the adsorption of male-specific filamentous deoxyribonucleic acid and isometric ribonucleic acid phages. J. Virol. 10: 869-871.

Pace, C.N., Vajdos, F., Fee, L., Grimsley, G., Gray, T. (1995). How to measure and predict the molar absorption coefficient of a protein. Protein Sci. 4: 2411-2423.

Paranchych, W. (1975). Attachment, ejection and penetration stages of the RNA phage infections process. In RNA phages, N.D. Zinder, ed. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), pp. 85-111.

Paranchych, W., Krahn, P.M., Bradley, R.D. (1970). Stages in phage R17 infection. Virol. 41: 465-473.

Paranchych, W., and Krahn, P.M. (1971). The fate of "A" protein during R17 phage infection. Fed. Proc. 30: 1264.

Park, T.P., Struck, D.K., Dankenbrind, C.A., Young, R. (2007). The pinholin of lambdoid phage 21: Control of lysis by membrane depolarization. J. Bacteriol. 189: 9135-9139.

Pascual, J.M., Shieh, C.C., Kirsch, G.E., Brown, A.M. (1995). K^+ pore structure revealed by reporter cysteins at inner and outer surfaces. Neuron. 14: 1055-1063.

Paukner, S., Kohl, G., Javala, K., Lubitz, W. (2003). Sealed bacterial ghosts – novel targeting vehicles for advanced drug delivery of water-soluble substances. J. Drug Target. 11: 151-161.

Poot, R.A., Tsareva, N.V., Boni, I.V., van Duin, J. (1997). RNA folding kinetics regulates translation of phage MS2 maturation gene. Proc. Natl. Acad. Sci. USA. 94: 10110-10115.

Puglsey, A.P., Lee, D.R., Schnaitman, C.A. (1980). Genes affecting the major outer membrane proteins of *Escherichia coli* K-12: Mutations at nmpA and nmpB. Molec. Gen. Genet. 177: 681-690.

Raynal, L.C., Carpousis, A.J. (1999). Poly(A) polymerase I of *Escherichia coli*: Characterization of the catalytic domain, an RNA binding site and regions for the interaction with proteins involved in mRNA degradation. Mol. Microbiol. 32: 765-775.

Remaut, E., Stanssens, P., Fiers, W. (1981). Plasmid vectors for high-efficiency expression controlled by the *pL* promoter of coliphage lambda. Gene. 15: 81-93.

Remaut, E., Waele, P.D., Marmenout, A., Stanssens, P., Fiers, W. (1982). Functional expression of individual plasmid-coded RNA bacteriophage MS2 genes. EMBO. 1: 205-209.

Robertson, H.D. (1975). Functions of replicating RNA in cells infected by RNA bacteriophages. In RNA phages, N.D. Zinder, ed. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), pp. 85-111.

Robichon, C., Vidal-Ingigliardi, D., Pugsley, A.P. (2005). Depletion of apolipoprotein N-acyltransferase causes mislocalization of outer membrane lipoproteins in *Escherichia coli*. J. Biol. Chem. 280: 974-983.

Roof, W.D. and Young, R. (1993). ϕX174 *E* complements λ *S* and *R* dysfunction for host cell lysis. J. Bacteriol. 175: 3909-3912.

Roof, W.D., Fang, H.Q., Young, K.D., Sun, J., Young, R. (1997). Mutational analysis of slyD, an *Escherichia coli* gene encoding a protein of the FKBP immunophilin family. Mol Microbiol. 25: 1031-1046.

Sahin-Toth, M., Frillingos, S., Bibi, E., Gonzales, A., Kaback, H.R. (1994). The role of transmembrane III in the lactose permease of *Escherichia coli*. Protein Sci. 3: 2302-2310.

Sambrook, J., Fritch, E.F., and Miniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2 Edition, (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Satta, G., Canepari, P., Botta, G., Fontana, R. (1980). Control of cell septation by lateral wall extension in a pH-conditional morphology mutant of *Klebsiella pneumoniae*. J. Bacteriol. 142: 43-51.

Schoulaker, R., and Engelberg-Kulka, H. (1978). *Escherichia coli* mutant temperature sensitive for group I RNA bacteriophages. J. Virol. 25: 433-435.

Schoulaker, R., and Engelber-Kulka, H. (1981). *Escherichia coli traD*(Ts) mutant temperature sensitive for assembly of RNA bacteriophage MS2. J. Virol. 38: 833-839.

Schoulaker, R., and Engelberg-Kulka, H. (1983). Effect of an *Escherichia coli traD*(ts) mutation on MS2 RNA replication. J. Gen. Virol. 64: 207-210.

Schmidt, B.F., Berkhout, B., Overbeek, G.P., van Strien, A., van Duin, J. (1987). Determination of the RNA secondary structure that regulates lysis gene expression in bacteriophage MS2. J. Mol. Biol. 195: 505-516.

Schwarz, U., and Asmus, A. (1969). Autolytic enzymes and cell division of *Escherichia coli*. J. Mol. Biol. 41: 419-429.

Scott, D.W. (1965). Serological cross reactions among the RNA-containing coliphages. Virology. 26: 85.

Shimura, Y., Moses, R.E., Nathans, D. (1967). Coliphage MS2 containing 5-fluorouracil II. RNA-deficient particles formed in the presence of 5-fluorouracil. J. Mol. Biol. 28: 95.

Silverman, P.M., Mobach, H.W., Valentine, R.C. (1967). Sex hair (F-pili) mutants of *E. coli*. Biochem. biophys. Res. Commun. 27: 412.

Silverman, P.M., Rosenthal, S., Mobach, H., Valentine, R.C. (1968). Two new classes of F-pili mutants of *E. coli* resistant to infection by the male-specific bacteriophage f2. Virology. 36: 142.

Skirpkin, E.A., Adhin, M.R., de Smit, M.H., and van Duin, J. (1990). Secondary structure of the central region of bacteriophage MS2 RNA: Conservation and biological significance. J. Mol. Biol. 211: 447-463.

Smilowitz, H. (1974). Bacteriophage f1 infection: Fate of the parental coat protein. J. Virol. 13: 94-99.

Stavis, R.L., and August, J.T. (1970). The biochemistry of RNA bacteriophage replication. Annu. Rev. Biochem. 39: 527-560.

Steitz, J. (1968). Identification of the A protein as a structural component of bacteriophage R17. J. Mol. Biol. 33: 923-936.

Stockley, P.G., Rolfsson, O., Thompson, G.S., Basnak, G., Francese, S., Stonehouse, N.J., Homans, S.W., Ashcroft, A.E. (2007). A simple, RNA-mediated allosteric switch controls the pathway to formation of a T=3 viral capsid. J. Mol. Biol. 369: 541-552.

Struck, D.K., Durica, D.S., Young, R. (1984). Gene fusion in vivo using transductional cointegrates. Gene. 47: 221-230.

Su, Q., Schuppli, D., Tsui, H.C.T., Winkler, M.E., Weber, H. (1997). Strongly reduced phage Qβ replication, but normal MS2 replication in an *Escherichia coli* K12 mutant with inactivated Qβ Host Factor (*hfq*) gene. Virology. 227: 211-214.

Sun, T.P., and Webster, R.E. (1986). *fii*, a bacterial locus required for filamentous phage infection and its relation to colicin-tolerant *tolA* and *tolB*. J. Bacteriol. 165: 107-115.

Sun, T.P., and Webster, R.E. (1987). Nucleotide sequence of a gene cluster involved in the entry of the E colicins and the single-stranded DNA of infecting filamentous phage into *Escherichia coli*. J. Bacteriol. 169: 2667-2674.

Sun, Z.P., Akabas, M.H., Goulding, E.H., Karling, A., and Siegelbaum, S.A. (1996). Exposure of residues in the cyclic nucleotide-gated channel pore: P region structure and function in gating. Neuron. 16: 141-149.

Suzuki, M., Hara, H., Matsumoto, K. (2002). Envelope disorder of *Escherichia coli* cells lacking phosphatidylglycerol. J. Bacteriol. 184: 5418-5425.

Tokunaga, H., Loranger, J.M., and Wu, H.C. (1984). Prolipoprotein modification and processing enzymes in *Escherichia coli*. J. Biol Chem. 259: 3825-3830.

Tomasz, A., and Waks, S. (1975). Mechanism of action of penicillin: Triggering of the pneumococcal autolyic enzyme by inhibitors of cell wall synthesis. Proc. Natl. Acad. Sci. USA. 72: 4162-4166.

Tomasz, A., and Holtje, J.V. (1977). Murein hydrolases and the lytic and killing action of penicillin. In Microbiology 1977, D. Schlessinger, ed. (Washington, D.C: American Society for Microbiology), pp. 209-215.

Tran, T.A.T., Struck, D.K., Young, R. (2005). Periplasmic domains define holin-antiholin interactions in T4 lysis inhibition. J. Bacteriol. 187: 6631-6640.

Tsui, H.C., Leung, H.C., Winkler, M.E. (1994). Characterization of broadly pleiotropic phenotypes caused by an hfq insertion mutation in *Escherichia coli* K-12. Mol. Microbiol. 13: 35-49.

van Duin, J. (1988). Single-stranded RNA bacteriophages. In The Bacteriophages, R. Calendar, ed. (New York, NY: Plenum Press), pp.117-167.

van Duin, J., and Tsareva, N.V. (2006). Single-stranded RNA phages. In The Bacteriophages, 2nd Edition, R. Calendar, ed. (New York, NY: Plenum Press), pp. 175-196.

Verbraeken, E., and Fiers, W. (1972). Further evidence on the role of the A protein in bacteriophage MS2 particles. FEBS Lett. 28: 89-92.

Von Heijne, G. (1995). Membrane protein assembly: Rules of the game. BioEssays. 17: 25-30.

Wahba, A.J., Miller, M.J., Niveleau, A., Landers, T.A., Carmichael, G.G., Weber, K., Hawley, D.A., Slobin, L.I. (1974). Subunit I of Qβ replicase and 30S ribosomal protein S1 of *Escherichia coli*: Evidence of the identity of the two proteins. J. Biol. Chem. 249: 2214-3316.

Walderich, B., Ursinus-Wossner, A., van Duin, J., Holtje, J.V. (1988). Induction of the autolytic system of *Escherichia coli* by specific insertion of bacteriophage MS2 lysis protein into the bacterial cell envelope. J. Bacteriol. 170: 5027-5033.

Walderich, B., and Holtje, J.V. (1989). Specific localization of the lysis protein of bacteriophage MS2 in membrane adhesion sites of *Escherichia coli*. J. Bacteriol. 171: 3331-3336.

Watanabe, I., Miyake, T., Sakurai, T., Shiba, T., Ohno, T. (1967). Isolation and grouping of RNA phages. Proc. Japan. Acad. 43: 204.

Waxman, D.J., Yu, W., Strominger, J.L. (1980). Linear, uncross-linked peptidoglycan secreted by penicillin-treated *Bacillus subtilis*. J. Biol. Chem. 255: 11577-11587.

Wessel, D., and Flugge, U.I. (1984). A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. Anal. Biochem. 138: 141-143.

Willets, N., and Achtman, M. (1972). Genetic analysis of transfer by the *Escherichia coli* sex factor F, using P1 transductional complementation. J. Bacteriol. 110: 843-851.

Willets, N., and Skurray, R. (1980). The conjugation system of F-like plasmids. Ann. Rev. Genet. 14:41-76.

Winter, R.B., Gold, L. (1983). Overproduction of bacteriophage Qβ maturation (A_2) protein leads to cell lysis. Cell. 33: 877-885.

Witte, A., Wanner, G., Blasi, U., Halfmann, G., Szostak, M., Lubitz, W. (1990). Endogenous transmembrane tunnel formation mediated by PhiX174 lysis protein E. J. Bacteriol. 172: 4109-4114.

Witte, A., Wanner, G., Sulzner, M., Lubitz, W. (1992). Dynamics of phiX174 protein E-mediated lysis of *Escherichia coli*. Arch. Microbiol. 154: 381-388.

Wong, K., and Paranchych, W. (1976). The polarity of penetration of phage R17 RNA. Virology. 73: 489-497.

Xu, M., Struck, D.K., Deaton, J., Wang, I.N., Young, R. (2004). A signal-arrest-release sequence mediates export and control of the P1 phage endolysin. Proc. Natl. Acad. Sci. USA 101: 6415-6520.

Xu, M., Arulandu, A., Struck, D.K., Swanson, S., Sacchettini, J.C., Young, R. (2005). Disulfide isomerization after membrane release of its SAR domain activates P1 lysozyme. Science. 307: 113-117.

Yagur-Kroll, S., and Amster-Choder, O. (2005). Dynamic membrane topology of the *Escherichia coli* β-glucoside transporter BglF. J. Biol. Chem. 280: 19306-19318.

Young, K.D. (2001). Approaching the physiological functions of penicillin-binding proteins in *Escherichia coli*. Biochimie. 83: 99-102.

Young, K.D., and Young, R. (1982). Lytic action of cloned ϕX174 gene *E*. J. Virol. 44: 993-1002.

Zinder, N.D., and Lyons, L.B. (1968). Cell lysis: Another function of the coat protein of the bacteriophage f2. Science. 159: 84-86.

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