THE ϕX174 LYSIS PROTEIN E: A PROTEIN INHIBITOR OF THE CONSERVED TRANSLOCASE MraY

A Dissertation

by

YI ZHENG

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 2009

Major Subject: Biochemistry

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Approved by:

Chair of Committee,	Ryland Young
Committee Members,	J. Martin Scholtz
	Vladislav Panin
	Jonathan Skare
Head of Department,	Gregory Reinhart

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ABSTRACT

The ϕX174 Lysis Protein E: A Protein Inhibitor of the Conserved Translocase MraY.

(May 2009)

Yi Zheng, B.S., Nankai University

Chair of Advisory Committee: Dr. Ryland Young

Most bacteriophages release progeny virions at the end of the infection cycle by lysis of the host. Large phages with double-stranded DNA genomes use a multigene strategy based on holins, small membrane proteins, and bacteriolytic enzymes, or endolysins. Holins mediate the control of endolysin activity and thus the timing of lysis. Phages with small genomes only encode a single protein for cell lysis. There are three known unrelated single protein lysis systems: the ϕX174 E protein, the MS2 L protein, and the Qβ A_2 protein. None of these phages encodes a cell wall degrading activity, and previous work has shown that the lytic activity of E stems from its ability to inhibit the host enzyme, MraY, which catalyzes the formation of lipid I, the first lipid intermediate in cell wall synthesis.

The purpose of the work described in this dissertation was to characterize the ϕX174 E-mediated inhibition of MraY using genetic and biochemical strategies. A fundamental question was why no large phages use the single gene system. This was addressed by constructing a recombinant phage, λE, in which the holin-endolysin based lysis cassette of λ was replaced with *E*. λE was compared with λ in genetic and

physiological experiments, with the results indicating that the holin-endolysin system increases fitness in terms of adjusting lysis timing to environmental conditions. Using λE, physiological experiments were conducted to characterize the interaction between E and MraY *in vivo*. Transmembrane domains (TMD) 5 and 9 have been identified as the potential E binding site by isolating MraY mutants resistant to E inhibition. The five E-resistant MraY mutants were found to fall into three classes, which reflect the apparent affinity of the mutant proteins for E. Finally, an assay for MraY activity employing the dansylated UDP-MurNAc-pentapeptide and phytol-P, was used to demonstrate the inhibition of MraY by purified E protein. It was determined that E is a non-competitive inhibitor for MraY in respect with both substrates. A model for E-mediated inhibition of MraY was proposed, in which E binds to TMDs 5 and 9 in MraY and thus inactivates the enzyme by inducing a conformational change.

DEDICATION

This dissertation is dedicated to my parents and to my husband Xiaomin Lin.

ACKNOWLEDGEMENTS

I would like to thank my committee members: Dr. J. Martin Scholtz, Dr. Vlad Panin, and Dr. Jon Skare, for their helpful suggestions and excellent outside guidance during the course of this work. I would especially like to thank my advisor, Dr. Ryland Young, for his continuant guidance, expertise, enthusiasm, and great teaching. Ry has always looked out for me and I could not have asked for a better graduate experience. Thanks Ry for everything you have done for me!

I would like to express my appreciation for Dr. Doug Struck, for his invaluable advice and daily supervision. He has taught me most of what I know about lab work in biochemistry and been absolutely essential to the biochemical part of my research. Special thanks go to Daisy for her assistance and encouragement. I would also like to thank all of the Young lab members, past and present, for their ideas, feedback and support. Min, Anh, Taehyun, Brenley and Rebecca have taught me so much.

Thanks also go to all my friends for being there for me, and the department faculty and staff for making my time at Texas A&M University a great experience. I can never thank my parents enough for their unconditional love and support every step of the way. Xiaomin, my lovely husband, I love you and sincerely appreciate your love, patience and support for all these years.

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CHAPTER I

INTRODUCTION

This dissertation is focused on the mechanism of action of the E lysis protein of the phage ϕX174. Both the *E* gene and the phage have a special place in the history of molecular biology. ϕX174 is a small bacteriophage with a circular, single-stranded (ss), positive polarity (+) DNA genome and an icosahedral protein capsid. It is the prototype for the *Microviridae*, a group characterized as small icosahedral phages with ss circular chromosomes, including members such as G4, S13 and α3. ϕX174 was the first organism whose DNA genome sequence was completely determined. Sanger *et al.* (SANGER *et al.* 1977; SANGER *et al.* 1978) sequenced the entire ϕX174 genome with the 5386 nucleotides using the "plus and minus" method and this earned Fred Sanger the chemistry Nobel prize in 1980. Michael Smith received the Nobel Prize in 1993 in chemistry for inventing a method for mutagenesis at a specific position in a DNA sequence, using the ϕX174 *am3* mutant as template (HUTCHISON *et al.* 1978), and as a result, gene *E,* lysis gene within which the nonsense mutation was located, was the first gene to be subjected to site-directed mutagenesis. Moreover, the *Microviridae* phages are ubiquitous, found for many if not all Gram-negative bacteria, even the intracellular bacterium *Chlamydia* (GARNER *et al.* 2004). Also, because of their simple nature, these phages were proposed as facile model systems in developing our current understanding

This dissertation follows the style of Genetics.

of DNA replication, transcription, gene expression, and morphogenesis.

ϕX174 biology

The ϕX174 genome harbors 11 genes, 9 of which are essential (HAYASHI 1978). Functions of the gene products are shown in Table 1.1. The sum of the estimated molecular weights of the protein products as identified by polyacrylamide gel electrophoresis is more than the coding capacity of the 5386 nucleotide genome (DENHARDT 1977), which initially indicated the existence of overlapping genes. It was later discovered that gene *A** is embedded in gene *A* in the same reading frame, while gene *B* is completely encoded within gene *A* in an alternate reading frame. Gene *E* is also embedded in an alternate reading frame within gene *D*, while gene *K* overlaps genes *A* and *C*. Figure. 1.1 shows the genetic map of ϕX174.

Table 1.1. Function of the ϕX174 gene products. The number of amino acid residues and the molecular weight of a protein are derived from the DNA sequence. Adapted from Fane *et al.* ((FANE *et al.* 2006) and Hayashi *et al*. (HAYASHI *et al.* 1988).

Protein	Function	# of amino acid residues	Molecular weight
A	Stage II and stage III DNA replication	513	58,650
A*	An unessential protein for viral propagation. It may play a role in the inhibition of host cell DNA replication and superinfection exclusion	341	38,700
B	Internal scaffolding protein, required for procapsid morphogenesis and the assembly of early morphogenetic intermediates. Sixty copies in the procapsid	120	13,830
C	Facilitates the switch from stage II to stage III DNA replication. Required for stage III DNA synthesis	86	10,050
D	External scaffolding protein, required for procapsid morphogenesis. Two hundred and forty copies present in the procapsid	152	16,920
E	Host cell lysis	91	10,370
F	Major coat protein. Sixty copies present in the procapsid and virion	427	48,400
G	Major spike protein. Sixty copies present in the procapsid and virion	175	19,020
H	DNS pilot protein needed for DNA injection, also called the minor spike protein. Twelve copies in the procapsid and virion	328	34,370
J	DNA binding protein, needed for DNA packaging. Sixty copies present in the virion	38	4,220
K	An unessential protein for viral propagation. It may play a role optimizing burst size in various hosts	56	6,380

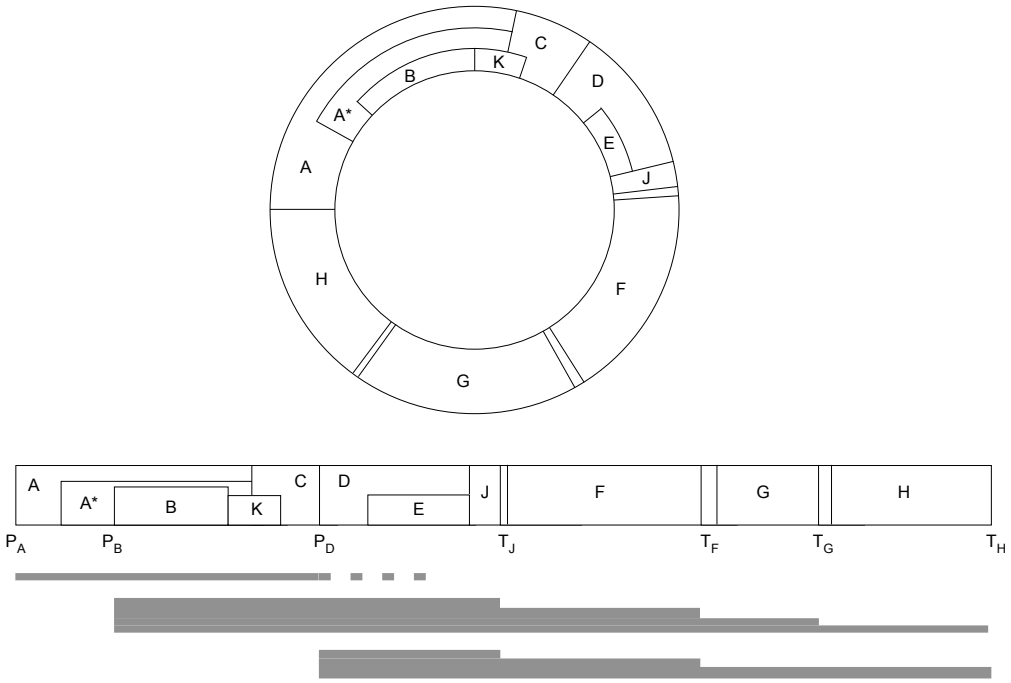
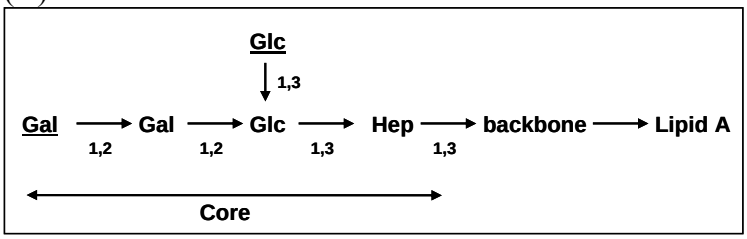


Figure 1.1. ϕX174 gene map. In the linear map, P and T are promoters and transcription terminators, respectively. Underneath the linear map are the mRNA transcripts, and line thickness represents the relative abundance. The gene *A* transcript is unstable and the termination site is not known. Adapted from Fane *et al*. (FANE *et al.* 2006).

ϕX174 infection of *E. coli* can be divided into three steps: adsorption of the phage to its receptor on the host surface, eclipse of the protein capsid and the ejection of the genomic DNA, and penetration of genome into the host cell (INCARDONA 1978). ϕX174 recognizes its host, which is the *Escherichia coli* C strain in the research laboratory, by binding to the outer membrane lipopolysacharide (LPS), specially the core polysaccharide of the LPS (INCARDONA and SELVIDGE 1973) (Fig. 1.2). Mutational analysis indicated that neither the heptose region nor the (1-3)-linked glucose residues are required for binding. However, the loss of the terminal (1-2)-linked galactose residue severely impairs adsorption (FEIGE and STIRM 1976). The common *E. coli* K-12

laboratory strain is naturally resistant to ϕX174 but can become sensitive with an altered LPS core (Fig. 1.2B) (OHKAWA 1980).

(A)



(B)

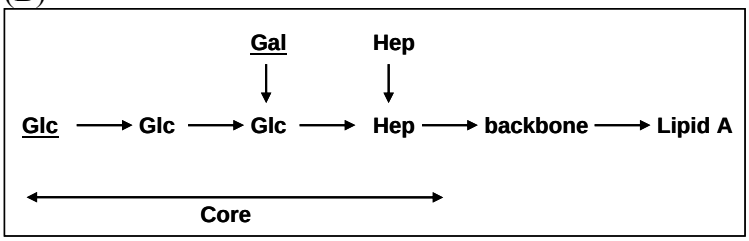


Figure 1.2. Structure of the core oligosaccharide. (A) from *E. coli* C required for phage binding. (B) from *E. coli* K-12. Gal, galactose; Glc, glucose; Hep, heptose. Adapted from Hayashi *et al*. (HAYASHI *et al.* 1988) and Gmeiner and Schlecht (GMEINER and SCHLECHT 1980).

After ϕX174 is attached to the host surface by reversible binding to LPS, the phage enters the eclipse phase of its life cycle, which is an irreversible reaction involving the DNA ejection from the protein capsid. The lipid A portion of the LPS (Fig. 1.2) is required for eclipse (JAZWINSKI *et al.* 1975b). The mechanism of how the eclipsed DNA penetrates the host cell is still unknown, but this step requires active metabolism in the host cell (HAYASHI *et al.* 1988) and the viral H protein as a pilot protein to guide the DNA into the cell (JAZWINSKI *et al.* 1975a).

DNA replication of the genome occurs after DNA ejection and penetration into the host cell. Replication of the ϕX174 (+) ss DNA genome can be separated into three stages. In stage I, the ss DNA is converted into RF I DNA (replicative form one DNA) which is a double-stranded (ds) circular molecule. During stage II, RF I DNA is amplified, and the (+) strand is replicated through a rolling circle mechanism (BENBOW *et al.* 1971). In Stage III, the viral circular DNA genome is synthesized and packaged into the procapsids. Stage I DNA replication does not require any phage-encoded proteins; however, in each of the three stages, a number of host proteins are required. For example, stage I requires at least 13 host proteins; in addition to the host proteins involved in stage I, stage II also requires the host *rep* gene product; DNA polymerase III holoenzyme is required in stage III replication.

During gene expression, the negative strand must first be synthesized before any transcription or translation can occur. There are promoters found upstream of genes *A*, *B*, and *D*, and terminators after genes *J*, *F*, *G* and *H* (HAYASHI *et al.* 1988) (Fig. 1.1). Since no temporal switch for transcription or translation has been found, viral proteins production is dependent on promoters, terminators, and mRNA stability. The relative abundance of mRNAs is shown in Figure 1.1.

The structure and morphogenesis of ϕX174 have been studied in great detail and the results must be viewed as one of the success stories in structural biology (DOKLAND *et al.* 1999; DOKLAND *et al.* 1997; MCKENNA *et al.* 1996; MCKENNA *et al.* 1994; MCKENNA *et al.* 1992). The mature ϕX174 virion consists of proteins F, G, H and J, while proteins B and D are internal and external scaffolding proteins, respectively, that

are required for formation of the procapsid. The ϕX174 assembly pathway is shown in Figure 1.3. During the first step of morphogenesis, the spike protein G and coat protein F each form pentamers. Secondly, the internal scaffolding protein B binds to the F pentamers and this allows interaction with the G protein pentamers and the external scaffolding protein D. Protein H is incorporated into this complex as part of the phage spike. The complex with proteins F, G, H, B and D is the procapsid. Protein J is an extremely basic protein and binds the genome and helps with DNA packaging into the procapsid. Maturation of the virion involves packaging of DNA and J proteins, extrusion of the B protein, and disassembly of the external scaffold by release of the D protein (CHEN *et al.* 2007). The higher resolution of ϕX174 procapsid structure solved with information provided by crystal structures has allowed the results from genetic and biochemical experiments to be interpreted in a structural context, and has made the ϕX174 system desirable for morphogenesis studies (FANE *et al.* 2006).

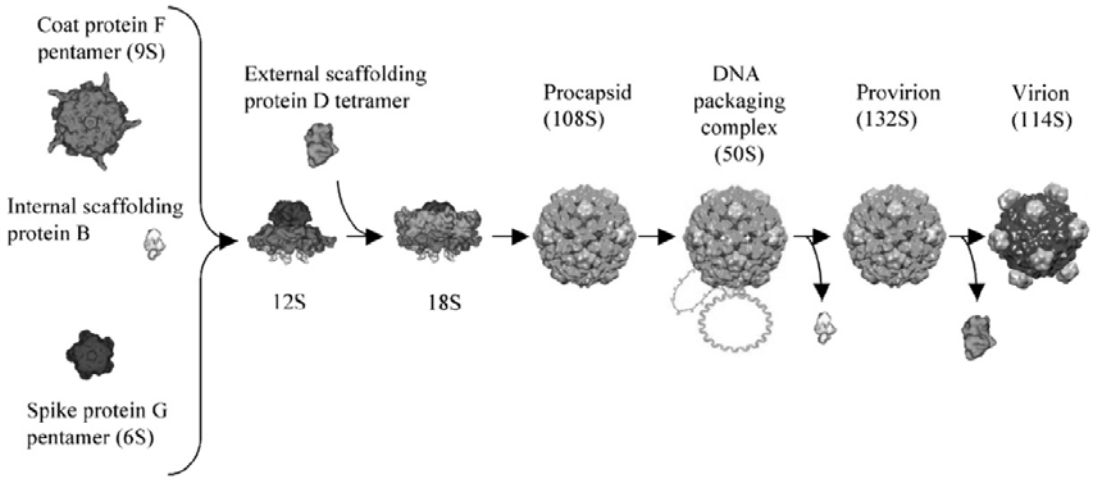


Figure 1.3. The pathway of ϕX174 assembly (Chen *et al.*, 2008).

The ϕX174 progeny virions, typically ~200 per infected cell, are released to the environment by lysis of the host cell, usually 20 min after infection. The essential gene *E*, a short reading frame embedded in an alternate reading frame within the essential gene *D*, is the gene responsible for this last step during the phage lifecycle. Protein E has 91 amino acids and an N-terminal hydrophobic stretch. The structure of the *E* gene and E-mediated lysis of the host will be discussed in detail later in this chapter.

Cell wall structure and synthesis

At the end of their life-cycle, bacteriophages must release their progeny from the host cell to find new prey. Filamentous bacteriophages, like M13, can extrude from their host without killing the cells utilizing their sophisticated secretion apparatus (RAKONJAC *et al.* 1999). All other bacteriophages escape by lysing their host. Most bacteria have a cell wall that is essential for them to resist high intracellular osmotic pressure and maintain their specific cell shape and integrity. This meshwork of peptidoglycan is the main barrier for bacteriophage progeny release. As a result, lytic bacteriophages have developed lysis systems that target the destruction of this structure. So a basic understanding of the bacterial cell wall structure and synthesis will be briefly reviewed using *E. coli* as a model system.

The cell wall consists of glycan strands and peptide chains, and these two chemically different threads form covalent bonds in two or three dimensions (HOLTJE 1998), so the cell wall material is often referred to as peptidoglycan or murein. The

covalent linkage results in a single-layered structure in most Gram-negative bacteria and a multi-layered structure in Gram-positive bacteria. The glycan strands are formed by repeating units composed of N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) linked by β-1,4 glycosidic bonds (Fig. 1.4A). The peptide chain is attached to the lactyl group of the muramic acid (Fig. 1.4A). In *E. coli*, this peptide is L-Ala-γ-D-Glu-m-diaminopimelic acid (DAP)-D-Ala-D-Ala in the nascent chain, and the terminal D-Ala is removed in the mature cell wall as part of the cross-linking reaction. The pentapeptide composition often varies in different bacterial species, unlike the glycan strand, which shows only few variations. However, a dibasic amino acid is always at the third position to enable formation of the cross-linking peptide bond with the adjacent glycan strands, usually between the amino group of the diamino-acid and the carboxyl group of D-Ala at position 4 (Fig. 1.4B).

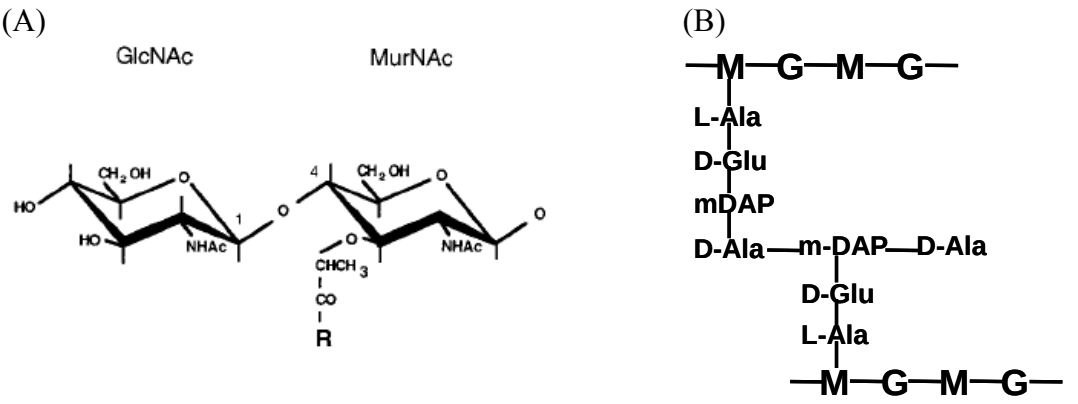


Figure 1.4. Cell wall structure. (A) Repeating unit of cell wall (HOLTJE 1998). R= L-Ala-γ-D-Glu-*m*-DAP-D-Ala-D-Ala in *E. coli*. (B) Cross-linking in cell wall. Adapted from Nanninga (NANNINGA 1998). G: GlcNAc; M, MurNAc.

The cell wall synthesis pathway involves many reactions and can be separated into three stages depending on where the reactions take place (Fig. 1.5) (for reviews see, (BARRETEAU *et al.* 2008; BOUHSS *et al.* 2008)). The first stage involves steps in the cytoplasm and can be divided into two major sets of reactions: (1) conversion of UDP-GlcNAc to UDP-MurNAc by enzymes MurA and MurB, (2) assembly of the pentapeptide from the lactyl moiety of UDP-MurNAc by enzymes MurC, D, E and F to form UDP-MurNAc-pentapeptide, the final cytoplasmic precursor. The second stage involves biosynthesis of the lipid-linked intermediates and occurs at the inner leaflet of the cytoplamic membrane. First, phospho-MurNAc-pentapeptide (P-MurNAc-pentapeptide) is transferred from UMP onto undecaprenol-phosphate (C_{55}-P), the carrier lipid, to form C_{55}-P-P-MurNAc-pentapeptide (lipid I), which is an essential intermediate molecule in the pathway. P-MurNAc-pentapeptide translocase (MraY), an integral membrane protein, catalyzes the formation of lipid I, and therefore links the cytoplamic and periplamic steps of cell wall biosynthesis. Then MurG, a membrane associated

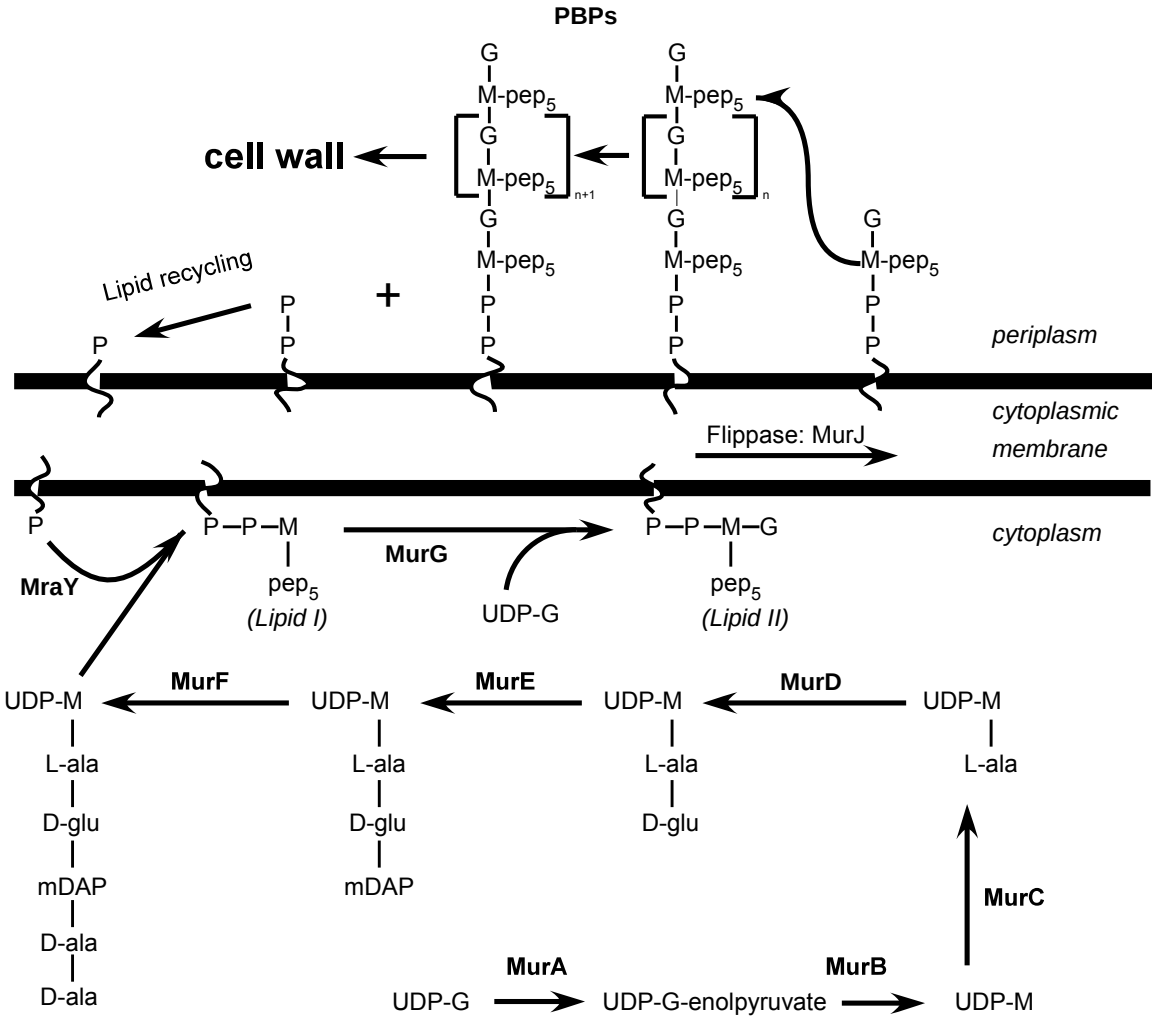


Figure 1.5. Cell wall biosynthesis pathway. C_{55}-P is represented as the curved line in the membrane. G, GlcNac; M, MurNAc; pep_5, pentapeptide; PBPs, penicillin-binding proteins; P, phosphate. Adapted from Nanninga (NANNINGA 1998).

protein, transfers the GlcNAc moiety from UDP-GlcNAc to lipid I thereby generating lipid II containing a β-linked disaccharide pentapeptide, the monomeric unit of the cell wall. The disaccharide pentapeptide is then flipped to the outer face of the cytoplamic membrane, where the last stage of cell wall synthesis occurs. The flippase responsible for this step has recently been identified as MurJ in *E. coli* by Ruiz (RUIZ 2008) using

bioinformatic and genetic approaches. After being flipped, the disaccharide pentapeptide is polymerized (transglycosylation) and cross-linked (transpeptidation) into the pre-existing cell wall catalyzed by the penicillin-binding proteins (PBPs). The 12 PBPs of *E. coli* have been subjected to various investigations (for reviews see Sauvage (SAUVAGE *et al.* 2008)).

Holtje (HOLTJE 1998) proposed a three-for-one model for murein growth. In this model, during elongation, one old glycan strand is degraded and replaced by three new glycan strands in the murein layer (Fig. 1.6). As a result of the degradation, there are pre-existing free single murein strands that act as primer strands. Two newly synthesized strands are cross-linked to one primer strand on each side. Then, in the stress-bearing murein layer, the triplet is attached to the docking strand, which is removed by murein hydrolases followed by the triplet insertion into the murein. During elongation, there is about 7% loss of the peptidoglycan components from the cell, referred to as peptidoglycan turnover, and peptidoglycan recycling then captures and reutilizes the turnover products (PARK and UEHARA 2008). It is clear that murein-degrading enzymes play important roles in the synthesis of the cell wall. Therefore, activities of these enzymes need to be carefully regulated. Unlike the stringent response induced upon amino acid starvation and other stresses, cells do not seem to have any feedback control to protect itself from sudden loss of precursors. Thus, uncontrolled action of these murein-degrading enzymes induced by insult to the cell, such as antibiotics that inhibit the synthesis of the cell wall precursors or inhibit the PBPs, causes cell lysis. The

resulting lysis all has similar phenotypic characteristics, such as requirement of growth and large blebs emerged from the mid-cell.

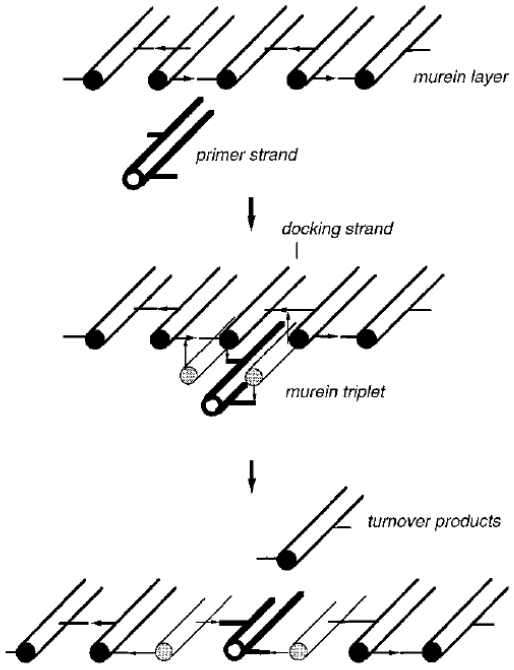


Figure 1.6. Murein elongation according to the three-for-one growth model (HOLTJE 1998).

Bacteriophage lysis strategies

Bacteriophages must release their progeny virions at the end of their vegetative cycle. Most of them accomplish this by causing lysis of the host cell. There are at least two distinct strategies employed by lytic bacteriophages (YOUNG 1992). All double-stranded (ds) nucleic acid bacteriophages with genomes larger than 20kb in size, among

which phage λ is the prototypic model, have a holin-endolysin system. Endolysins are enzymes with cell wall degrading activity. The prototypical endolysins such as λ R or T4 E are cytoplasmic proteins with no secretory signals to cross the cytoplamic membrane and thus gain access to their substrate. Holins are small proteins that accumulate in the cytoplasmic membrane, and at a genetically programmed time, form membrane lesions that allow the release of endolysin molecules into the periplasm where they degrade the cell wall and cause cell lysis within seconds (Fig. 1.7 C and D). Recently, a new group of endolysins have been indentified called SAR (signal-arrest-release)-endolysins (Xu *et al.* 2005), of which the endolysins from bacteriophages P1 and 21 are considered the prototype. The SAR-endolysins are exported by the host *sec* system and reach to periplasmic space but are tethered to the cytoplasmic membrane by their SAR domain. Importantly, the exported SAR endolysins are inactive in their tethered forms, so the infected cell does not begin to undergo lysis when the endolysins are expressed. The SAR domain exits the membrane when holin depolarizes the membrane, and this releases and activates the endolysin, resulting in cell wall degradation and cell lysis (Fig. 1.7 A and B).

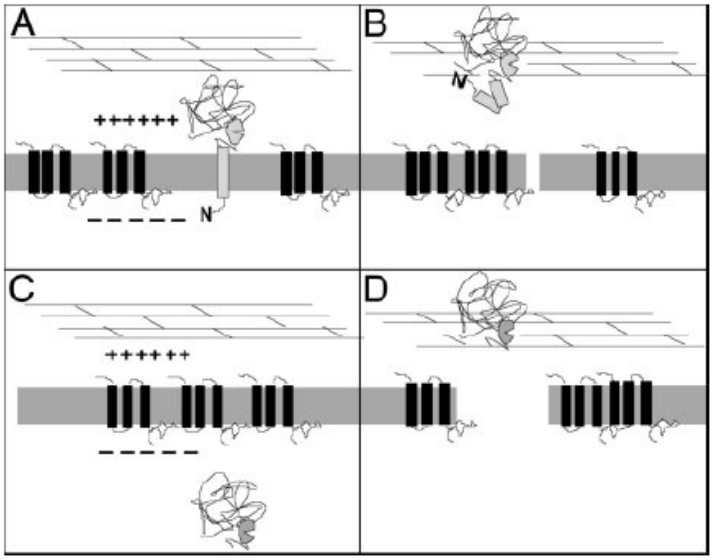


Figure 1.7. Model of holin-endolysin mediated lysis. (A) and (B) with SAR-endolysins. (C) and (D) with cytoplasmic endolysins (XU *et al.* 2004).

In contrast, lytic bacteriophages with genomes composed of ss nucleic acid and smaller than 6 kb, which cannot afford to encode many proteins, have been found to encode a single lysis protein ("single-gene lysis", (YOUNG *et al.* 2000)). Three types of small phages with either ssDNA or ssRNA genomes have been isolated. As noted above, ϕX174 is the prototype of small isometric phages with circular, ssDNA genomes, the *Microviridae*. There are also ssRNA male-specific phages of two morphologically similar classes, *Leviviridae* and *Alloleviviridae*, with MS2 and Qβ as the prototypes, respectively. None of these phages encodes cell wall degrading activity, which indicates they must use a mechanism distinct from the holin-endolysin system. In fact, these three types of small phages comprise three unrelated single gene lysis systems (Fig. 1.8): ϕX174 *E*, MS2 *L* protein and Qβ A_2.

Genetic and biochemical experiments have demonstrated that both E and A_2 inhibit cell wall synthesis at different steps (BERNHARDT *et al.* 2001a; BERNHARDT *et al.* 2001b), and lysis induced by these phages share a lot of characteristics with the lysis caused by the antibiotic penicillin, such as dependence on cell growth and the terminal phenotype of lysis observed by microscopy (discussed in detail later in this chapter). Penicillin causes lysis by inhibiting cell wall synthesis at the step of cross-linking between peptidoglycan strands (TOMASZ 1979). The mechanism of L-mediated lysis is as yet unknown. Below, the existing information about these single-gene lysis systems is reviewed.

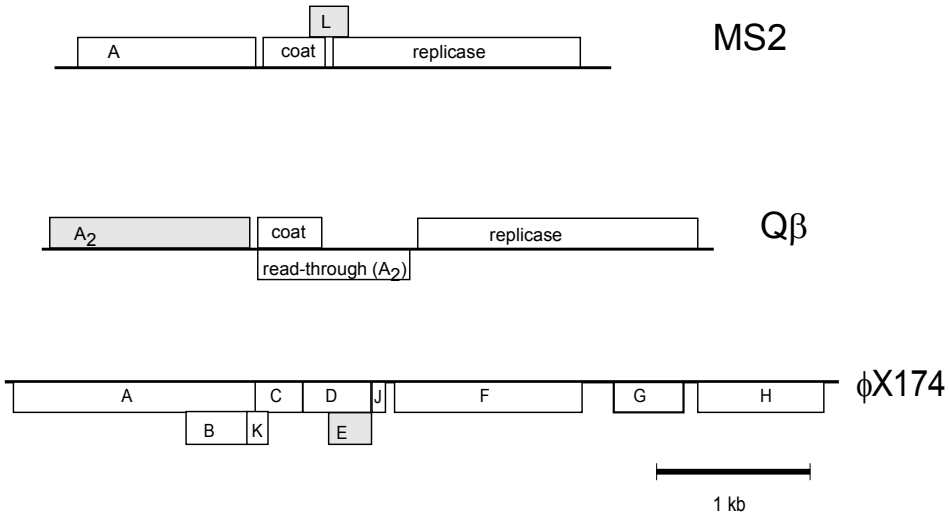


Figure 1.8. Small lytic phages with single-gene lysis systems. The phage lysis genes are shaded. ϕX174 is shown in a linear representation starting at gene *A*. Adapted from Bernhardt *et al.* (BERNHARDT *et al.* 2002b).

Lysis of the host by ϕX174

Initial characterization of ϕX174-induced lysis

The mechanism of release of ϕX174 has long been of interest. There were initially three possible hypotheses on the mechanism of lysis mediated by ϕX174: (1) existence of a phage encoded cell wall degrading enzyme like endolysin in phage λ; (2) blockage of cell wall synthesis; (3) induction of a bacterial lytic system. The bacterial lytic system includes cellular enzymes that are capable of cleaving covalent bonds in the peptidoglycan and causing lysis. These lytic enzymes must be tightly regulated, to avoid ectopic and lethal degradation of cell wall. In some bacteria, cell wall degradation can be induced, either by physiological stress, chemical antibiotics or as normal part of the growth cycle (i.e., in sporulation) (VOLLMER *et al.* 2008). The various forms of this process are called autolysis, and the lytic enzymes involved are referred to as the autolytic system (HOLTJE and TUOMANEN 1991). Autolysis plays very important roles in genetic transformation (allolysis) in Gram-positive bacteria (for bacterial lytic enzyme or hydrolases, see review (VOLLMER *et al.* 2008)). However, in *E. coli*, autolysis is merely a failure to maintain normal cell wall functions, which is always induced by factors such as antibiotics and chemicals.

Several unsuccessful attempts were made to try to identify a lytic enzyme (EIGNER *et al.* 1963; MARKERT and ZILLIG 1965). Together with the fact that phage release took a relatively long time, longer than the minimum latent period, this led

Eigner *et al.* (EIGNER *et al.* 1963; HUTCHISON and SINSHEIMER 1963) to question whether these phages cause lysis at all and suggest that ϕX174 release occurred before lysis, and phage leakage leading to cell wall damage might be the cause of lysis. However, Hutchison and Sinsheimer (HUTCHISON and SINSHEIMER 1963) developed a method to observe phage release from a single infected cell and demonstrated that all of the mature phages were released by a single burst that occurred in within 30 seconds, and prior to this burst/lysis, there was no free phage found. Thus, the long release time in bulk culture is merely a reflection of different lengths of time required by individual infected cells to enter the lytic process (YOUNG 1992). Since the isolation of a ϕX174 encoded lytic enzyme was not successful, the lysis mechanism of ϕX174 was proposed to be either blocking cell wall synthesis or inducing host autolytic systems or both (MARKERT and ZILLIG 1965).

Bradley *et al.* (BRADLEY *et al.* 1969) studied the infective process of phage α3, a ϕX174 type phage that is morphologically indistinguishable from ϕX174 but with a slightly different host range. By investigating infected *E. coli* cells with phase contrast and electron microscopy, it was observed that during the lytic event, blebs of membrane protrusions emerged from the cell midpoint (Fig. 1.9) where a lesion in the cell wall had occurred. Rupture of these blebs allowed phage release. It was also found that the process of phage induced lysis was very similar to penicillin induced lysis. ϕX174 infected cells were also examined by phase-contrast microscopy, and the morphological changes during lysis were very similar to that of α3 observed by Bradley *et al.* (BRADLEY *et al.* 1969; YOUNG 1992).

(A) (B)

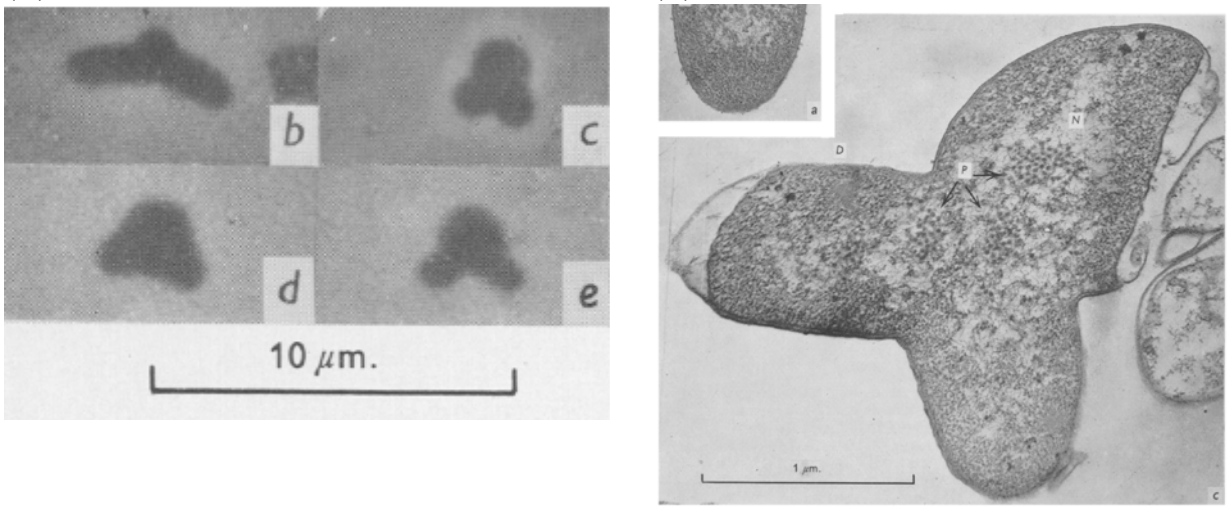


Figure 1.9. Morphologies of ϕX174 related phage α3 induced lysis. (A) phase contrast micrographs of α3 infected *E. coli* ((b) and (c)) and typical penicillin spheroplasts of *E. coli* ((d) and (e)). (B) α3 infected cells investigated by electron microscopy (BRADLEY *et al.* 1969).

Discovery and characterization of the ϕX174 lysis protein

It has been shown that addition of chloramphenicol no later than 12 minutes after infection inhibited lysis, and this suggested that a ϕX174 encoded protein was likely required for lysis (MARKERT and ZILLIG 1965). Hutchison and Sinsheimer (HUTCHISON and SINSHEIMER 1966) found a class of *amber* (*am*) mutants that were defective in cell lysis but were able to replicate normally in the non-permissive host. These mutants were shown to compose a single complementation group that was later designated gene *E*. They then studied a particular mutant, ϕX174 *am*3, in detail. Infection with this mutant yielded about 10 times more progeny than the wt ϕX174 phage. Less than 2% of these

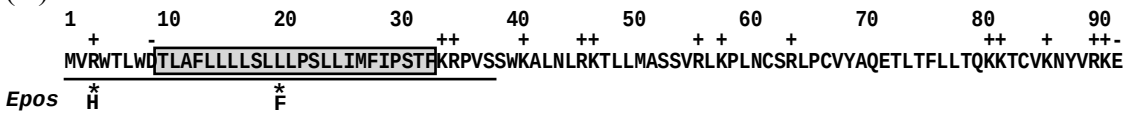
phage progeny were spontaneously released, while the majority of the phage progeny remained inside the cells until artificially lysed by the addition of lysozyme and EDTA. EDTA chelates divalent cations and thus destabilizes the bacterial outer membrane, and lysozyme degrades the cell wall. Treatment with EDTA and lysozyme to bacterial cells leads to rupture of the cell envelop and release of the intracellular contents including the phage progeny. In 1977, Sanger *et al.* (SANGER *et al.* 1977) reported the sequencing of the whole ϕX174 genome. Prior to that, Barrell *et al.*(BARRELL et al. 1976), studied the nucleotide sequence of the gene *D* region, including the DNA sequence from the promoter before gene *D* to gene *J*, and analyzed the amino acid sequence of the D protein. Unexpectedly, they found that all the gene *E* mutants were located within gene *D*. Sequence analysis of the *E* mutations showed that gene *E* was translated from the +1 reading frame within the essential gene *D*. This was the first time that an overlapping gene had been identified. When *E* was expressed from a plasmid, the expression was sufficient to cause lysis of *E. coli*, suggesting that *E* was the only gene required for lysis (HENRICH *et al.* 1982; YOUNG and YOUNG 1982).

The *E* gene encodes a 91 amino acid protein (Fig. 1.10A) with a 10 kD molecular weight, which was identified by labeling experiments in ϕX174 infected cells (POLLOCK *et al.* 1978). It was shown to be a membrane protein (ALTMAN *et al.* 1985), with a putative N-terminal transmembrane domain (TMD). The predicted membrane topology is shown in Figure 1.10B. Deletion and gene fusion analyses were carried out in order to define the lytic domain of E. Fusions $E_{29}\Phi cat$, $E_{59}\Phi lacZ$, and $E_{35}\Phi gfp$ were able to lyse the host cells and indicated that the TMD of E contains its lytic activity (BERNHARDT *et*

al. 2000; BUCKLEY and HAYASHI 1986; MARATEA *et al.* 1985). Buckley and Hayashi (BUCKLEY and HAYASHI 1986) observed that *E* fusion to *trpE*, which encodes a monomeric protein, was not lytic. Since *E* fusions with both the *cat* and *lacZ* genes that encode homotetrameric proteins were functional, it led them to propose a model that the oligomerization by the C-terminal domain of E was required for lytic activity. This was a formal possibility, however, there were other possible explanations; for example, the E-TrpE fusion protein might not be stable. Bläsi *et al.* (BLÄSI *et al.* 1989) isolated E from membranes and detected SDS-resistant oligomers of E by immunoadsorption studies. However, because of the use of a low affinity anti-peptide antibody for E, and thus the extensive sample manipulation required to detect the E protein, it is not clear if these results are a sample preparation artifact.

The *E* genes from 30 independently isolated *Microviridae* have been identified, and alignment between the ortholog E proteins (Fig. 1.9C) indicates that the hydrophobic core of the putative N-terminal TMD which contains the lytic activity is highly conserved. Many of these genes have a His at position 3, and G4 also has a Phe at position 19, so the *pos* changes (BERNHARDT *et al.* 2002a) are fairly wide-spread at least among sequenced *Microviridae*.

(A)



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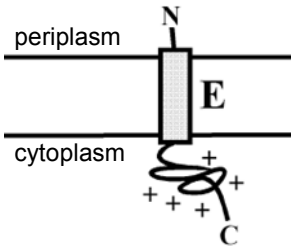

(C)

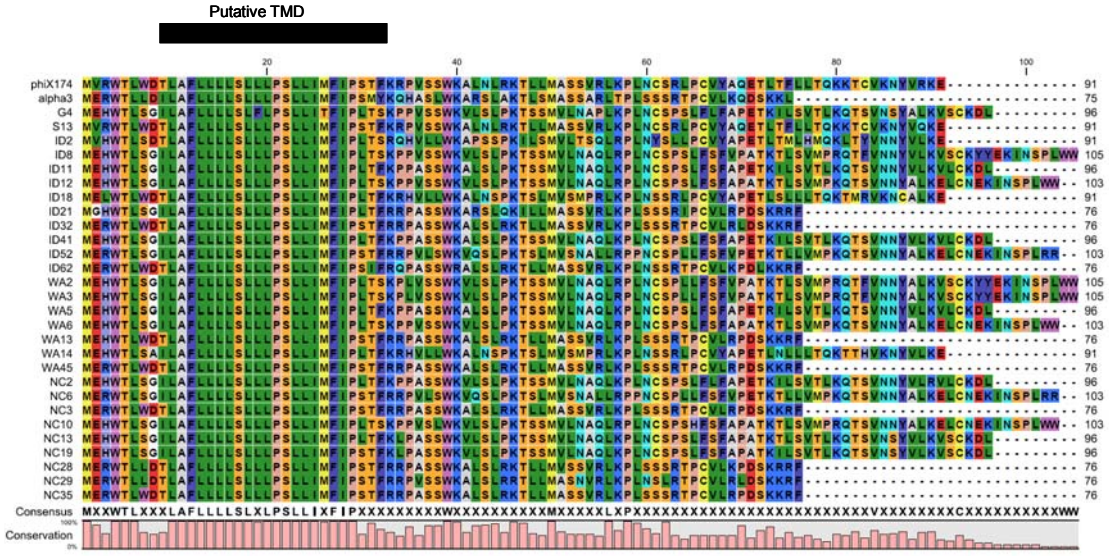

Figure 1.10. Features of protein E. (A) The primary structure of protein E. The position of the two missense changes in Epos (BERNHARDT *et al.* 2002a) is shown below the E sequence. The putative TMD is shown in the shade rectangles. Underlined are the amino acids in E_{pep} (MENDEL *et al.* 2006) (See later in this chapter). (B) Proposed topology of E. (C) Alignment of the E proteins from the related phages. The putative TMDs in the E proteins are indicated by the black rectangle. The amino acid residues are colored by RasMol color scheme according to traditional amino acid properties.

Studies on the mechanism of E lysis

As mentioned earlier, the mechanism of ϕX174 mediated lysis was proposed to be either induction of the host autolytic enzymes or inhibition of cell wall synthesis, since no ϕX174 encoded cell wall degrading activity could be isolated (MARKERT and ZILLIG 1965). Later studies of penicillin-induced lysis showed these two mechanisms are related (TOMASZ and WAKS 1975). Both microscopic and physiological studies showed that ϕX174 induced lysis shared great similarities with penicillin-induced lysis (BRADLEY *et al.* 1969; LUBITZ *et al.* 1984b). The question was then whether ϕX174 caused lysis by affecting host autolytic system directly or indirectly through inhibition of cell wall synthesis.

Lubitz and co-workers investigated the role of autolytic system in E-mediated lysis (BLÄSI *et al.* 1984; LUBITZ *et al.* 1984a; LUBITZ *et al.* 1984b). Lubitz and Plapp (LUBITZ and PLAPP 1980) suggested that a mechanism involving cell wall synthesis inhibition was not compatible with the rapid onset of cell wall degradation they observed. Lubitz *et al*. (LUBITZ *et al.* 1984b) used a temperature-sensitive mutant defective in autolysis and demonstrated that both phage ϕX174 and a cloned *E* gene induced lysis only at the permissive temperature, and concluded host lysis by ϕX174 required a functional host cell autolytic enzyme system. It was found that ϕX174 induced lysis could not occur with starved cells grown in minimal medium, although the phage progeny were produced, and lysis of *E. coli* by both ϕX174 infection and expression of a cloned *E* gene was affected by pH (LUBITZ *et al.* 1984a). They

considered these penicillin-like characteristics further support for involvement of host autolytic system in E-mediated lysis. Later, the same group, by comparing the cell wall composition before and during lysis by either ϕX174 or cloned *E*, concluded that induction of the host autolytic system was more modest than previous studies had suggested (WITTE *et al.* 1998b).

As described earlier in this chapter, Bradley *et al.* (BRADLEY *et al.* 1969) observed large blebs that emerged from the cell mid-point during α3 mediated lysis and these forms were indistinguishable from those induced by penicillin. Young (YOUNG 1992) obtained similar results from ϕX174 infected cells. E-mediated lysis was also shown to be dependent on continued host cell division (WITTE *et al.* 1998). These observations suggested that E might effect lysis by interfering with the host cell wall synthesis in a manner similar to penicillin, which causes lysis by inhibiting cross-linking between peptidoglycan strands during cell wall synthesis. According to this view, the synthesis of new peptidoglycan at the developing septum was compromised in the presence of E, which then resulted in weakened region at the septal area and led to the formation of the blebs and, ultimately, lysis of the cell. Thus it was most likely that E functioned by targeting some host protein involved in cell wall synthesis.

A genetic approach was taken to identify the putative host target of E-mediated lysis. Initially, recessive mutations in the host gene *slyD* (*s*ensitivity to *ly*sis) that can block E-mediated lysis were isolated (ROOF *et al.* 1994). SlyD is a FK506 binding protein (FKBP)-like peptidyl-prolyl *cis-trans* isomerase (PPIase). This enzyme has been shown *in vitro* to catalyze the folding of proteins whose peptidyl-prolyl isomerization is

the limiting step in their folding pathway (for review see (ERDMANN and FISCHER 2007)). E has 5 prolines, and experiments have shown that none of the prolines are essential for its lytic function. However, a C-terminal proline is required for the stability of E, possibly acting to recruit SlyD, though the position is not significant (BERNHARDT 2001). *Epos* (*p*lates on *slyD*) mutants (with mutations in *E*) that bypass the SlyD requirement were isolated by selecting for plaque-forming ϕX174 phage mutants on a *slyD* mutant host. The mutants all contain the missense changes R3H or L19F or both (BERNHARDT *et al.* 2002a) (Fig. 1.10A). In a *slyD* host, both E and Epos are highly unstable, but the expression level for Epos is significantly higher than that for E (BERNHARDT *et al.* 2002a), so the Epos mutants increase synthesis not stability. This indicates that SlyD is involved in the E protein folding or membrane insertion and not essential for the lytic activity of E. Unlike Epos mutants which are not completely independent on SlyD, E_{59}*ΦlacZ* was found to lyse both wt and *slyD* mutant strains (MARATEA *et al.* 1985). These observations suggest that SlyD cannot be the target of E.

Using scanning electron microscopy, Lubitz and co-workers studied the morphological changes of *E. coli* with E expression (WITTE *et al.* 1990a; WITTE *et al.* 1990b). They observed large holes enriched at cell mid point that spanned both the inner and outer membranes. Witte and Lubitz (WITTE and LUBITZ 1989) also observed the release of cytoplasmic enzymes but very few periplasmic enzymes by E-mediated lysis. These observations led them to propose that E functioned by forming a "transmembrane-tunnel". In this model, it was hypothesized that E protein formed a localized tunnel structure that spanned both membranes, and this tunnel structure led to a rapid

decompression of the cytoplasm, which eventually resulted in lysis. They also concluded that formation of the tunnel structure required a limited amount of host autolysis to explain their previous observations that E-mediated lysis depended on the host autolytic system. However, fluorescence microscopy studies of EΦGFP have shown that the fusion protein, which is fully functional, is evenly distributed in the membrane instead of localized only to the lytic lesions (YOUNG *et al.* 2000). As mentioned previously, that as little as the N-terminal 29 amino acids of E protein are required for lysis, making it difficult to imagine how such a small protein can span the entire cell envelop. Lubitz and co-workers proposed that the fusion proteins did not factor into the model because they were able to lyse the E-resistant *slyD* mutant host and thus functioned by a completely different mechanism. This was a formal possibility, but a simpler explanation would be the fusion proteins are able to bypass the requirement of SlyD. Witte *et al*. (WITTE *et al.* 1997) proposed a role of SlyD in the "transmembrane-tunnel" model. In their proposal, the PPIase activity of SlyD was required to catalyze a dramatic conformational change of E so that its C-terminus was brought from inside the cytoplasm to span the rest of the cell envelop while the N-terminus stayed embedded in the inner membrane. However, isolation and characterization of the Epos mutants show that SlyD is not essential for lysis and instead is only required for the stability of the E protein. Witte *et al*. (WITTE *et al.* 1997) observed that when proline at position 21 (Pro21) in E was substituted with several different residues, lysis mediated by E was impaired. Therefore, they proposed that the Leu20-Pro21 bond must be isomerized by SlyD to induce the conformational change essential for E function. Bernhardt and co-workers (BERNHARDT 2001) observed

that E mutant with P21A change was defective in protein accumulation. When the same change was made into the Epos mutant (EposP21A), lysis was restored to wt levels, and these observations support that Pro21 is not essential for E function. All these data seem to preclude the "transmembrane-tunnel" model. The model becomes even more difficult to believe after the isolation of E-resistant mutants in *mraY* (see below).

Since SlyD is clearly not the target of E, pursuit of the E target was continued by selecting spontaneous mutants of *E. coli* C *slyD* that were resistant to *Epos* expression and the mutations were mapped to the essential gene *mraY* (BERNHARDT *et al.* 2000; BOYLE and DONACHIE 1998). *mraY* encodes translocase I (MraY), which catalyzes the formation of lipid I in the cell wall biosynthetic pathway (Fig. 1.5). E was then demonstrated biochemically to inhibit cell wall synthesis at the MraY-catalyzed step by monitoring peptidoglycan synthesis *in vivo* (BERNHARDT et al. 2001a). MraY activity in membranes isolated after E expression was reduced dramatically, but activity of Rfe, an enzyme that belongs to the same UDP-GlcNAc/ MurNAc1: polyisoprenyl-P GlcNAc/MurNAc 1-P transferase (GPT) family as MraY and catalyzes the formation of C_{55}-P-P-GlcNAc in enterobacterial common antigen synthesis, was unchanged (BERNHARDT *et al.* 2001a). This suggests that E does not inhibit cell wall synthesis by complexing with C_{55}-P and making it unavailable to MraY, and thus is a specific inhibitor of MarY. These data support the contention that MraY is the cellular target of E, and the mechanism of E-mediated lysis is inhibition of cell wall synthesis. The model of E-mediated cell lysis is shown in Figure 1.11.

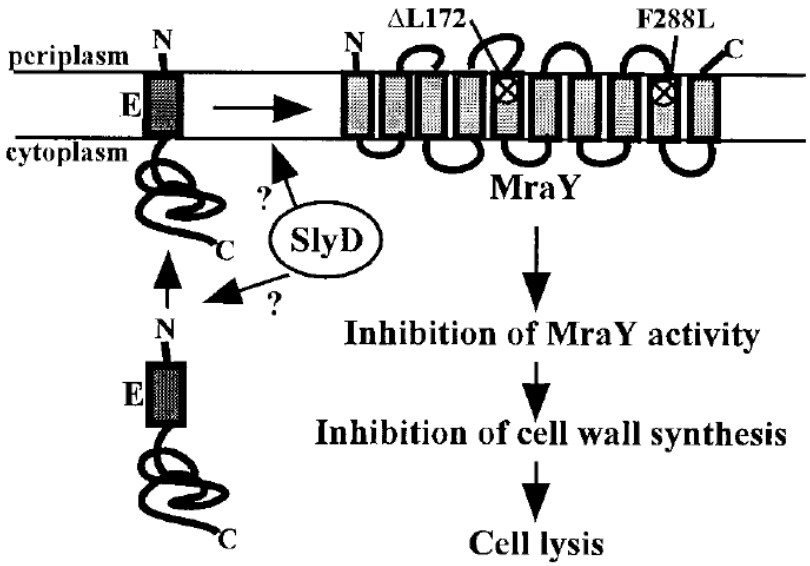

Figure 1.11. Model of E-mediated cell lysis. Both genetic and biochemical experiments have provided evidence that E inhibits cell wall synthesis at the step catalyzed by MraY. SlyD is a host PPIase that is required for accumulation of E protein either before or after E insertion into the cytoplasmic membrane. Two of the E-resistance mutations in MraY isolated from the initial study are indicated (BERNHARDT *et al.* 2001a).

Characteristics of MraY

The P-MurNAc-pentapeptide translocase (MraY) (E.C. 2.7.8.13) belongs to the GPT family. *In vivo*, MraY catalyzes the formation of lipid I by transferring P-MurNAc-pentapeptide from UDP-MurNAc-pentapeptide to C_{55}-P. Lipid I is an essential intermediate in the cell wall synthesis pathway and was first reported by Chatterjee and Park (CHATTERJEE and PARK 1964). The complete *mraY* gene was identified and cloned into pUC119 in 1991 by Ikeda *et al.* (IKEDA *et al.* 1991). *mraY* is located at 2 min on the *E. coli* chromosome within a cluster of genes called *mra* (*m*urein *r*egion *A*). Genes in this cluster encode proteins involved in cell wall synthesis and cell division. It has been

identified that the *mraY* gene exists in all bacterial genomes sequenced to date, but is absent from archaebacteria and eukaryotes (an exception for this is that an *mraY* homolog, whose product had putative plastid-targeting signals, was discovered in *Arabidopsis thaliana* (MACHIDA *et al.* 2006; MONDEGO *et al.* 2003)) both of which are lacking a cell wall. Boyle and Donachie (BOYLE and DONACHIE 1998) demonstrated that *mraY* is an essential gene by achieving insertional inactivation of the chromosomal copy of *mraY* only when a plasmid borne wild-type *mraY* gene under the control of inducible promoter P_{BAD} was present. In the absence of arabinose, cells are not viable; in the presence of arabinose, they grow as well as the parental strain.

MraY is an integral membrane protein, spanning the cytoplasmic membrane with both N- and C-termini located in the periplasm. It has been proposed to adopt a ten-TMD topology (Fig. 1.12) with four periplasmic loops and five cytoplamic loops, by studies of β-lactamase fusions with MraY (BOUHSS *et al.* 1999). Recently, Bouhss *et al.* (BOUHSS *et al.* 2004) reported for the first time the over-expression and purification of MraY from *Bacillus subtilis*.

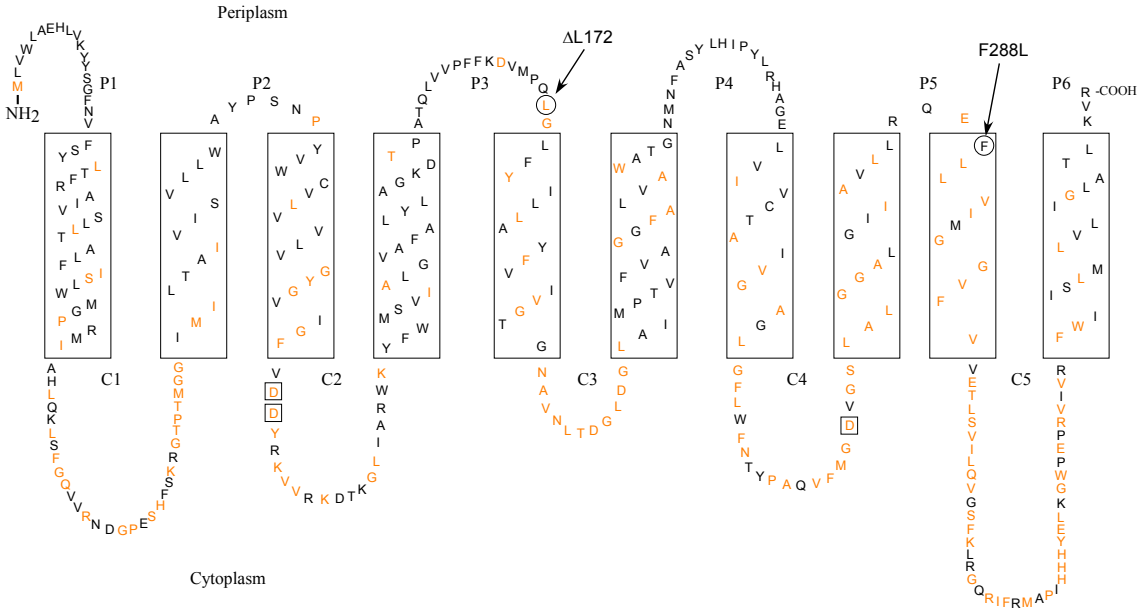

Figure 1.12. Proposed topology of MraY. The three conserved Asp residues essential for MraY enzymatic activity are labeled in rectangles on the cytoplasmic loops below TMD3 and TMD8 (LLOYD *et al.* 2004). The E-resistance mutations isolated in MraY (BERNHARDT *et al.* 2000), ΔL170 and F288L are labeled in the circles. C1-C5, cytoplamic loops 1-5. P1-P6, periplasmic portions 1-6. The amino acid residues that are conserved between the *E.coli* and *B. subtilis* MraY proteins are labeled in gold. Adapted from Bouhss *et al*. (BOUHSS *et al.* 1999).

The transfer of the P-MurNAc-pentapeptide moiety onto C_{55}-P was demonstrated for the first time using membrane preparations from *S. aureus* and *M. luteus* in 1965 (ANDERSON *et al.* 1965; STRUVE and NEUHAUS 1965). Enzymatic activity of MraY can be measured by either the transfer assay or the exchange assay (HEYDANEK *et al.* 1969). The transfer assay measures the transfer of P-MurNAc-pentapeptide from UDP-MurNAc-pentapeptide to C_{55}-P. The exchange assay measures the exchange of labeled UMP with the unlabeled UMP moiety of UDP-MurNAc-pentapeptide. A two-step mechanism involving a covalent MraY-P-MurNac-pentapeptide has been proposed (HAMMES and NEUHAUS 1974) as shown in Figure 1.13. The MraY activity additionally

requires a divalent metal ion, particularly Mg^{2+}, though Mn^{2+} can replace Mg^{2+} with a decreased activity (BOUHSS *et al.* 2004; HEYDANEK *et al.* 1970).

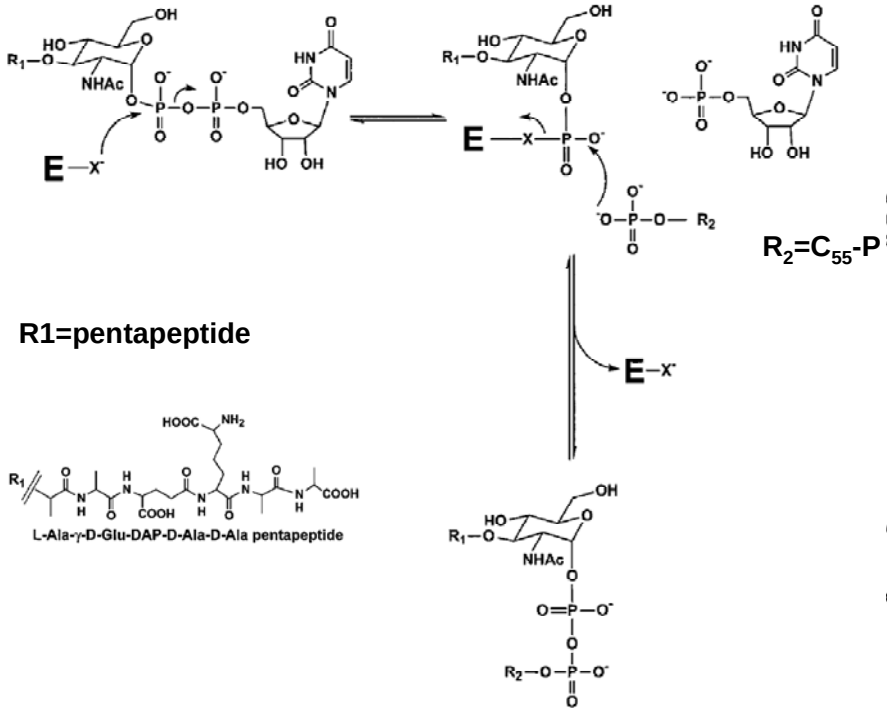

Figure 1.13. Reaction mechanism of MraY. The MraY-catalyzed reaction has been proposed to be a two-step process (HEYDANEK *et al.* 1969). The first step generates a MurNAc-pentapeptide-phospho enzyme intermediate and free UMP. In the second step, C_{55}-P attacks the intermediate, and results in formation of lipid I and release of the free enzyme. E-x represents MraY. Adapted from Lloyd *et al*. (LLOYD *et al.* 2004).

Alignments of members of the translocase I family and the related transmembrane phosphosugar transferase superfamily have shown that among all of the conserved residues, D115, D116, and D267 (Fig. 1.12) are the only ones in the cytoplasmic loops with nucleophilic side chains (LLOYD *et al.* 2004). In a model

proposed by Lloyd *et al.* (LLOYD *et al.* 2004), D115 and D116 coordinate the Mg^{2+}, involved in pyrophosphate binding, with the UDP-MurNAc-pentapeptide substrate, and D267 is the active-site nucleophile. In accordance with their model, they also showed that the MraY mutant, $MraY_{D267N}$, completely abolishes the enzymatic activity *in vitro*. Al-Dabbagh *et al.* (AL-DABBAGH *et al.* 2008) tried to map the active site of purified *B. subtilis* MraY, and showed that 14 residues might be essential for its *in vivo* activity and D98 in *B. subtilis* MraY might also be involved in deprotonation of the lipid substrate.

For both purified and partially purified MraY protein, Michealis-Menten kinetics were observed (BOUHSS *et al.* 2004; BRANDISH *et al.* 1996a; STRUVE *et al.* 1966). *In vitro*, the UDP-MurNAc-pentapeptide substrate can be replaced by a dansylated UDP-MurNAc-pentapeptide, UDP-MurNAc-L-γ-D-Glu-*m*-DAP(Nε-dansyl)-D-Ala-DAla (UDP-MurNAc-pp-DNS) (WEPPNER and NEUHAUS 1977). The detailed specificity of MraY for the nucleotide substrate was reviewed by Price and Momany (PRICE and MOMANY 2005). MraY can also accept *in vitro* a broad range of substitutes for its lipid phosphate substrate, such as heptaprenyl phosphate (C35), dodecaprenyl phosphate (C60), phytol phosphate (C20), and water-soluble prenyl phosphates (BRANDISH *et al.* 1996b; BREUKINK *et al.* 2003). It was indicated that with larger substrates that were closer in chain length to the natural substrate, C_{55}-P, MraY worked more efficiently (BRANDISH *et al.* 1996b).

There are three different groups of known inhibitors of MraY (VAN HEIJENOORT 2001): (1) nucleosides (tunicamycin, ribosamine-uridines, uridylpeptides, and capuramcins); (2) lipopeptide amphomycin; (3) ϕX174 lysis protein E (for a detailed

review see (BUGG *et al.* 2006)). Inhibition of MraY by tunicamycin and liposidomycin B has been studied by Brandish *et al.* (BRANDISH *et al.* 1996b). Their study showed that tunicamycin is a competitive inhibitor for the sugar nucleotide substrate, and a noncompetitive inhibitor for the lipid substrate; whereas, liposidomycin B is a noncompetitive inhibitor for the sugar nucleotide substrate, and a competitive inhibitor for the lipid substrate.

Prior to studies discussed in this dissertation, two MraY dominant mutants, providing resistant to E-mediated lysis and impaired in plaque-formation by ϕX174, have been isolated (BERNHARDT *et al.* 2000). One was a single deletion mutation, ΔL172, in proposed TMD 5 and the other one was a missense mutation, F288L, in proposed TMD 9 (Fig. 1.12). Since the lytic domain of E is contained within its TMD, it led to an attractive hypothesis that the most likely mode by which E could inhibit MraY would be through direct interactions between the two proteins via their TMDs.

Lysis mechanisms by small phages

After MraY was demonstrated to be the target of E both biochemically and genetically, similar experiments were carried out to determine the targets of Qβ A_2 protein and MS2 L protein to see if small bacteriophages share the E-mediated lysis mechanism.

An A_2-resistant allele of *murA, murA*^{rat1} (resistance to *A*-two), was isolated in a genetic selection and screen that was nearly identical to that used for the analysis of E

function (BERNHARDT *et al.* 2001b). *murA* encodes UDP-N-acetylglucosamine 1-carboxyvinyltransferase (MurA), the enzyme that catalyzes the first and committed step in cell wall biosynthesis (Fig. 1.5). This suggests that like E, A_2 also induces lysis by inhibiting the cell wall synthesis, but targets a different enzyme from E. In a key experiment, peptidoglycan synthesis in the presence of A_2 expressed from a plasmid, was monitored by measuring the incorporation of [^{3}H]-DAP into the SDS-insoluble material (murein). Similar results were obtained as those from E (Fig. 1.14A and B), with incorporation inhibited shortly after the induction of A_2, before any detectable effect on growth, and at least 20 min before detectable lysis. Qβ particles, which contain one copy of A_2 per particle, were demonstrated to be capable of inhibiting MurA enzymatic activity *in vitro* (BERNHARDT *et al.* 2001b). These biochemical experiments provide further support that A_2 induces cell lysis by inhibiting cell wall synthesis at the MurA-catalyzed step.

Including the E-mediated inhibition of MraY, these results mean that two of the three prototypes of small phages effect host lysis by inhibiting cell wall synthesis. To date, a protein target of MS2 lysis protein L has not been identified using similar genetic approaches as used for E and A_2. Moreover, unlike A_2 and E, incorporation of [^{3}H]-DAP into the SDS-insoluble material is not inhibited during *L* expression (Fig. 1.14C, (BERNHARDT 2001)), remaining normal up to the onset of lysis. The mechanism of L-mediated lysis is still unknown. So, for small phages with single gene lysis syetems, there are two basic strategies: one is the production of an inhibitor of precursor synthesis, and the other, L-mediated lysis, that remains mysterious and, presumably,

results from either induction of a cryptic autolysis system or somehow blocking an essential step beyond the first step of incorporation of the disaccharide precursor into the murein.

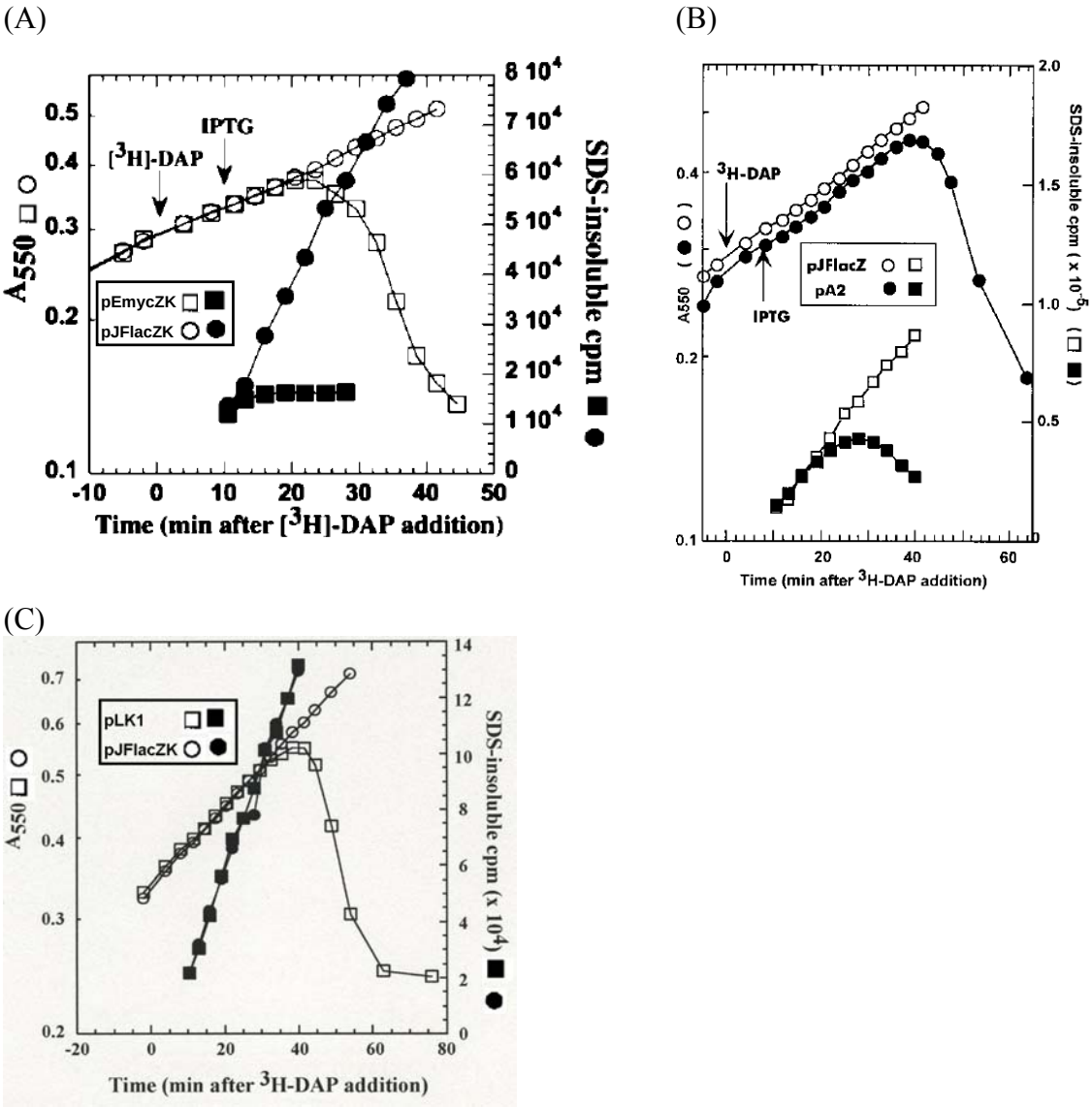

Figure 1.14. [^{3}H]DAP incorporation into the cross-linked cell wall. In the presence of E (A), A_2 (B) and L (C). Adapted from Bernhardt *et al*. (BERNHARDT 2001; BERNHARDT *et al.* 2001a; BERNHARDT *et al.* 2001b).

Questions to be addressed

As described above, for the microviruses and the leviviruses, bacteriophage with small ss nucleic acid genomes, host lysis is accomplished by expression of a single gene that encodes an inhibitor of cell wall synthesis. In contrast, phage with ds DNA genomes use a more complex system involving, at minimum, an endolysin, which degrades peptidoglycan, and a holin, which permeabilizes the membrane in a temporally-programmed manner. What is the basis of this difference? How do small phages with single gene lysis systems control timing? Is there an evolutionary advantage for utilizing the holin-endolysin system over the single gene lysis system? In Chapter II, the lysis characteristics are compared between λ and λE, a chimera created in which lysis gene *E* of the microvirus, ϕX174, replaced the entire lysis cassette of phage λ, and the results are interpreted in an evolutionary view.

Protein E is a specific inhibitor of MraY. The original evidence for this inhibition was the isolation of two spontaneous E-resistant *mraY* mutants. In Chapter III, further genetic studies aimed at dissecting the interaction between E and MraY are reported. A genetic strategy is described, that is facile, rapid and does not depend on the availability of purified E, purified MraY, or its substrates.

The precise mechanism of E-mediated inhibition of MraY is still not known. Purification of full length E has not been reported. Mendel *et al.* (MENDEL *et al.* 2006) prepared a synthetic polypeptide, E_{pep}, with the N-terminal 37 residues of E containing the putative TMD (Fig. 1.8A). They showed that this SDS-solubilized E pep was only

able to inhibit membrane-bound MraY (IC_{50}=0.8μM) and did not inhibit the solubilized form of the enzyme. This led to a model in which E effects lysis via a protein-protein interaction with MraY preventing its assembly into an essential heteromultimeric integral membrane complex with high MraY activity, a complex which would not be formed in detergent (Fig. 1.15). This is a formal possibility. However, in Chapter IV, biochemical studies of E-mediated inhibition of MraY and its mutants are reported, the results are discussed in terms of a new model for E action.

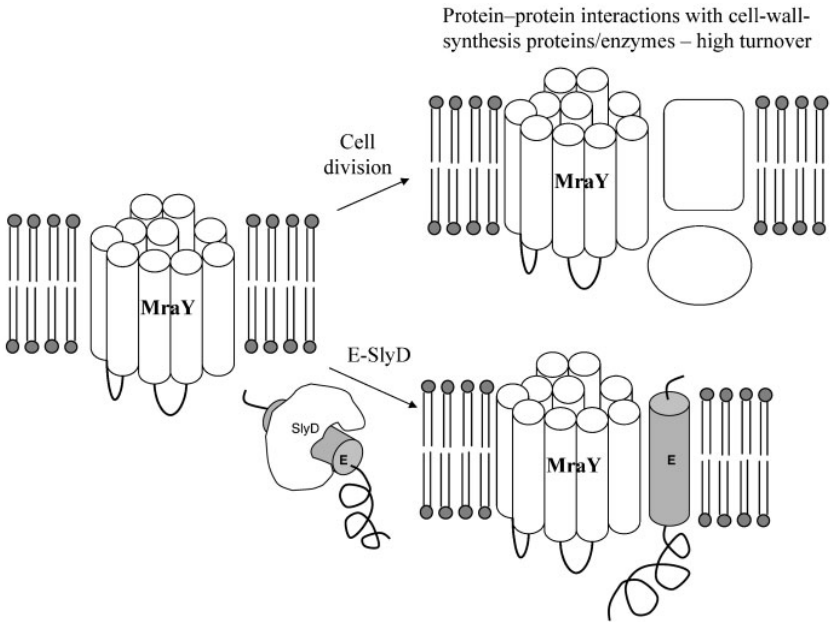

Figure 1.15. Model of E-mediated inhibition proposed by Mendel *et al*. (MENDEL *et al.* 2006). In their model, SlyD binds E, protects it from proteolysis, and maybe assists the formation of a protein-protein interaction between E and MraY; the binding of E to MraY then blocks the formation of a complex between MraY and other proteins involved in cell wall synthesis, and this results in inhibition of MraY.

CHAPTER II

EVOLUTIONARY DOMINANCE OF HOLIN LYSIS SYSTEMS DERIVES FROM SUPERIOR GENETIC MALLEABILITY*

Introduction

Phages use two fundamentally different host lysis strategies (YOUNG and WANG 2006). Double-strand DNA (dsDNA) and double-stranded RNA (dsRNA) phages use a holin-endolysin system (YOUNG *et al.* 2000). The endolysin, or lysozyme, is an enzyme that has one or more murein-degrading activities. The holin is a small membrane protein that controls the activation of the endolysin or its access to the murein (WANG *et al.* 2000; XU *et al.* 2005; XU *et al.* 2004) and thus constitutes the "clock" of the bacteriophage infection cycle. The best-studied holin, the S product of the λ *S* gene (Fig. 2.1), accumulates in the membrane throughout the late gene expression period, without affecting membrane integrity or energization, until suddenly, at an allele-specific time, it triggers "hole-formation" (GRÜNDLING *et al.* 2001). This allows the endolysin, protein R, which accumulates fully folded and active in the cytoplasm, to escape across the bilayer and attack the cell wall. Lysis follows within seconds. In addition to the holin and endolysin, dsDNA phages often encode other lysis proteins, including antiholins that regulate holin function (YOUNG and WANG 2006) and, in phages of Gram-negative

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hosts, Rz and Rz1 proteins, which form a complex that connects the inner and outer membranes (SUMMER *et al.* 2007).

In contrast, lytic single-strand nucleic acid (ssDNA, ssRNA) phages like the microviruses (e.g., ϕX174; 5.4kb), the leviviruses (e.g., MS2; 3.5kb), and the alloleviruses (e.g., Qβ; 4.2kb) accomplish lysis of the host without encoding a muralytic enzyme. Instead, in all three cases, lysis is accomplished by expression of a single gene: gene *E* (Fig. 2.1), gene *L* and gene A_2, in ϕX174, MS2, and Qβ, respectively. The mechanism by which lysis is effected has been elucidated for the E and A_2 proteins, which have been shown to be specific inhibitors of the conserved murein biosynthesis enzymes MraY and MurA, respectively (BERNHARDT *et al.* 2001a; BERNHARDT *et al.* 2001b).

Although the small genome size of the ssRNA and ssDNA phages undoubtedly favored the evolution of these single gene systems, it is unclear why they are not employed by any known dsDNA. One notion was that the lytic lesion supported by the single gene system was sufficient for the release of small phages like ϕX174 but not for the much larger, more complex dsDNA phages. This idea was based on a model for E-mediated lysis in which the E protein would oligomerize into a "transmembrane tunnel" that spanned the entire envelope of the host cell (WITTE *et al.* 1997; WITTE *et al.* 1990b). The tunnel would be adequate for release of the small ϕX174 virion but not for a large, complex dsDNA phage like λ. However, the elucidation of the molecular mechanism of E, as an inhibitor of MraY, and the complete cell lysis that derives from it, leaves the

transmembrane tunnel hypothesis untenable. Thus there is no fundamental functional limitation that disqualifies single gene lysis systems for the context of dsDNA phages.

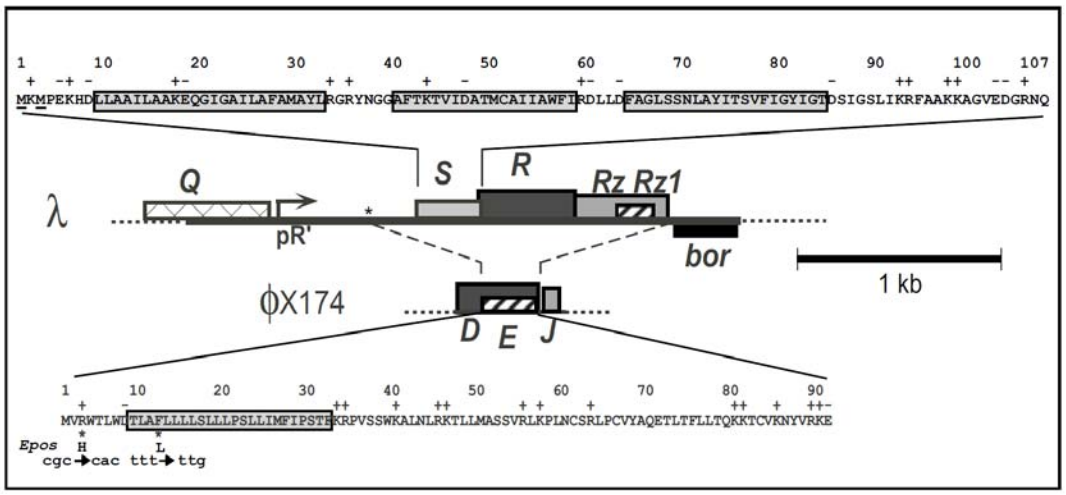

Figure 2.1. Sequences and genes of the λ S and ϕX174 E lysis proteins and the context of the λ lysis cassette. Top: the amino acid sequence of the S107/S105 dual start sequence is shown; S105, the holin, starts at the second methionine. The 3 transmembrane domains (TMDs) are boxed. Bottom: the amino acid sequence of E is shown, with its putative TMD boxed. The *E* gene is embedded out of frame within the *D* gene, which encodes the ϕX174 morphogenetic scaffolding protein. Below the E protein sequence, the two mutations in *Epos* are shown. Middle: the λ lysis cassette is depicted, transcribed from the late promoter, pR', and flanked by the *Q* and *bor* genes; the dashed lines indicate the region of the cassette replaced by ϕX174 DNA in the *E* chimeras. The asterisk indicates the position (coordinate 44953) of the upstream single nucleotide mutation in λ**E* and λ**Epos*.

Three holin genes have been subjected to extensive mutational analysis: λ *S* (GRÜNDLING *et al.* 2000a; JOHNSON-BOAZ *et al.* 1994; RAAB *et al.* 1988), T4 *t* (RAMANCULOV and YOUNG 2001b) and PRD1 *XXXV* (RYDMAN and BAMFORD 2003). In all three cases, the timing of holin-mediated lysis was found to be extraordinarily allele-specific, with missense mutations throughout each of the holin genes conferring

profound changes in the timing of lysis, either shortening or lengthening the latent period. It has been suggested that the genetic plasticity of holins confers a selectable advantage on dsDNA phages because it allows them to rapidly evolve towards optimum lysis timing for any particular environmental condition and host character (BULL *et al.* 2004). The existence of such an ideal lysis time, for a given host and environmental milieu, has been predicted on a theoretical basis (WANG *et al.* 1996). This mathematical model suggests that, to effect the best exponential increase in phage titer, the length of the infection cycle should increase when host cell concentration, host quality or the affinity of the phage for the host is reduced, and should decrease if these parameters improve. Support for this idea was obtained in competition experiments with mutants of the T4-like phage RB49 differing only in the holin allele (ABEDON *et al.* 2003).

To test the notion that the functional plasticity of holins confers a fitness advantage to holin-endolysin systems when compared to single gene lysis systems, we have constructed chimeric phages in which the lysis gene *E* of ϕX174 replaces the entire lysis gene cassette of λ. The chimeric phages were compared to λ in terms of the timing and extent of lysis in bulk liquid culture, and the dispersion of burst sizes within populations of cells. In addition, the mutational plasticity of lysis timing was assessed for *E* and *S*, starting with alleles of comparable lysis kinetics. The results are discussed in terms of a model for the contribution of holin-mediated timing to the evolutionary fitness of bacteriophage.

Methods

Media, chemicals and general methods

Standard Luria-Bertani (LB) borth was used for all bacterial cultures growth and agar plates. When indicated, media was supplemented with ampicillin (100 μg/ml), chloramphenicol (10 μg/ml), and kanamycin (40 μg/ml). Isopropyl-β-D-thiogalactopyranoside (IPTG) and arabinose were used for induction at a final concentration of 1mM and 0.2%, respectively. Lysis profiles were obtained by monitoring A550 after thermal or IPTG and arabinose induction, as described previously (TRAN *et al.* 2005). Phage plating was performed as previously described (BLÄSI *et al.* 1999). To permit meaningful comparison of plaque sizes, phage and indicator bacteria were pre-incubated together for 30 min at 25°C to insure complete pre-absorption prior to initiating infection.

Bacterial strains, bacteriophages, and plasmids

The prototroph MDS12 *tonA::*Tn*10* (KOLISNYCHENKO *et al.* 2002; TRAN *et al.* 2005) carrying deletions of all the cryptic prophage sequences of *E. coli*, was used as a non-complementing indicator strain for phage plating and host for all constructions and lysogenic inductions. The same strain carrying the plasmid pS105 (see below) was used for complementing lysis defects. Substitution of the various *E* alleles for the λ lysis

cassette was done by recombination of the indicated plasmid constructs with λΔ*(SR)*, which is λΔ(*stf tfa)::cat cI857 Δ(SR)*, as previously described (GRÜNDLING *et al.* 2000b). The isogenic phage designated λ*S* was constructed in the same way, using the plasmid pSwt for recombination (see below), and thus has the genotype λΔ(*stf tfa)::cat cI857* (R. White, unpublished). Lysogenization with these phages was accomplished by infecting MDS12 *tonA::*Tn*10* at low multiplicity, plating for survivors on LB-Cam at 30°C, and screening candidate lysogens for single copy prophages using PCR (POWELL *et al.* 1994).

The plasmid pQ, a low copy plasmid carrying *Q*, which encodes the λ late gene activator, has been described (GRÜNDLING *et al.* 2001). Inductions were done with an isogenic $lacI^Q$ derivative of MDS12 *tonA::*Tn*10*, carrying pQ to supply the Q protein for transactivation of pRW and pRE derivatives. pRW was derived from pRE (PARK *et al.* 2006), a medium copy plasmid which has a multiple cloning site under the control of the λ late promoter, by inserting the λ *bor* gene (λ nt 46421 to 46772) after the multiple cloning site. pRW was the backbone used for construction of pRWE and pRWEpos, carrying the parental and *Epos4B* alleles of the ϕX174 *E* lysis gene. In these plasmids, nt 544 to nt 840 of ϕX174, spanning the 273 bp of *E*, were inserted into the RI and BamHI sites of pRW. The coding sequence for the *c-myc* epitope tag (EQKLISEEDL) was added at the end of the *E* or *Epos* sequence in pRWE or pRWEpos, using PCR with primers carrying an EcoRI site (EEcoRIFor: GAGCAGGAATTCGTCGCTGCGTTGAGG) at the 5' end of the coding sequence and the c-myc sequence and a BamHI site (EcmycBamHIRev:

GACGAGGGATCCTTACAGATCTTCTTCAGAGATCAGTTTCTGCTCCTTCCGCA CGTA) at the 3' end. The amplified products were digested with EcoRI and BamHI and ligated into the corresponding sites on the expression vectors, generating pRWEcmyc and pRWEposcmyc, respectively. The plasmid pS105 has the *S105* allele of the *S* gene in the context of the complete λ lysis cassette and the wt λ late promoter region (SMITH *et al.* 1998). The plasmid pSwt was made by site-directed mutagenesis of pS105 to restore the wt *S* gene dual start motif.

Standard DNA manipulations, PCR, and DNA sequencing

Plasmid DNA isolation, DNA amplification by PCR, DNA transformation, DNA sequencing, and Quikchange (Stratagene) site-directed mutagenesis were performed as previously described (TRAN *et al.* 2005). Primers were from Integrated DNA Technologies, Coralville, IA, and were used without further purification. All enzymes were purchased from New England Biolabs, except for *Pfu* polymerase, which was from Stratagene. The Laboratory for Plant Genome Technology at the Texas Agricultural Experiment Station performed the automated fluorescent sequencing.

SDS-PAGE and Western blotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were performed generally as described previously (TRAN *et al.* 2005).

Quantification of the production of cmyc-tagged E proteins from induced lysogens was done by growing designated thermo-inducible cultures in LB-Cam at 30°C to A_{550}~0.5, aerating at 42°C for 15 minutes and then aeration at 37°C for an additional 5 min. At this time, well before lysis of any of the cultures, 5 ml aliquots were withdrawn and the cells collected by centrifugation for 30 minutes in clinical benchtop centrifuge at 4°C. Pellets were resuspended in SDS-PAGE buffer (4% SDS, 110 mM Tris-HCl (pH6.8), 10% glycerol, 9% β-mercaptoethanol); volumes were chosen to normalize final A_{550} units per μl. The samples were boiled for 5 minutes prior to SDS-PAGE. 100 μl of each sample were resolved on a 10% Tris-Tricine gel at 100 volts for 4-5 h. Gels were blotted to nitrocellulose overnight using a semi-dry blotting apparatus. Blots were washed, incubated with antibodies (cmyc monoclonal antibody 9E10 from Covance, at 1:1000 dilution; Goat anti-mouse-HRP, from Pierce, at 1:1000 dilution), and developed as described previously (BERNHARDT *et al.* 2002a). For the analysis of cmyc-tagged E production in the collection of *E* mutants, MDS12 *lacI*^{Q} *tonA::*Tn*10* carrying pQ and the indicated pRW derivative were grown in LB-Amp-Kan at 37°C, induced with IPTG+arabinose at A550 = 0.5, and aerated for 20 min before 5 ml samples were harvested by centrifugation in the cold. Processing of the samples for immunoblotting was the same as for the lysogenic inductions, except that PVDF membranes were used for E blots.

Burst size dispersion

Lysogenic cultures were grown at 30°C to A_{550} = 0.2 and diluted 10^6-fold in PBS buffer (120mM NaCl, 10mM NaH_2PO_4, pH 7.5) to give ~100 cfu/ml. Then, 3 μl aliquots of this dilution were added to each well of a 96 well microtiter plate, which already contained 100 μl pre-warmed LB. The lysogens were induced by incubating the microtiter dishes at 42°C for 20 minutes and the plates were further incubated at 37°C for 2 hours to allow all cells to lyse. The total pfu in each well was determined by plating its entire contents on a lawn of MDS12 *tonA::*Tn*10*.

Results

Generation of λE constructs

To explore the functionality of single gene lysis in comparison to the well-characterized holin-endolysin lysis system of phage λ, it was necessary to replace the λ lysis cassette (Fig. 2.1) with the lysis gene *E* of ϕX174. To accomplish this substitution, λΔ*(SR)* was induced in trans to the plasmid pRWE, which carries the *E* gene flanked by the sequences proximal and distal to the lysis cassette, immediately downstream of the λ late promoter. Plaque-forming recombinants were detected in the lysate, but the frequency was extremely low approximately 10^4-fold lower than observed with an isogenic S^+R^+ plasmid (Table 2.1). This result suggested that, in addition to the

recombination event, a mutation was required to generate the plaque-forming phenotype. Sequencing revealed a single base change, C to T, at position 44953 in the λ DNA, 49 nt upstream from *E*. When this mutation was incorporated into the initial pRWE clone, plaque-forming recombinants were recovered at a high frequency, confirming that this mutation (indicated by "*"; e.g., pRW*) is necessary for the ability of λ*E* to form plaques on lawns of *E. coli*. Accordingly, the plaque-forming λ*E* construct is designated λ**E*. An identical experiment starting with the *Epos* allele, which carries two point mutations in the 5' end of the *E* gene that are known to increase synthesis of the E protein (BERNHARDT *et al.* 2002a), also yielded plaque-forming recombinants at high frequency, irrespective of the presence of the mutation at 44953 (Table 2.1). This suggests that the upstream mutation in λ*E* confers plaque-forming ability on the recombinant phage by increasing the expression level of the wt *E* gene.

Table 2.1. Frequency of plaque-forming recombinants.

plasmid	PFU[1]	PFU[2] (complemented)	Frequency of plaque-forming recombinants
pRWE	4×10^{2}	1.2×10^{10}	3.3×10^{-8}
pRWEpos	3.6×10^{6}	7.6×10^{9}	4.7×10^{-4}
pRW*E[3]	3.0×10^{6}	5.8×10^{9}	5.0×10^{-4}
pRW*Epos[3]	3.7×10^{6}	1.1×10^{10}	3.4×10^{-4}
pSwt	1.3×10^{7}	7.8×10^{9}	1.6×10^{-3}

[1]titer on non-complementing lawn, MDS12 *tonA::*Tn*10*.
[2]titer on complementing lawn, MDS12 *tonA::*Tn*10* pS105.
[3]Asterisk designates plasmids with C to T mutation at [1] coordinate 44953; see text.

Lysis phenotypes of the chimeric λE phages

To assess the overall lysis function of the *E* single-gene system in the λ context, several phenotypes associated with lysis were examined for the three recombinant phages, λ**E*, λ*Epos*, and λ**Epos*, in comparison with the parental phage, which will be designated λ*S* for clarity. This set of four phages was first compared in terms of lysis in bulk liquid culture, using thermal induction of single-prophage lysogens to permit synchronous induction of the infection cycle throughout the subject cultures (Fig. 2.2). As expected, all the phages effected lysis of their hosts. Moreover, the presence of the *pos* and the upstream mutations accelerated lysis synergistically in the E constructs, with λ*Epos lysing before λS, and the two other E constructs, λEpos, and λ*E, lysing later. Western blot analysis confirmed that the shorter latent periods correlated with increased E production (Fig. 2.2, inset).

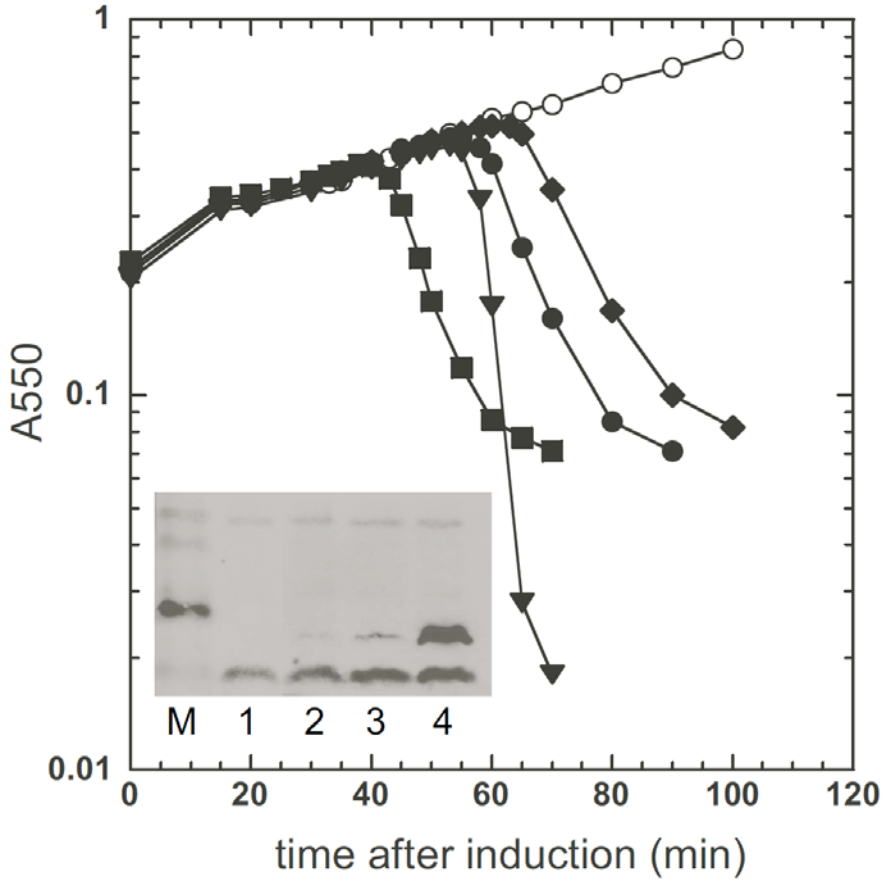

Figure 2.2. Induced lysis of λ*S* and the λE chimeras. MDS12 *tonA*::Tn*10* cells with the indicated prophages were thermally induced by shifting the aerating cultures from 30°C to 42°C at time zero for 15 minutes and then to 37°C for the duration of the experiment. ○, λ*Δ(SR)*; ♦, λ**E*; ●, λ*Epos*; ■, λ**Epos*; ▾, λ*S*. Inset: E protein production in inductions of chimeric prophages. Inductions of chimeric phages carrying *cmyc*-tagged versions of the three *E* alleles were analyzed for production of the E protein by immunoblotting with anti-cmyc antibody. Lanes: M, mass standards; 1, λ*Δ(SR)*; 2, λ**E-cmyc*; 3, λ*Epos-cmyc*; 4, λ**Epos-cmyc*.

Unlike with the λS culture, cultures lysed by any of the chimeric phage retained a significant fraction (~20%) of the pre-lysis turbidity. This was due to the presence of large numbers of non-refractile "ghosts" in the latter cultures; no ghosts or any large cellular remnants were visible in the λS culture. This is consistent with the previous

observation that rod-shaped, empty cell ghosts are produced in ϕX174 infections and with inductions of the cloned *E* gene (WITTE *et al.* 1990a), reflecting the mechanism of E-mediated lysis which involves the inhibition of peptidoglycan synthesis rather than its degradation (BERNHARDT *et al.* 2000; BERNHARDT *et al.* 2002a; BERNHARDT *et al.* 2001a). Despite the production of ghosts, no more than 11% of the total virions produced were trapped inside sedimentable debris in the λ*E* constructs (Table 2.2). Moreover, λ virion production was inversely related to the length of the latent period for the three lytic E constructs. This finding, coupled with the calculated average burst sizes, also shows that there is nothing about the E-mediated lysis pathway that impairs the efficiency of λ morphogenesis (Table 2.2).

Table 2.2. Characteristics of phages with S and E lysis functions.

Phage	Burst[1] (pfu/cell)	Trapped[2] (%)	EOP[3]	Plaque size[4]
λS	125	-	1.19 (± .16)	1.00(± 13%)
λ*E	143	7	0.70 (± .13)	0.32(± 38%)
λEpos	112	7	0.67 (± .08)	0.33(± 33%)
λ*Epos	44	11	1.06 (± .23)	0.45(± 24%)

[1] Calculated average burst size per induced lysogenic cell; >1000 plaques were counted in each case.
[2] Fraction of pfu retained in sedimental cells and released by mechanical cell disruption.
[3] Relative efficiency of plating, as calculated from titer on non-complementing lawn divided by titer on complementing lawn.
[4] Average plaque size, relative to λS (=2.25 mm), and standard deviation, based on measuring 100, 198, 220 and 64 plaques for λS, λ*E, λEpos, and λ*Epos, respectively, as a percent of the average diameter.

Plaque morphology variance and burst size dispersion

While the efficiency of plating of the λE constructs was not significantly different from that of λS (Table 2.2), there was a dramatic difference in plaque morphology. Under conditions where virions were quantitatively preadsorbed to the indicator cells, λS formed large plaques of nearly uniform size (Fig. 2.3; Table 2.2). In contrast, all three lytic λ*E* constructs formed plaques of smaller and more variable size (Table 2.2). Moreover, when replated, phages isolated from both small and large plaques generated the same variable size distribution (not shown). This suggested to us that, at the level of an individual cell, there was significant variability in the latent period with any of the λE constructs. It is not practical to test this notion by direct observation of infected cells. However, since progeny virions accumulate linearly shortly after the beginning of late gene expression (READER and SIMINOVITCH 1971; WANG 2006), the burst size should reflect the length of the latent period. Thus, measurement of the dispersion in burst size for individual cells should allow us to detect a signficant variation in time of their lysis. Cells lysogenic for λS or λEpos, which have approximately the same lysis time, were distributed to wells of a microtiter plate at an average of 0.3 cells per well, induced, and the total progeny in each well measured by plating. At this average number of cells per well, only 3.7% of the wells should contain two or more cells, so the titers in the non-zero wells should mostly reflect the bursts from individual lytic events. As shown in Fig. 2.4, it is clear that the distribution of burst sizes from the λEpos lysogen was significantly more disperse that from λS, consistent

with a greater cell-to-cell variation in latent period or time of lysis. If the same data are re-plotted with the assumption that the burst size per cell varies linearly with the lysis time (Fig. 2.4B), it is clear that the lysis of the induced λS culture is significantly more saltatory than that of λEpos.

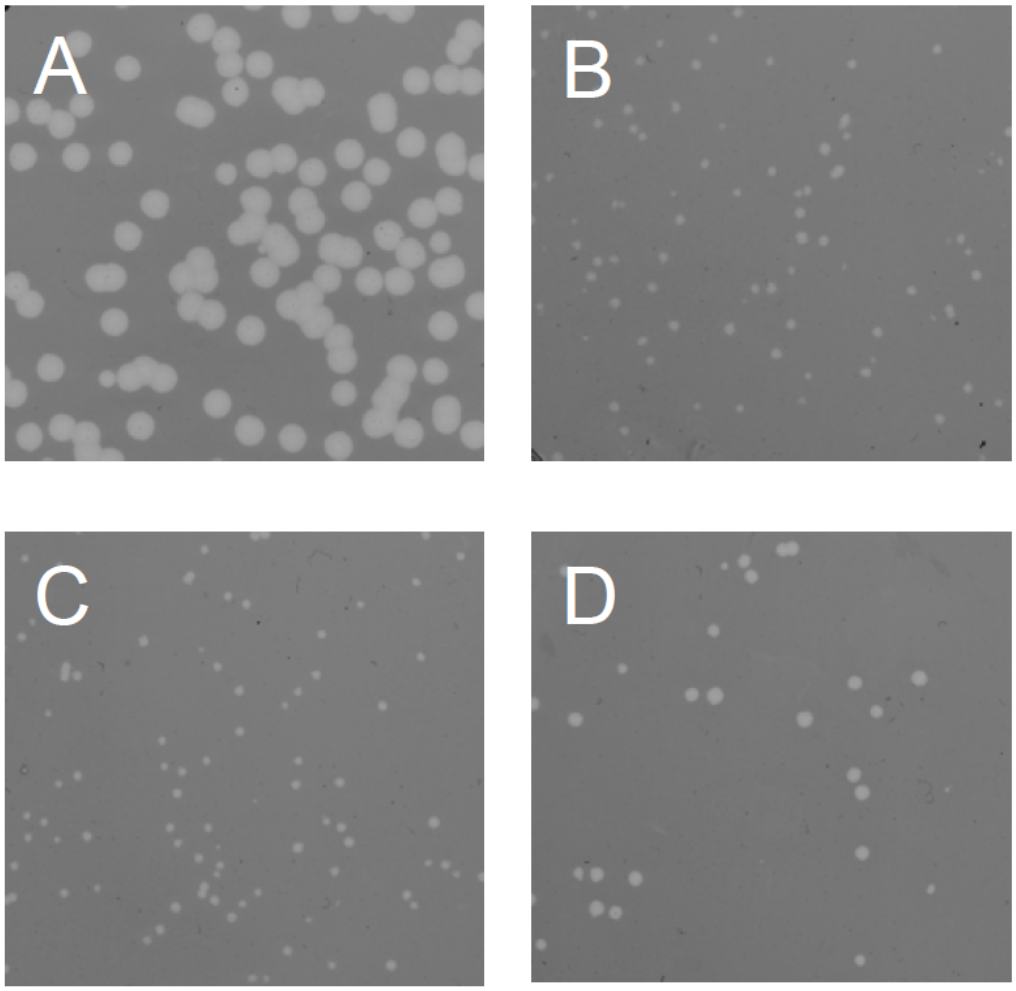

Figure 2.3. Plaque morphology. Top: Lawns of cells of MDS12*tonA*::Tn*10* plated with (A) λ*S*; (B) λ**E*; (C) λ*Epos*; (D) λ**Epos*.

Lysis time plasticity of S and E

In a previous study aimed at identifying transmembrane domains, the S holin was subjected to cysteine-scanning, in which multiple locations throughout the protein were substituted with a Cys residue. Remarkably, nearly every substitution resulted in altered lysis timing, with mutant timing phenotypes both advanced and retarded from the parental lysis time (GRÜNDLING *et al.* 2000a). To see if this functional plasticity extended to E, 14 missense changes in E were chosen by a randomizing computer algorithm and constructed in the vector pRW*. In parallel, 10 missense changes in S were chosen in the same way and constructed in the vector pRE. The complete sets of *E* and *S* mutant plasmids were characterized for their lysis timing phenotype by transformation into a host carrying pQ and induction with IPTG and arabinose (see Materials and Methods). For this experiment, the *S105* allele, which encodes only the holin product S105 (Fig. 2.1), was used as the parental *S* allele to avoid potential complications stemming from effects on the S107 antiholin.

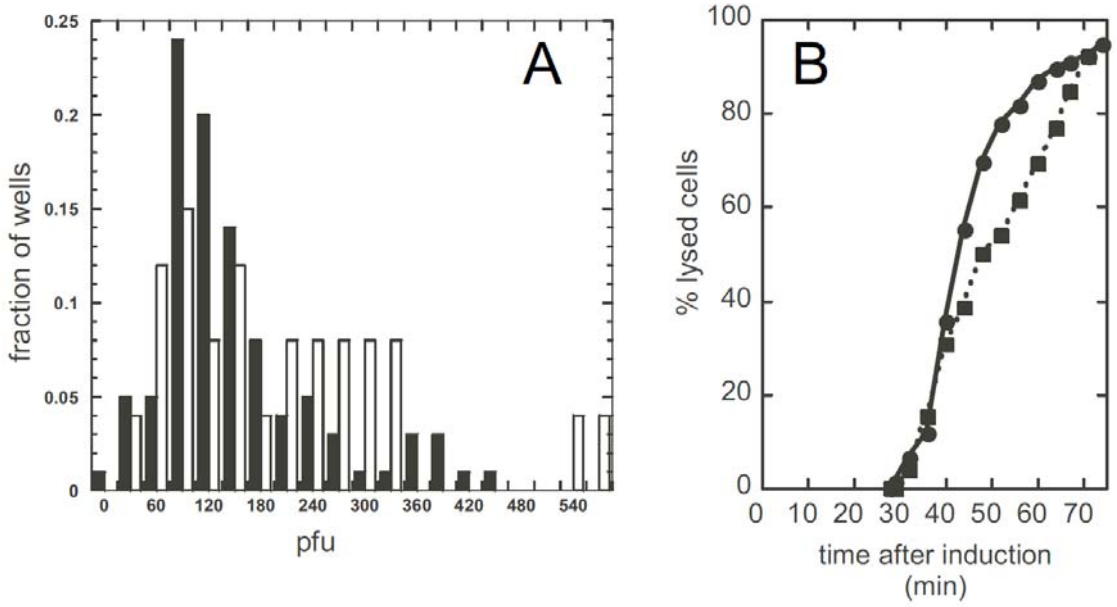

Figure 2.4. Burst size dispersion of λS and λEpos. A. Values on the x-axis are bins with the total number of pfu in a well; except for the "0" bin, which represents 0 to 15 pfu, each bin spans 30 pfu and is labeled with the midpoint of the bin. For example, '60' represents 45 - 75 pfu. For clarity, only alternate bins are labeled. Filled bars: λS. Open bars: λEpos. B. Data from A are converted into lysis times, with the assumptions that phage accumulate intracellularly at 7.7 pfu/min, beginning at 28 min after induction and that samples with less than 360 pfu contained a single lysogenic cell at the time of induction. Filled circles: λS. Squares: λEpos.

Table 2.3. Lysis time of random missense *S* and *E* alleles. Cells carrying plasmid-borned clones of the indicated *E* or *S105* alleles and plasmid pQ were induced and monitored for A550. Change in lysis time is relative to the parental allele in each case: pRW*E for *E* and pS105 for *S*. -, allele gave a lysis time within 3 min of the wt. Asterisk indicates mutant *E* alleles previously isolated by selecting for plaque-formation by ϕX174 on a *slyD* lawn (BERNHARDT *et al.* 2002a) and included here for comparative purposes. The G83I allele of *S105* exhibited a very delayed triggering phenotype, with a gradual decline in A550 beginning approximately 50 min after wt lysis.

E allele	Change in lysis time (min)
wt	wt
R3H L19F (*Epos4b*)*	-12
R3H (*Epos6*)*	-12
L19F (*Epos5*)*	-
R3D	-7
T5S	-
L16A	-
S17I	-
C61S	-
N43R	-
S53R	-
K57A	-
K90F	-
P21S	Non lytic
L24P	Non lytic
L42P	Non lytic
P65R	+10
T72P	+25
S allele	
wt	wt
A12T	-
L25G	+42
Y31I	-10
M50G	-5
I53Y	+20
G66E	-23
V77T	-10
G83I	(+50)
I87Y	+12
V101T	-

As shown in Table 2.3, 8 of the 14 *E* alleles were indistinguishable from the wild type and three others conferred an absolute lysis defect. One change, R3D, advanced lysis by 7 min, and two others, P65R and T72P, retarded lysis by 10 min and 25 min, respectively. When these alleles were analyzed by Western blot in samples taken at 20 min after induction (before the earliest lysis time), there was clearly increased accumulation of E protein for the R3D allele (Fig. 2.5), as previously reported for another early lysing allele, E_{R3H}, which was isolated in the context of the ϕX174 phage (BERNHARDT *et al.* 2000). Also, much less E protein accumulated in the alleles with retarded lysis. Thus lysis time was largely invariant, and what variation could be achieved was correlated with the net production of E protein. In contrast, 9 of the 10 randomly chosen missense changes in *S* affected lysis timing, four alleles advancing lysis between 5 and 23min, four alleles retarding lysis between 10 and 42 min, and one allele losing the ability to support saltatory lysis. Moreover, Western blot analysis of samples taken at 20 min after induction revealed that the the wt and mutant S proteins accumulated to similar levels (Fig. 2.5). Thus not only were timing mutants much more frequent in the panel of random *S* missense alleles than in the *E* panel, the timing differences for the *S* mutants could not be correlated with altered expression levels.

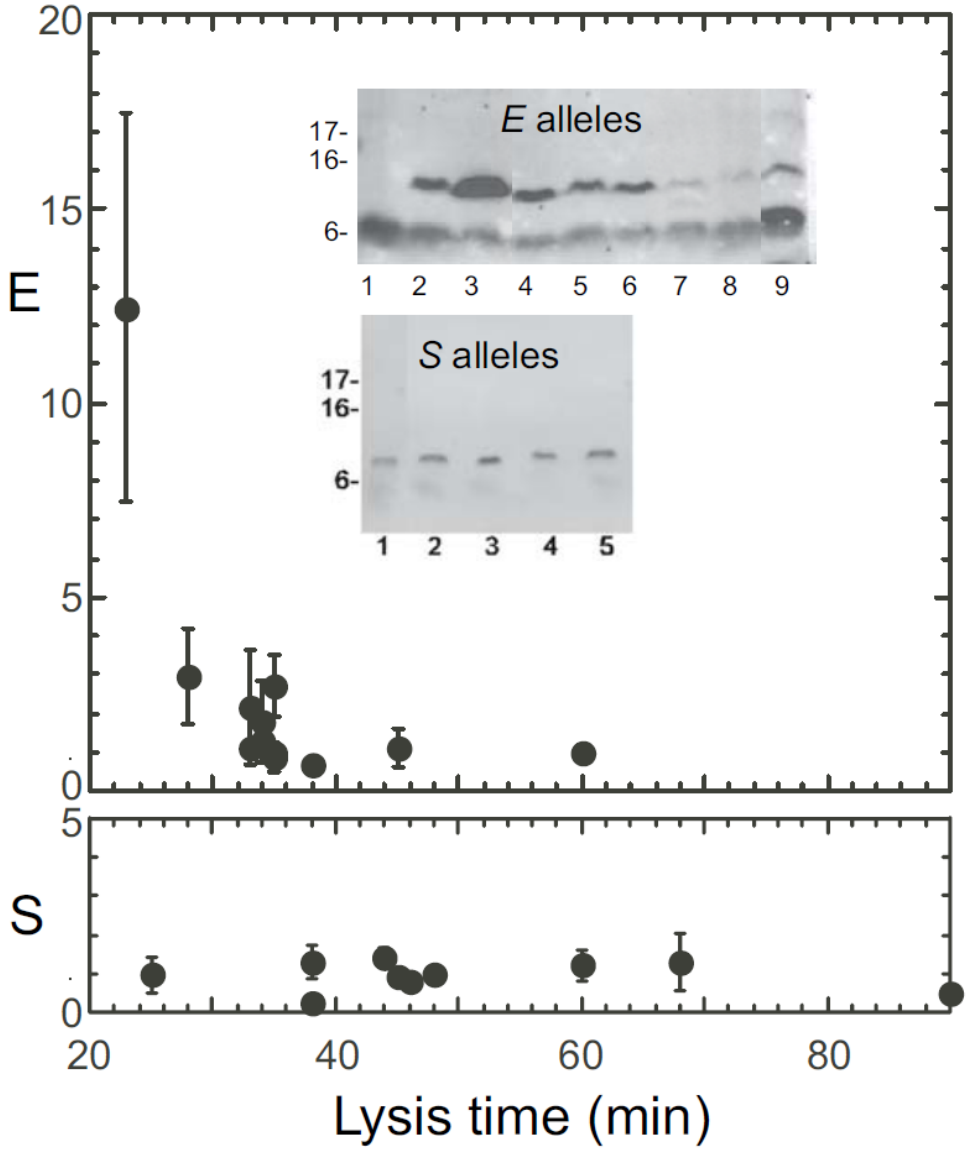

Figure 2.5. Expression of *E* and *S* mutants with different lysis times. Inset: Representative immunoblots of induced cultures of MDS12 *tonA*::Tn*10* pQ hosts carrying the pRW* clones of mutants described in Table 2.3. Upper gel, *E* alleles, blotted with anti-c-myc antibody, lanes: 1, pRW vector; 2, pRW*E; 3, pRWEpos; 4, R3D; 5, T5S; 6, S17I; 7, P21S; 8, L24P; 9, P65R. Lower gel, *S* alleles, blotted with anti-S, lanes: 1, Swt; 2, M50G; 3 I53Y; 4 G66E; 5 V77T. Graph: Relative amount of *E* or *S* expression, compared to parental allele (*E or Swt), versus time of lysis. Only alleles that supported clear lysis are plotted.

Discussion

The experiments reported here were aimed at determining if single-gene lysis systems had features that placed them at a competitive disadvantage when compared to the holin-endolysin systems universally found in dsDNA phage. λ was chosen as a test-bed because synchronous culture-wide lysogenic induction allows precise discrimination of lysis kinetics unlike infection which is inherently asynchronous. To compare the functionality of *S* and *E*, the ϕX174 *E* gene was substituted for the entire lysis cassette of λ. We found that an additional mutation in the λ DNA upstream of the *E* gene was required for plaque formation by the λE construct. This mutation dramatically increases the amount of E protein produced in infected cells or induced lysogens allowing host lysis. In any case, this upstream mutation found in λ*E or the previously identified *pos* mutation, known to increase the expression level of *E*, or a combination of both, resulted in λE chimeras that effect host lysis with kinetics that are within the range of known λ*S* alleles (GRÜNDLING *et al.* 2000a; JOHNSON-BOAZ *et al.* 1994; RAAB *et al.* 1988) and generate plaques with comparable efficiency. Presumably this reflects the fact that in ϕX174 transcription of the *E* gene, which occurs constitutively from all the known phage promoters throughout the infection cycle, is significantly higher than in the λ chimera, where late genes are expressed from the single late promoter. In addition, in the λ infection cycle, but not in ϕX174 infections, the dsDNA templates are steadily packaged into proheads, thus depleting the transcribable pool during late gene expression. This set

of *E*-dependent dsDNA phages were examined for features that might explain why dsDNA phages with single-gene lysis systems are not found in nature.

Phenotypic comparison of single-gene and holin-endolysin lysis

In terms of gross lysis, no compelling defect is evident for the λE constructs, compared to λS. Lysis is complete in both cases (Fig. 2.2), and ~90% or more of the virions produced are released into the medium. This suggests that any fitness advantage conferred by holin-dependent systems is not due to the inability of the E protein to allow release of phage particles that are much larger than ϕX174. Moreover, the efficiency of plating for the λE constructs was identical to that for λS indicating that reliance upon the E protein for host lysis did not result in a significant number of unproductive infections. The most striking phenotypic differences between λS and λE were the highly variable plaque size and the greater burst size dispersion found for the E chimeras (Figs. 2.3, 2.4).

Changing the lysis time

Cysteine-scanning mutagenesis used to determine the topology of S also suggested that a large fraction of missense mutations in *S* would alter the triggering time of the holin protein rather than be without effect or result in its inactivation. Here, we have provided additional evidence in support of this conclusion by measuring the triggering time of 10 randomly chosen *S* missense alleles. Triggering times were altered

in eight of these mutants, ranging from an advance of 25 min to delays of 50 min (Table 2.3). The ease at which the triggering time for S can be altered by single mutations presumably reflects the fact that most of the residues in this protein are involved in intra- and inter- molecular interactions necessary for its oligomerization into a "hole".

Unlike the holins, the lysis proteins encoded by ssRNA and ssDNA phage do not disrupt the cytoplasmic membrane of the host. The E protein brings about host lysis due to its ability to inhibit MraY, an enzyme required for peptidoglycan synthesis. Thus, the timing of E-mediated lysis can only be adjusted by altering the time at which the concentration of lipid I, the product of MraY, is reduced below the level necessary for the viability of the dividing host cell. In principle, this could be achieved by mutations that change either the concentration of E in the infected cell or the affinity of E for its target, MraY. In λE, the scope for mutational changes affecting the level of E is limited to those that affect the total translatability of the mRNA or the decrease the stability of the E protein. Transcriptional effects are not likely since varying the activity of pR', which as the single λ late promoter serves all of the late genes, would also cause profound changes in the levels of the many proteins involved in morphogenesis. The previously described *Epos* mutants (BERNHARDT *et al.* 2002a) are examples of alleles which cause earlier lysis due to increased levels of the E protein (Fig. 2.2, inset).

The frequency at which missense changes in the E protein altering its interaction with MraY result in physiologically relevant changes in lysis timing is unknown. In this study, we have found that when a collection of randomly chosen mutations in *E* was constructed, more than half of the missense changes had no effect on lysis time, and 20%

were lysis-defective. Only one advanced lysis timing, and this mutation, R3D, was in the same codon as *Epos* (R3H) and, like *Epos*, resulted in an increase in E accumulation. Two mutations retarded lysis: P65R, by ~ 10 min, and T72P, by ~25 min. In both cases, the E protein was found to accumulate to lower levels as is the case with the previously described slow lysing allele E_{P29A}. Thus, missense mutations in *E* that result in an altered time of lysis appear to be rare. It is worth noting that the greater latent period dispersion for the λE constructs would be predicted to compound the defect associated with less plasticity, relative to the holin-mediated system. For example, if a population of λ*E phages were growing under media and host conditions where the parental lysis time was ideally suited for the current environment, and that environment was then changed, such that the average host density was dramatically lowered, it is predicted that a new, delayed lysis time would confer optimum fitness (WANG 2006; WANG *et al.* 1996). However, even if a mutation did occur that caused a change the level of *E* translation without ablating lysis completely, the intrinsic dispersion of the latent period in a population of infected cells would retard the selection of this mutant. In contrast, mutations that retard S triggering are relatively easy to obtain, and, with the tighter latent period dispersion (Fig. 2.4), they would be more rapidly selected from the population of parentals. Thus, compared to single-gene lysis, holin-mediated lysis timing shows greater phenotypic plasticity but much less phenotypic variance and, the fitness contributions of these features would be expected to be synergistic. Based on these observations, we propose that apparently universal reliance of dsDNA phage on holin-endolysin systems derives from the malleability of holins with respect to their triggering

time. The lack of such malleability in single-gene lysis systems led to their disappearance from phage with sufficient coding capacity to accommodate holin-endolysin systems. Of course, the difference in malleability reflects the fundamental difference in the mechanism of lysis. E causes lysis by inhibiting MraY, thus stopping the flow of murein precursors. Most frequently this results in lysis when septation is attempted, since entirely new murein is required to complete cell division. Thus another reason for the predominance of the holin-endolysin systems is that the lytic function is independent of the host cell cycle.

Recently, it was shown that a large class of phage endolysins have intrinsic export signals, designated as SAR (signal-anchor-release) domains, which confer the capacity to effect host lysis without holin function (XU *et al.* 2005; XU *et al.* 2004). Phages carrying a SAR endolysin gene make large, uniform plaques, but inactivation of the holin gene results in a heterogeneous plaque size phenotype (PARK *et al.* 2007). In effect, SAR endolysins represent another mode of single-gene lysis. In this case, holin-mediated control has been apparently been super-imposed on the lysis system, presumably conferring a fitness advantage analagous to that which has prevented a λE-equivalent from evolving.

CHAPTER III

GENETIC ANALYSIS OF MraY INHIBITION BY THE ϕX174 PROTEIN E*

Introduction

In infections of double-strand DNA phages, host lysis is a strictly regulated, precisely timed, multigenic event, involving up to five proteins, including a holin to permeabilize the cytoplasmic membrane and an endolysin to degrade the cell wall(YOUNG and WANG 2006). In contrast, host lysis by the much simpler single-strand RNA (ssRNA) and DNA (ssDNA) phages is accomplished by expressing a single gene with no known relationship to any of the lysis genes encoded by more complex phage (BERNHARDT *et al.* 2002b; COLEMAN *et al.* 1983; HENRICH *et al.* 1982; KARNIK and BILLETER 1983; WINTER and GOLD 1983; YOUNG and YOUNG 1982). There are three unrelated lysis genes encoded by these small phages: *E*, in the prototype microvirus (ssDNA) ϕX174; A_2, in the prototype allolevirus (ssRNA) Qβ, and *L*, in the prototype levivirus (ssRNA) MS2. Although the mechanism of lysis mediated by L remains obscure, it has been established that both E and A_2 operate by inhibiting cytoplasmic steps in cell wall synthesis (BERNHARDT *et al.* 2000; BERNHARDT *et al.* 2001a; BERNHARDT *et al.* 2001b).

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* Reprinted with permission from Zheng, Y., D. K. Struck, T. G. Bernhardt, and R. Young, 2008 Genetic analysis of MraY inhibition by the ϕX174 protein E. Genetics **180**:1459-1466.

E has had a prominent role in the history of molecular biology. It was the first gene shown to be completely embedded within another gene in a different reading frame (SANGER *et al.* 1977) (Fig. 3.1) and was the first gene to be subjected to site-directed mutagenesis(HUTCHISON *et al.* 1978). *E* encodes a 91 aa protein that is encoded by more than 90% of ϕX174 mRNAs (HAYASHI *et al.* 1976) and is localized to the cytoplasmic membrane, presumably by virtue of its putative N-terminal transmembrane domain (TMD) (ALTMAN *et al.* 1985; BLÄSI *et al.* 1983). Gene fusion experiments have shown that only the N-terminal 35 amino acids of E, including its putative TMD, are required for its lytic activity (BUCKLEY and HAYASHI 1986; MARATEA *et al.* 1985). Moreover, EΦβ−galactosidase fusions are lytically active and exhibit β-galactosidase activity, indicating that E has an N-out, C-in topology. We have shown that E causes lysis in growing cells by blocking cell wall synthesis, and that this blockage is effected by specific inhibition of MraY, a conserved enzyme in the pathway for murein biosynthesis (BERNHARDT *et al.* 2000; BERNHARDT *et al.* 2001a). MraY, also known as translocase I, catalyzes the formation of the precursor Lipid I by transfering phospho-MurNAc-pentapeptide from UDP-MurNAc-pentapeptide to undecaprenol-P. MraY has been proposed to have ten TMDs and to adopt an N-out, C-out topology (BOUHSS *et al.* 1999)(Fig. 3.1). Lloyd *et al.* have shown that aspartate residues at positions 115, 116 and 267 are essential for MraY activity in vitro (LLOYD *et al.* 2004). All three of these residues would reside in cytoplasmic loops of MraY given its predicted topology. D115 and D116 are thought to coordinate the Mg^{2+} ion involved in binding the pyrophosphate

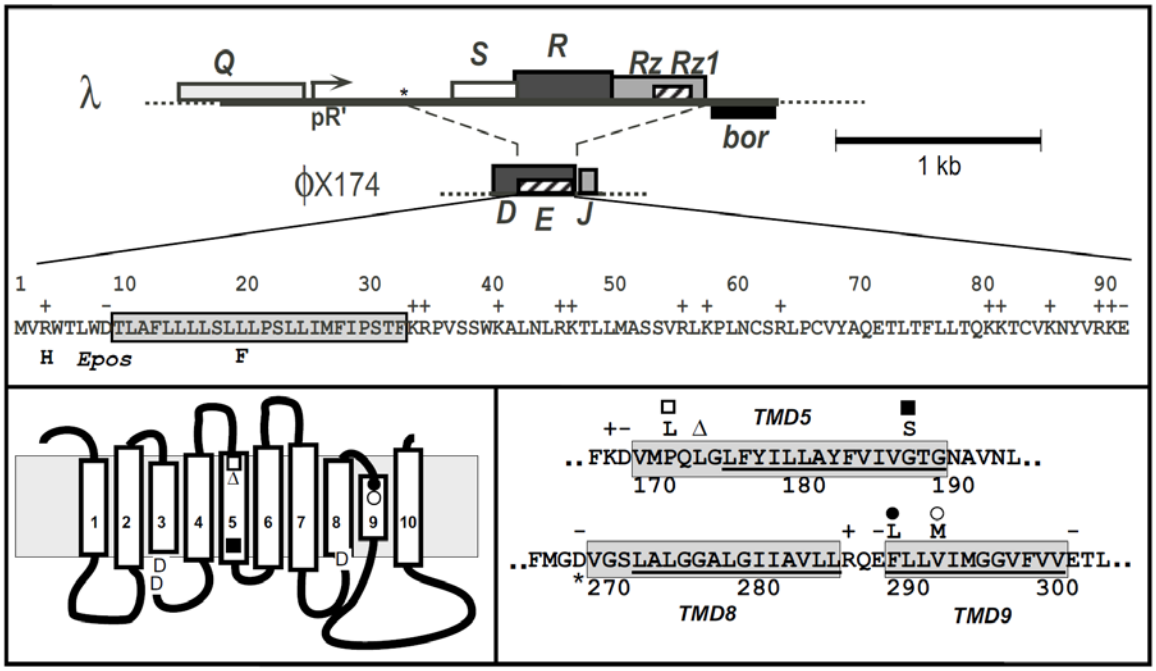

Figure 3.1. Features of E and MraY. Top panel: Structure of the lysis gene regions of the phages lambda and ϕX174, showing the replacement of *SRRzRz1* with *E* in the chimera λ*E used in this study. The position of a mutation that increases expression of *E* in this chimera is indicated by an asterisk (ZHENG *et al.* 2008b) and the two missense changes in Epos are shown below the primary structure of E(BERNHARDT *et al.* 2002a). Lower panel, left: proposed topology of MraY, based on primary structure analysis using the MemBrain algorithm(SHEN and CHOU 2008), is slightly modified from the topology of Bouhss *et al.* (BOUHSS *et al.* 1999), mainly in the the placement of TMDs 1, 2 and 5. This topology is till consistent with the results of the β–lactamase fusion study upon which the original topology was based. The beginning and ending residues for each TMD in this model, compared, where different, with those of the previous model, in brackets, are as follows: TMD1: 25 - 42 [19 - 45]; TMD2: 70 - 92 [77 - 90]; TMD3: 97 - 113; TMD4: 134 - 153 [134 - 156]; TMD5: 168 - 188 [174 - 188]; TMD6: 200 - 220; TMD7: 234-- 257 [239 - 251]; TMD8: 268 - 284 [271 - 284]; TMD9: 288 - 299; TMD10: 342 - 358 [343 - 358]. The positions of three conserved Asp residues important for enzyme activity are indicated on cytoplasmic loops below TMD3 and TMD8(LLOYD *et al.* 2004). The positions of *mraY* mutations conferring resistance to E are indicated by the following symbols: Δ, ΔL172 and ●, F288L reported previously(BERNHARDT *et al.* 2000)and three described in this work: □, P170L; ■, G186S; ○, V291M. Lower panel, right: The sequences of two regions of MraY in which E-resistance mutations have been isolated are shown, with the proposed catalytic Asp267 residue indicated by an asterisk. The extent of the TMDs proposed in this study and in the previous work are indicated by shaded rectangles and underlining, respectively.

moiety of the UDP-MurNAc-pentapeptide substrate, while D267 is predicted to be an active-site nucleophile that attacks its β-phosphate.

In our original study, MraY was identified as the target of E by the isolation of two dominant *mraY* mutations conferring resistance to this lysis protein. One of these was a single-codon deletion, ΔL172, in putative TMD5 and the other a missense change, F288L, in putative TMD9 (Fig. 3.1). Here we report studies extending the mutational analysis of the E-MraY interaction and discuss the results in terms of a model for the E-mediated inhibition of MraY.

Methods

Media, chemicals and general methods

Cultures were grown in standard LB media supplemented with appropriate antibiotics, as described (TRAN *et al.* 2005). Inductions were performed by addition of arabinose to a final concentration of 0.2% and, for lysogenic cultures, beginning 2 min after arabinose induction, by aerating at 42°C for 15 min and at 37°C thereafter. Lysis profiles were obtained by monitoring A_{550} after induction, as described previously (RAMANCULOV and YOUNG 2001a). β-galactosidase activity was assayed according to Miller (MILLER 1972b), except that the cells are pelleted and resuspended in assay buffer, as described by M. Price-Carter (http://rothlab.ucdavis.edu/protocols/beta-galactosidase-3.html). Plasmid DNA isolation, DNA amplification by PCR, DNA

transformation, DNA sequencing, and Quikchange (Stratagene) site-directed mutagenesis were performed as previously described (TRAN *et al.* 2005).

Bacterial strains, bacteriophages, and plasmids

The prototroph MDS12 *tonA::*Tn*10* (KOLISNYCHENKO *et al.* 2002; TRAN *et al.* 2005), carrying deletions of all the cryptic prophage sequences of *E. coli*, was used as the host for all lysogenic inductions. The phages λ**E* and λ*Epos* (Fig. 3.1) and the construction of single-copy lysogens have been described (ZHENG *et al.* 2008b). The medium-copy plasmid pMY30 has the *E. coli mraY* gene (^{Ec}*mraY*) inserted between the *Sma*I and *Hin*dIII sites of pBAD30 (GUZMAN *et al.* 1995) placing it under the control of the p_{araBAD} promoter (BERNHARDT *et al.* 2000). The plasmid pBAD30-BsMraY was constructed similarly except the *mraY* gene was from *B. subtilis* W23 (nt 1587210 to 1588202 of the *B. subtilis* genome). A strain with a chromosomal *ΔmraY* was constructed using the protocol of Link et al.(1997). Briefly, the plasmid pKOMY3 was constructed by inserting a DNA fragment spanning nt 95015 to 98343 of the *E. coli* K-12 genome into the unique *Bam*HI site of pKO3, a vector with a *ts*-replicon. This construct carries *mraY* as well as portions of the upstream and downstream genes *murF* and *murD*. Next the plasmid pKOMY3ΔmraY was constructed, in which the entire *mraY* sequence was deleted (nt 96025 to 97051 of the *E. coli* genome), leaving 1 and 1.3 kb of homology upstream and downstream of *ΔmraY*, respectively. The strain RY3316 was constructed from MG1655 (*F⁻ ilvG rfb50 rph1*; obtained from the *E. coli* Genetic Stock

Center http://cgsc.biology.yale.edu/) by exchanging the deletion from pKOMY3ΔmraY into the chromosome, as described (LINK *et al.* 1997). The strain RY3321, which is RY3316 *recA srl::*Tn*10* pKOMY3, was constructed by P1 transduction and used as the host strain for all complementation experiments. To test the ability of each allele of *mraY* to functionally replace the wild type gene in *E. coli*, we first placed it under the control of the *ara* promoter in the vector pBAD30 and transformed the resulting plasmid into RY3321. The ability of the transformants to grow at 42ºC in the presence, but not the absence, of arabinose was taken as proof that the *mraY* gene on the pBAD30 plasmid was able to complement a chromosomal *mraY* deletion.

Selection of mraY *mutants resistant to E*

Mutants resistant to E-mediated lysis were isolated as previously described (BERNHARDT *et al.* 2000), except that the cells were mutagenized with ethylmethanesulfonate (EMS), essentially as described by Miller (MILLER 1972a), prior to the selection. The only differences in the protocol used here were that the exposure to EMS was limited to 15 to 30 min, instead of 2 h, and that, after the EMS treatment, the cells were washed twice in minimal salts, grown in LB overnight, and stored at -80°C after addition of dimethylsulfoxide (85 µl per ml of culture). Individual treated cultures were tested for the frequency of rifampicin-resistance as a measure of mutagenesis. Cultures treated for 15 and 30 min exhibited approximately 60-fold and 100-fold

increases in rifampicin-resistance, respectively, and were subcultured and used for selection. The selection of *mraY* mutants were performed by Dr. Tom Bernhardt.

Detection of MraY

Bethyl Laboratories (Montgomery, TX) prepared the antibody used for detecting MraY by affinity purification of antisera raised against the peptide RGQRIFRMAPIHHHYEL (residues 314 to 330 of MraY). For the detection of MraY, logarithmic cultures of MDS12 *tonA::*Tn*10* were induced at A_{550} ~ 0.6. After 1 h, cells were harvested by centrifugation and 1 A_{550} unit was analyzed by SDS-PAGE on a 12% separating gel and immunoblotting, as described (ZHENG *et al.* 2008b).

Results

Over-expression of active and inactive alleles of E. coli mraY *protects against E-mediated lysis*

Since E is an inhibitor of MraY, it seemed likely that the over-expression of *mraY* would prevent E-mediated lysis. This hypothesis was tested in a "protection assay" using a host with a copy of *mraY* gene on the chromosomal carrying the *E. coli mraY* gene (^{Ec}*mraY*) on an arabinose-inducible plasmid, pBAD30 in trans to a thermally inducible lambda prophage, λ**E,* in which the lysis gene cassette is replaced by the *E*

gene (ZHENG *et al.* 2008b). When a culture of this strain was sequentially induced with arabinose and a thermal shift in early logarithmic phase (A_{550} = 0.2), the $^{Ec}mraY$ plasmid had no effect on lysis (Fig. 3.2). However, when cultures were induced at a higher culture density, lysis was prevented. Presumably, this reflects increased $^{Ec}mraY$ expression from the plasmid at higher culture densities because of higher cAMP levels and the catabolite-sensitive character of the arabinose promoter of pBAD30 (GUZMAN *et al.* 1995). This interpretation is supported by the fact we found *lacZ* expression to be 2.5-fold higher at A_{550} = 0.5, than at A_{550} = 0.2 (not shown).

Next, we tested a catalytically inactive allele of $^{Ec}mraY$ for its ability to similarly prevent lysis of the induced λ*E lysogen. For this experiment, we used the $^{Ec}mraY_{D267N}$ allele, which encodes an inactive protein thought to be defective because the D267N missense change eliminates a putative active site nucleophile (LLOYD *et al.* 2004). Once again, arabinose-induction at low cell densities had no effect on lysis, but at higher cell densities, lysis was completely blocked (Fig. 3.2). This suggests that, if produced in sufficient quantity, an inactive MraY protein can bind enough of the E protein produced by the induced λ*E lysogen to spare functional MraY, produced from the chromosomal *mraY* gene, from E-mediated inhibition.

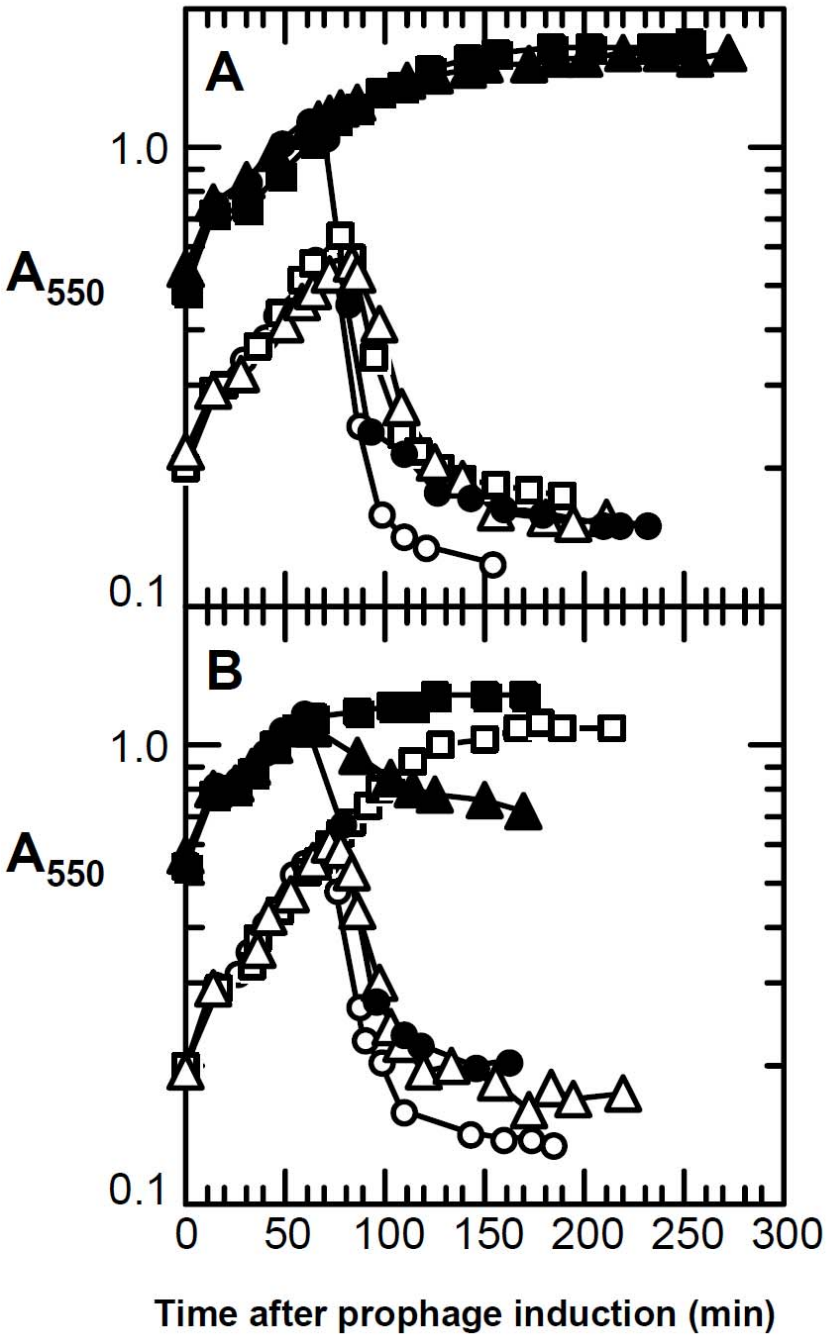

Figure 3.2. Induction of plasmid-borned *mraY* alleles can protect against E-mediated lysis. Cultures of MDS12 *tonA::*Tn*10* (λ*E), bearing derivatives of the plasmid pBAD30 carrying the indicated alleles of *mraY* of *E. coli* (panel A) or *B. subtilis* (panel B) were induced at either A_{550} = 0.2 (empty symbols) or A_{550} = 0.5 (filled symbols) and monitored for culture turbidity, as described in Materials and Methods. Symbols: ○●, pBAD30 vector; □■ pBAD30 carrying the wt *mraY* gene; Δ▲pBAD30 carrying the inactivated gene ($^{Ec}mraY_{D267N}$ or $^{Bs}mraY_{D231N}$).

A heterologous MraY protein does not interact with E

Since the MraY proteins from Gram-positive bacteria diverge significantly when compared to ^{Ec}MraY, the specific protein-protein contacts necessary for E-sensitivity might not occur with enzymes from the former. In fact, it has been reported that the cloned *E* gene is not lytic when expressed in *Staphylococcus carnosus* (HALFMANN *et al.* 1993). To assess the ability of the plasmid-based system to discriminate between MraY proteins on the basis of their interaction with E, we decided to repeat the experiments described above using plasmids carrying active and inactive alleles of the *mraY* gene from *B. subtilis* (^{Bs}*mraY*). As a first step, we tested the ability of the ^{Bs}*mraY* gene to complement a chromosomal deletion of *mraY.* In this experiment, the only source of MraY protein is from the transformed plasmid, unlike the protection assay where the chromosomal *mraY* is kept intact for testing the ability to protect by the enzymatically inactive allele. As can be seen in Figure 3.3, the essential function(s) of MraY in *E. coli* can be fulfilled by the *B. subtilis* enzyme. Since sequence alignment indicates that Asp231 of the ^{Bs}MraY is equivalent to the proposed catalytic Asp267 of the ^{Ec}MraY (AL-DABBAGH *et al.* 2008; LEHRMAN 1994), we tested both ^{Ec}*mraY*$_{D267N}$ and ^{Bs}*mraY*$_{D231N}$ genes for their ability to complement the *mraY* deletion. As can be seen in Figure 3.3, neither allele allowed cell growth at the restrictive temperature, consistent with a catalytic role for the altered aspartate residues protein.

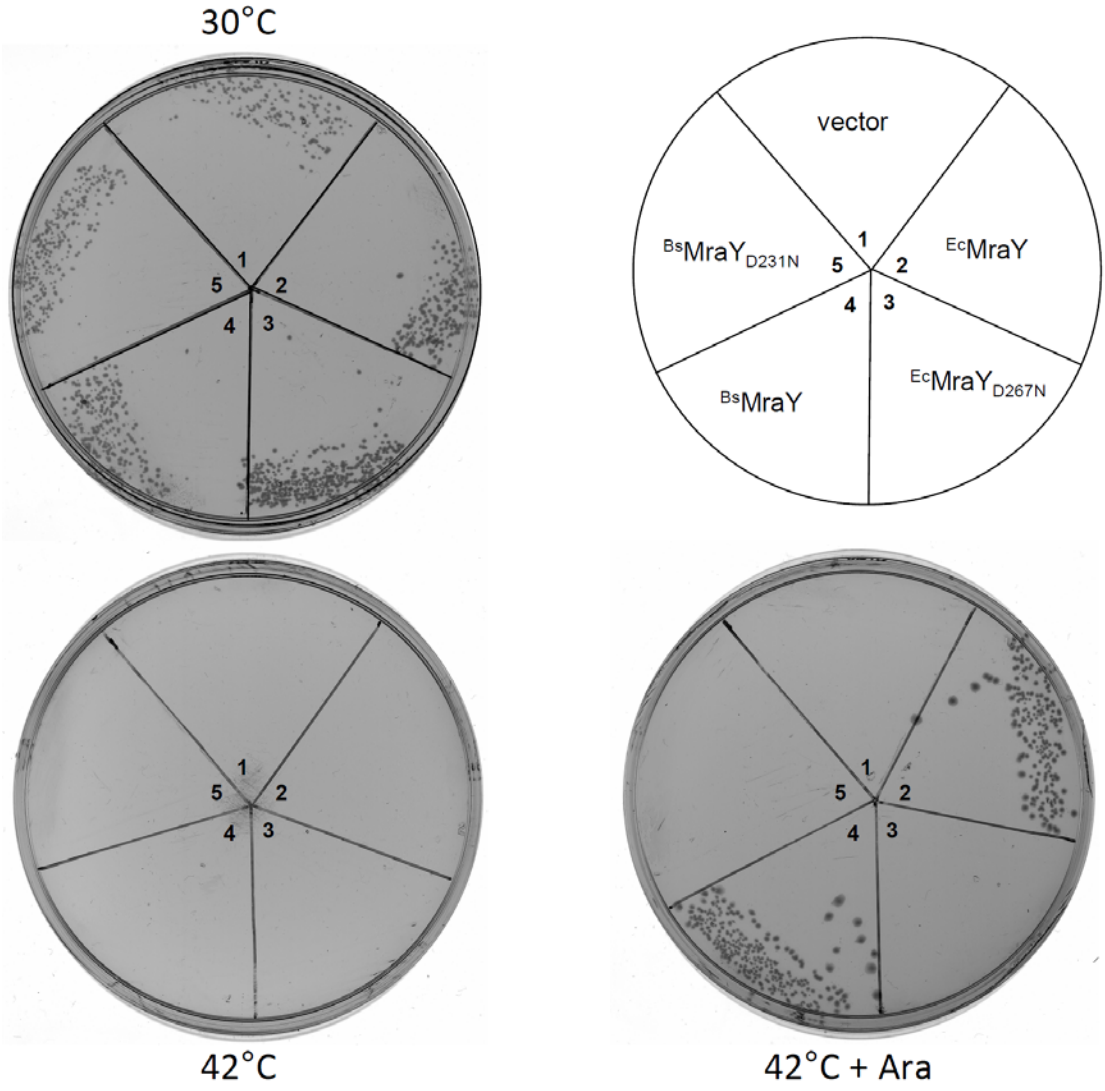

Figure 3.3. ^{Bs}*mraY* complements *Δ*^{Ec}*mraY*. RY3321 which has a chromosomal deletion of *mraY* and carries the wt ^{Ec}*mraY* gene on a low copy, ts-replicon, was transformed with the indicated plasmids and tested for growth under the indicated conditions, as described in Methods.

We next examined the ability of ^{Bs}*mraY* and ^{Bs}*mraY*$_{D231N}$ to prevent lysis of an induced λ*E lysogen. In contrast to what was observed with ^{Ec}*mraY*, the ability of the active allele, ^{Bs}*mraY*, to block E-mediated lysis was independent of culture density (Fig. 3.2) suggesting that fewer molecules of the *Bacillus* enzyme are required to protect cells

from lysis after induction of λ*E. However, over-expression of the catalytically-inactive allele, $^{Bs}mraY_{D231N}$, afforded little or no protection against E-mediated lysis in this system. Taken together, these results suggest that the active *B. subtilis* enzyme, even at the lower level of production, can provide sufficient lipid I, despite inhibition of the host MraY by E, but the inactive ^{Bs}MraY, even at the higher level of expression, cannot titrate out E and thus allow lipid I production by the host MraY. Thus, in our protection assay, the $^{Ec}mraY$ and $^{Bs}mraY$ genes are useful as controls encoding proteins that, respectively, do and do not interact strongly with E. For the experiments described below, the ability of the inactivated version of any *mraY* allele to protect against lysis is taken as evidence for the ability of its product to bind E, and thus spare the host MraY activity.

The E-resistant alleles of ^{Ec}mraY *encode proteins with different apparent affinities for E*

The original E-resistant mutants were obtained by inducing a plasmid-borne allele of *E* and then screening the spontaneous survivors for resistance to ϕX174 (BERNHARDT *et al.* 2000). More than 99% of the survivors harbored alterations in the *E*-plasmid, and only two ϕX174^R alleles of *mraY* were found, ΔL172 and F288L. To increase the pool of ϕX174^R alleles, we used EMS mutagenesis to increase the total frequency of survivors by approximately 20-fold. Every phage-resistant isolate was found to have a missense change in the *mraY* gene. However, from three independent mutagenesis pools in which a total of 13 mutants were sequenced, only three more alleles were obtained, all multiple times (not shown). Together with the original mutants

used to identify MraY as the target of E, the five E-resistance mutations mapped to only two of the 10 TMDs of MraY (Fig. 3.1). The clustering of these mutations in the two TMDs and their repeated isolation suggested that this mutant selection was at or near saturation.

We examined the behavior of the five E-resistant alleles of $^{Ec}mraY$ in our protection assay. As can be seen in Figure 3.4, these alleles fall into three classes. Two of these *mraY* mutants, G186S and V291M, are indistinguishable from the wild type. In their active or inactive (D267N) forms, neither protects at low culture density but both do at high culture density. Alleles encoding the P170L and ΔL172 variants protect only in their active forms and only at high culture density. Finally, the $^{Ec}MraY_{F288L}$ protein appears to be similar to ^{Bs}MraY in that protection is observed only with the active enzyme but occurs at both low and high culture densities. Although the low levels of MraY even from the plasmid-borne alleles preclude accurate quantitation by immunoblot, nevertheless it is clear that at least for the $mraY_{P170L,D267N}$ and $mraY_{F288L,D267N}$ alleles, the amount of MraY protein in these protection experiments is as high as or higher than the parental $mraY_{D267N}$ (Fig. 3.5).

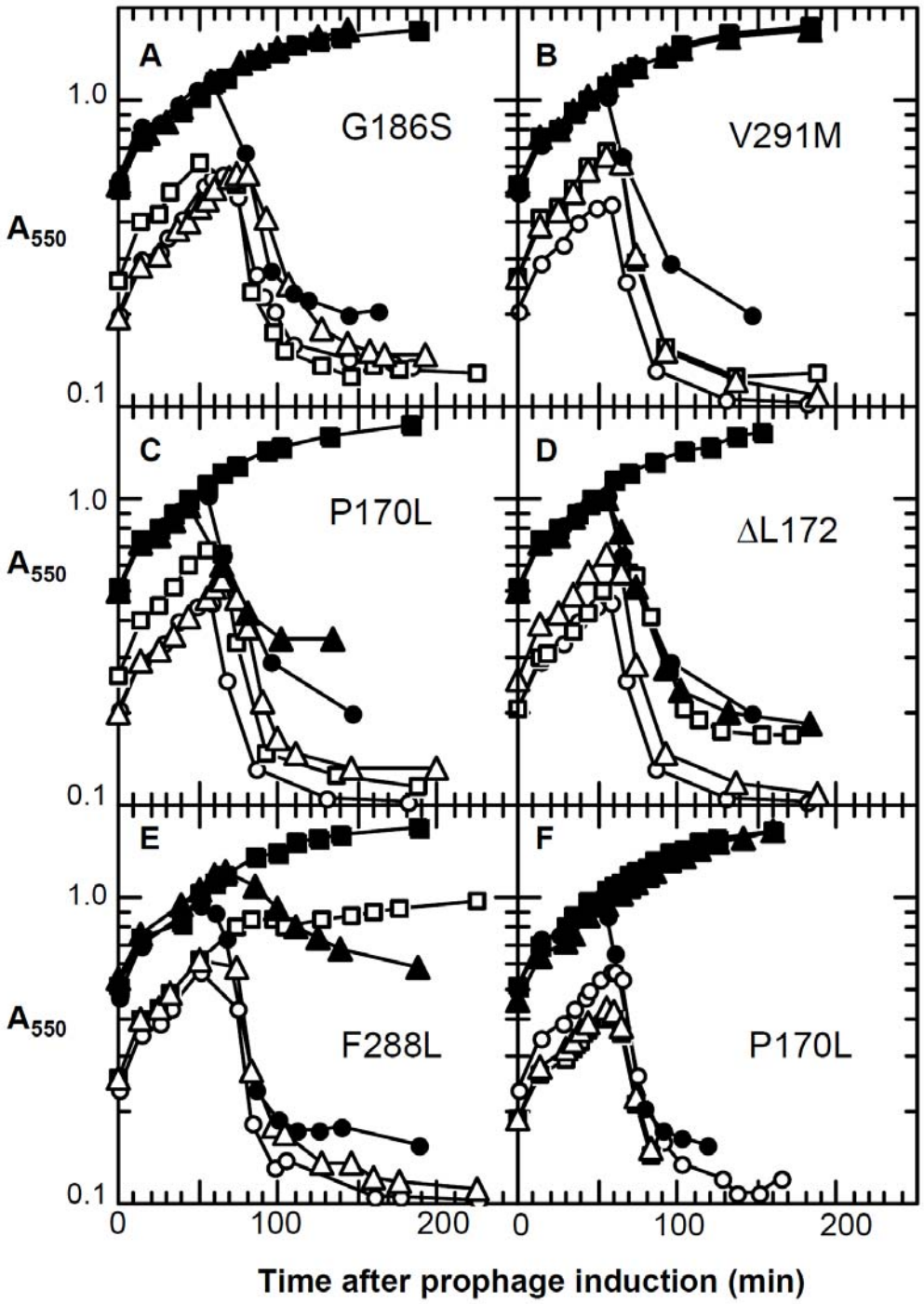

Figure 3.4. E-resistant alleles of *mraY* show different protection against E-mediated lysis. Inductions and symbols are the same as in Fig. 3.2, except that in each panel, the indicated ^{Ec}*mraY* allele is used and in panel F, the prophage is λEpos. Symbols: ○●, pBAD30 vector; □■ pBAD30 carrying *mraY*; Δ▲pBAD30 carrying the inactivated gene ($mraY_{D267N}$).

$MraY_{P170L}$ interacts with Epos more strongly than E

The alleles of ^{Ec}*mraY* that provide resistance to E were originally selected using a plasmid encoding the *Epos* gene (BERNHARDT *et al.* 2000). This was necessary because otherwise frequent knockout mutations in *slyD*, which encodes an abundant cytoplasmic peptidyl-prolyl isomerase required for the stability of the E protein, would overwhelm the selection for E-insensitivity (BERNHARDT *et al.* 2002a; MARATEA *et al.* 1985; ROOF *et al.* 1994). The *Epos* allele does not require *slyD* for function. The protein encoded by the *Epos* gene has two missense changes, R3H and L19F (Fig. 3.1). For this reason, we repeated the protection assays using a λEpos lysogen. The only significant difference from the experiments using the λ*E lysogen was found with the ^{Ec}*$mraY_{P170L}$* plasmid. While the inactive form (D267N) of $^{Ec}MraY_{P170L}$ did not protect against lysis by E under any condition tested, it did provide complete protection against Epos at high culture densities (Fig. 3.4). The simplest interpretation of this result is that $^{Ec}MraY_{P170L}$ binds Epos more tightly than E.

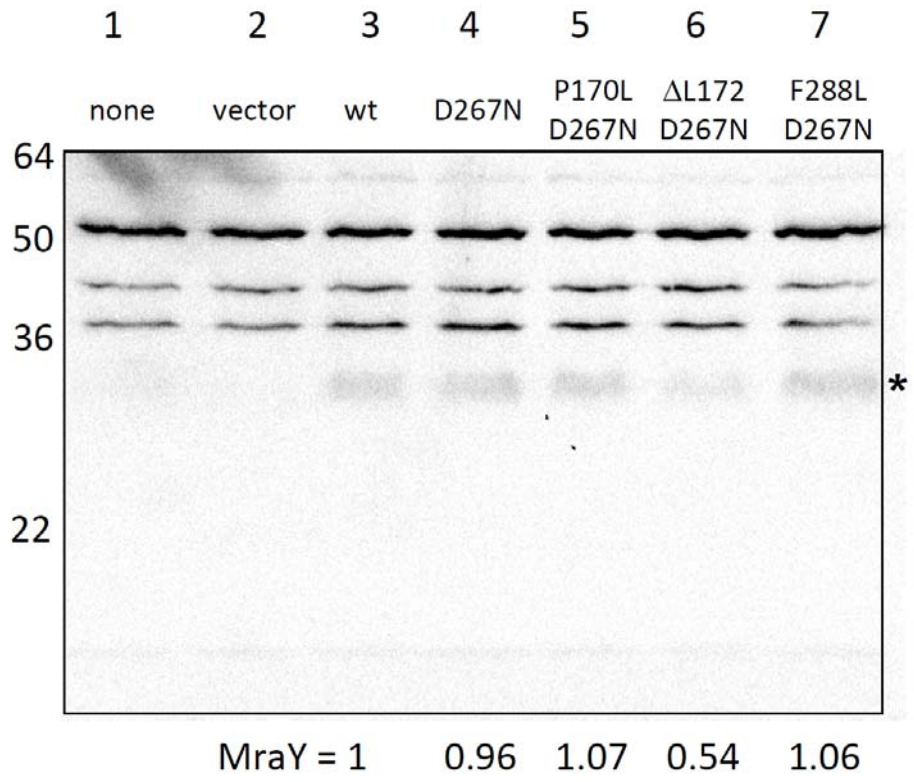

Figure 3.5. Accumulation of MraY proteins. Membranes from induced cultures bearing either no plasmid (lane 1), pBAD30 (lane 2) or pBAD30 carrying the indicated allele of ^{Ec}*mraY* (lanes 3 - 7) were analyzed by immunoblot with antibodies raised against a peptide of MraY. The position of molecular mass standards are indicated to the left and the relative amount of MraY, by integration of the band indicated by an asterisk, is given below lanes 4 through 7, relative to the amount in lane 3.

Discussion

Genetic systems for assessing mraY *function and interaction with E*

Here, we present two genetic systems for the further analysis of MraY and its interaction with E, the lysis protein of ϕX174. First, using a host with a deletion of *mraY*

on the chromosome and a functional copy of *mraY* on a *ts*-replicon, we were able to test the ability of any given *mraY* allele to substitute for the ^{Ec}*mraY* gene. Somewhat unexpectedly, we found that the highly divergent ^{Bs}*mraY* gene was able to complement a chromosomal ^{Ec}*mraY* deletion. Given the high degree of divergence in the primary structures of ^{Ec}MraY and ^{Bs}MraY, this argues that the single, essential function of MraY is to convert UDP-MurNAc-pentapeptide into lipid I. It has been suggested that MraY might participate in the formation of a multi-enzyme complex or "machine" that is essential for the biosynthesis of peptidoglycan (BOUHSS *et al.* 2008; BUGG *et al.* 2006; MENDEL *et al.* 2006). While our results do not rule out this possibility, they do suggest that MraY is, at best, a peripheral and not essential for the assembly of such a machine. Finally, we found that the ^{Ec}*mraY*$_{D267N}$ allele was unable to complement the chromosomal *mraY* deletions, providing additional support for the proposal that Asp267 is an essential residue for ^{Ec}MraY, as reported by Lloyd and colleagues (2004). The fact that this system provides a robust, low-background read-out on solid medium for functional determination makes it ideally suited for high throughput analysis of randomly mutated *mraY*, which, to date, has been subjected only to limited site-directed mutagenesis of conserved residues(LLOYD *et al.* 2004). The adjustability of the p_{araBAD} vector, using alleles of *pcnB* to alter copy number (LOPILATO *et al.* 1986) and numerous agents that exert different levels of catabolite repression also may allow this system to be used for a chemical biology approach in screening for small molecule inhibitors of MraY.

In a second type of assay, the ability of a plasmid-borne allele of *mraY* under p_{araBAD} control is tested for its ability to prevent the lysis by an induced λ*E lysogen. The chromosomal *mraY* gene of the host is left intact, so that even enzymatically inactive *mraY* alleles could be examined for their ability to protect against E-mediated lysis. When $^{Ec}mraY$ was present on the plasmid, we found that inductions of a culture in early logarithmic phase had no effect on lysis, but when cultures were allowed to grow to a higher culture density before induction, lysis was prevented. Presumably, this dependence on culture density reflects the 2.5-fold higher level of expression at high culture density from the catabolite-sensitive p_{araBAD} promoter in the plasmid vector. Essentially identical results were obtained when the $^{Ec}mraY_{D267N}$ allele was present on the plasmid. We interpret this as indicating that the inactive $MraY_{D267N}$ protein bound E and, thus, spared a smaller pool of active MraY produced from the chromosomal *mraY* gene from E-mediated inhibition. Strikingly different results were obtained using plasmids carrying $^{Bs}mraY$ or a variant encoding an inactive protein, $^{Bs}mraY_{D231N}$. First, the protection seen with $^{Bs}mraY$ was independent of culture density, suggesting that the ^{Bs}MraY protein protected against E-mediated lysis, even when present at low levels, by catalyzing the formation of Lipid I. Furthermore, in contrast to what was seen with $^{Ec}mraY_{D267N}$, induction of $^{Bs}mraY_{D231N}$ did not block lysis at either high or low cell densities. Together, these results indicate that the ^{Bs}MraY protein has very low affinity, if any, for E. Thus, the protection assay we have developed is a genetic tool that can be used to distinguish between genes encoding MraY proteins with differing apparent affinities for E.

Next, we assessed the behavior of five ^{Ec}*mraY* alleles which provide resistance to ϕX174 in our protection assay (Fig. 3.4; Table 3.1). Two of these alleles were previously identified (BERNHARDT *et al.* 2000), while the isolation of the other three is described in this study. The protection assay allowed these five alleles to be divided into three classes, based on their apparent affinity for E. The G186S and V291M variants behaved identically to wild type ^{Ec}*mraY*, in that their catalytically active and inactive forms protect at the higher expression level, but not at the lower expression level (Fig. 3.4, panel A, B). In contrast, the F288L mutant was indistinguishable from ^{Bs}*mraY*, protecting at low expression when catalytically active but unable to protect in the catalytically inactive form even when produced at the higher level (Fig. 3.4, panel E, open squares). We interpret this as indicating that F228L abrogates or severely reduces E binding, because the active form can provide Lipid I even in the presence of excess E and an excess of its inactive form does not titrate E. The other two alleles, P170L and ΔL172, exhibited an intermediate behavior, since their active forms resemble wt ^{Ec}*mraY* in requiring the higher expression level to prevent lysis, but their inactive forms, like ^{Bs}MraY$_{D231N}$, cannot titrate E when over-expressed (Fig. 3.4, panel C, D, closed triangles). Thus, in this interpretation, P170L and ΔL172 would have an intermediate affinity for E. All five of the ϕX174^{R} alleles of *mraY* were initially isolated by selecting for cells that survived the induction of the cloned *Epos* gene, which has two changes, R3H and L19F, relative to wild type *E*. Thus, it was satisfying to find that the protection assay indicated that one of the ϕX174^{R} variants, ^{Ec}MraY$_{P170L}$, interacts more strongly with Epos than it does with E. Although preliminary in nature, this observation might

indicate that one or both of the residues altered in Epos interacts with Leu170 of $^{Ec}MraY_{P170L}$. Moreover, since all five E-resistance mutations map to predicted TMDs 5 and 9 of MraY, it is tempting to speculate that they interact directly with the single TMD of E. In the continued absence of any structural information about MraY, using a genetically-malleable probe like E may be an effective way to make progress towards mechanistic understanding. From this perspective, we note that the detailed topology that we have presented for MraY (Fig. 3.1) differs from that proposed by Bouhss et al (1999) in the positions of the TMDs, most specifically to allow the sites that give rise to E-resistance to be contained within domains predicted to span the bilayer.

Table 3.1. The ability of plasmid-borned *mraY* alleles to protect against E-mediated lysis defines different levels of E-binding.

MraY protein	Position of E-resistance mutation	Protection at A_{550}=0.2	Protection at A_{550}=0.5	Apparent affinity for E
MraY		No	Yes	+++
$MraY_{D267N}$		No	Yes	
BsMraY		Yes	Yes	-/+
$BsMraY_{D231N}$		No	No	
$MraY_{G186S}$	TMD5	No	Yes	+++
$MraY_{G186S, D267N}$		No	Yes	
$MraY_{V291M}$	TMD9	No	Yes	+++
$MraY_{D267N, V291M}$		No	Yes	
$MraY_{\Delta L172}$	TMD5	No	Yes	+
$MraY_{\Delta L172, D267N}$		No	No	
$MraY_{P170L}$	TMD5	No	Yes	+
$MraY_{P170L, D267N}$		No	No	
$MraY_{F288L}$	TMD9	Yes	Yes	-/+
$MraY_{D267N, F288L}$		No	No	

Implications for the mechanism of E inhibition

The ability of ^{Bs}*mraY* to complement chromosomal *ΔmraY* casts doubt on the notion that MraY plays an integral role in the formation of a multiprotein machine required for murein synthesis and suggests, instead, that its sole essential role is to catalyze the formation of Lipid I. This perspective is also inconsistent with the model proposed by Mendel et al. (2006) where E acts by binding MraY and preventing its incorporation into such a complex. Our results do put constraints on models for the E-mediated inhibition of MraY. First, the ability of modest increases in expression of *mraY* to block E-mediated lysis indicates that E does not function catalytically, like some bacteriocins of approximately the same size. Host lysis by λ*E occurs in approximately the same time scale after infection as occurs with ϕX174, so the level of E produced is likely to be comparable in the two cases. This suggests that ϕX174 does not produce E in large excess over its target, MraY, presumably because the phage never encounters situations where the level of MraY is dramatically different. Together, the protection conferred by the catalytically inactive protein in sparing the chromosomal MraY and the existence of three classes of E-resistant MraY mutants may indicate that the ϕX174^{R} alleles of *mraY* encode proteins with different affinities for E. In this view, the F288L mutant is resistant because it binds E poorly whereas the G186S and V291M mutants bind E with an affinity that is not distinguishable, at least in our assay, from that of the wt protein. These classes resemble the different classes of inducer-insensitivity that have been observed in the Lac repressor, in which some mutations block inducer binding but

others interfere with the inducer-mediated conformational change (PACE *et al.* 1997). It will be interesting to exploit this system to select *E* mutants that overcome the *mraY* mutations, with the aim of using allele-specific suppression to map out point to point interactions between E and MraY.

CHAPTER IV

THE ϕX174 E LYSIS PROTEIN IS A NON-COMPETITIVE INHIBITOR OF MraY

Introduction

Bacteriophages with small genomes accomplish lysis with a single lysis protein lacking muralytic activity. There are three unrelated "single gene" lysis systems in the three common classes of small phages (BERNHARDT *et al.* 2002b; YOUNG and WANG 2006). The *Microviridae*, for which ϕX174 is the prototype, are small icosahedral phages with ~5 kb single-stranded circular chromosomes. The ϕX174 lysis gene *E*, embedded in the +1 reading frame within the unrelated essential gene *D*, encodes a membrane protein of 91 residues (Fig. 4.1) (ALTMAN *et al.* 1985; BLÄSI *et al.* 1983; SANGER *et al.* 1977). The two classes of male-specific single-stranded RNA phages, the *Leviviridae* and the *Allolevivirida*e, represented by the classic phages MS2 and Qβ, effect lysis differently. The MS2 lysis gene *L* is a 75 codon reading frame overlapping the end of the *coat* and beginning of the *rep* gene (ATKINS *et al.* 1979). In contrast, Qβ lacks a separate lysis gene. Instead, its A_2 protein, present in single copy on the surface of the virion and responsible for attachment to the host pili, also provides its lysis function (WINTER and GOLD 1983). Although the mechanism by which L effects lysis is unknown, both E and A_2 are specific inhibitors of the conserved pathway of murein precursor biosynthesis (BERNHARDT *et al.* 2000; BERNHARDT *et al.* 2001b). A_2 inhibits

MurA, the committed step of the pathway, whereas E inhibits MraY, which is responsible for synthesis of Lipid I.

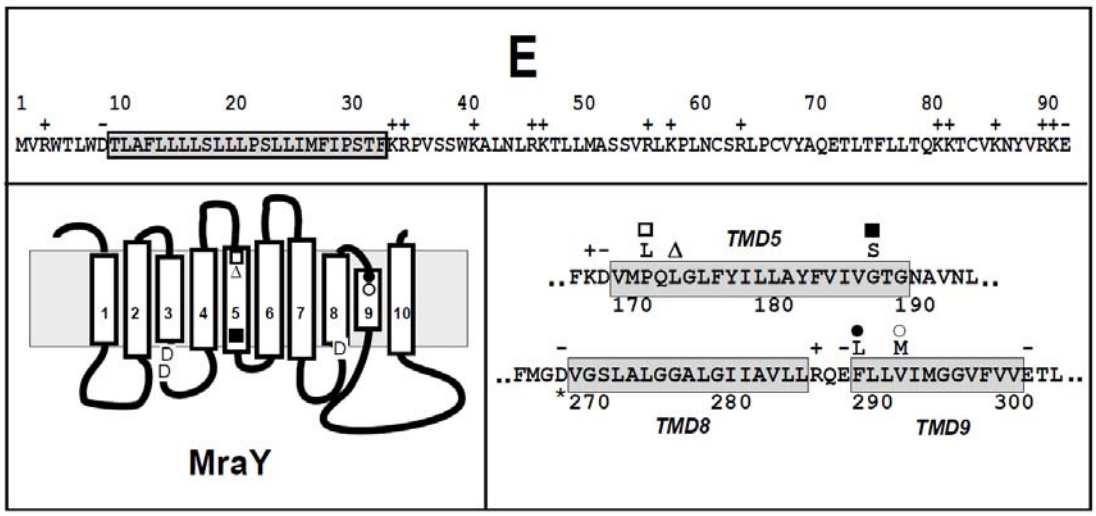

Figure 4.1. Features of E and MraY. Top panel: primary structure of E with the putative TMD in the shaded rectangles. The positively and negatively charged residues are indicated. Bottom panel, left: proposed topology of MraY (ZHENG *et al.* 2008a). The three conserved Asp residues essential for MraY enzymatic activity are labeled on cytoplasmic loops below TMD3 and TMD8 (LLOYD *et al.* 2004). The E-resistance mutations isolated in *mraY* (BERNHARDT *et al.* 2000; ZHENG *et al.* 2008a) are indicated by the following symbols: □, P170L; Δ, ΔL172; ■, G186S; ●, F288L; ○, V291M. Bottom panel, right: The sequences of three TMDs (TMD 5, 8 and 9) in MraY. In TMD 5 and 9, the E-resistance mutations are shown. The sequences of the TMDs proposed (ZHENG *et al.* 2008a) are shown in the shaded rectangles. The proposed catalytic Asp267 residue is indicated by an asterisk.

E function has had considerable study. Gene fusion experiments have shown that only its N-terminal 35 amino acids, including the sole predicted transmembrane domain (TMD), are required for lytic activity (BERNHARDT *et al.* 2000; BUCKLEY and HAYASHI 1986; MARATEA *et al.* 1985), which fits well with its target, MraY, a multi-spanning

integral membrane protein (Fig. 4.1). *In vivo*, MraY catalyzes the formation of lipid I by transferring phosphate-N-acetylmuramic acid-pentapeptide (in *E. coli*, phosphate-N-acetylmuramic acid-L-Ala-γ-D-Glu-*meso*-diaminopimelic acid (DAP)-D-Ala-D-Ala) (P-MurNAc-pentapeptide) from UDP-MurNAc-pentapeptide to undecaprenol-phosphate (undecaprenol-P). A two step mechanism involving a covalent MraY-P-MurNAc-pentapeptide has been proposed (HAMMES and NEUHAUS 1974) (Fig. 4.2). MraY enzymatic activity has been reported using either a transfer assay, measuring the transfer of P-MurNAc-pentapeptide from UDP-MurNAc-pentapeptide to undecaprenol-P and an exchange assay, measuring the exchange of labeled UMP with the unlabeled UMP moiety of UDP-MurNAc-pentapeptide (HEYDANEK *et al.* 1969). Using the transfer assay, tunicamycin, a general inhibitor of glycotransferases, was reported to be a competitive inhibitor of MraY in respect with the UDP-MurNAc-pentapeptide substrate (BRANDISH *et al.* 1996b). Three conserved aspartate residues in cytoplasmic loops have been implicated in MraY catalysis (LLOYD *et al.* 2004) (Fig. 4.1).

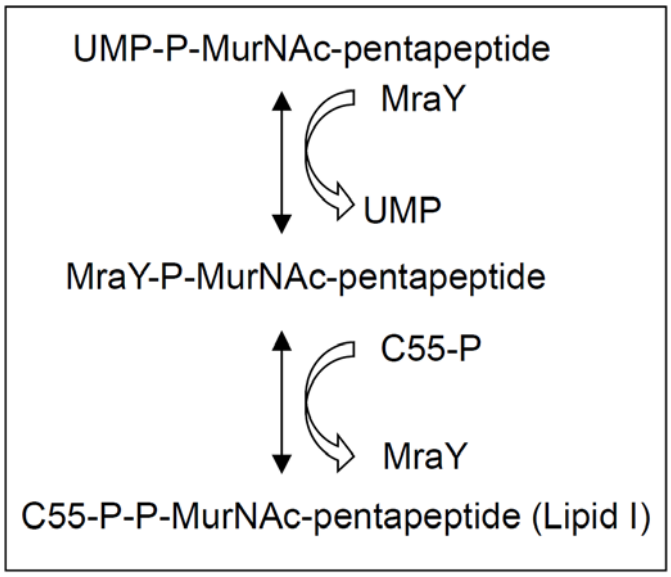

Figure 4.2. Proposed reaction mechanism of MraY. The MraY-catalyzed reaction has been proposed to be a two-step process (HEYDANEK *et al.* 1969). In this model, the first step generates a MurNAc-pentapeptide-phosphoenzyme (MraY-P-MurNAc-pentapeptide) intermediate and free UMP. The second step, undecaprenol-P (labeled as C55-P) attacks the intermediate, and results in formation of lipid I and release of the free enzyme.

The mechanism of E-mediated inhibition of MraY function was initially addressed by genetics and physiological experiments. *In vivo*, lysis by the wt E protein requires functional SlyD, a cytoplasmic FKBP (FK506 binding protein)-type peptidyl-prolyl cis-trans isomerase (ROOF and YOUNG 1995). This requirement is not shared by EΦlacZ fusions and can be overcome by N-terminal mutations in *E*, designated as *Epos* (BERNHARDT *et al.* 2002a; MARATEA *et al.* 1985). Labeling studies indicated that in the absence of SlyD, the E protein is very unstable, and that the effect of the Epos mutations was to increase the level of synthesis, presumably at the translational level, rather than stabilizing the protein (BERNHARDT *et al.* 2002a). The original studies identifying MraY as the target were based on the isolation of dominant mutants in *mraY* resistant to the

expression of a cloned *E* gene (BERNHARDT *et al.* 2000). To date, five missense mutants of *mraY* providing resistance to E-mediated lysis, and impaired in plaque-formation by ϕX174, have been isolated. These mutations map to TMDs 5 and 9 of the proposed topological map of MraY (Fig. 4.1) (ZHENG *et al.* 2008a). Using the exchange assay on membranes containing E and MraY, Bernhardt *et al.* (BERNHARDT *et al.* 2001a) showed that activity of MraY was drastically reduced, whereas in the same membranes, the activity of Rfe, a member of the same super-family of translocases, was unaffected. Experiments in which inductions of plasmid-borne *mraY* genes have been used to protect the host from E lysis have indicated that E binds to MraY *in vivo* and that the five E-resistant *mraY* alleles can be separated into three classes based on apparent affinities for E (ZHENG *et al.* 2008a). Moreover, E was found to be incapable of causing lysis when the active form of *B. subtilis* MraY (^{Bs}MraY) was produced in *E. coli*, indicating that the great disparity between the sequences of ^{Bs}MraY and ^{Ec}MraY prevent E binding.

In vitro studies of E inhibition has been hampered by the fact that, like other membrane proteins, E and MraY are problematical in terms of over-expression and, of course, that E is lethal to *E. coli*. Experiments with quantifiable *EΦlacZ* gene fusions indicated that lysis is brought about when only a few hundred chimeric molecules were present, suggesting that the *in vivo* levels of MraY must also be very low (MARATEA *et al.* 1985). Moreover, MraY has unusual N-out, C-out topology (BOUHSS *et al.* 1999), so appending purification tag domains to either terminus might interfere with proper integration into the membrane. Nevertheless, Bouhss *et al.* (BOUHSS *et al.* 2004) reported

substantial purification of an N-terminally oligohistidine-tagged variant of ^{Bs}MraY over-expressed from a T7-promoter clone.

Purification of E has not been reported. However, Mendel *et al.* (MENDEL *et al.* 2006) prepared a synthetic polypeptide, E_{pep}, corresponding to the N-terminal 37 residues of the predicted E sequence. Consistent with the earlier finding that only the N-terminal portion of E is required for lysis, inhibition was observed when SDS-solubilized E_{pep} was added to membranes containing over-expressed MraY. Unexpectedly, it was found that E_{pep} was not able to inhibit the MraY activity in detergent-solubilized membrane extracts. This led to a model in which E effects lysis by preventing the assembly of MraY into an essential hetero-multimeric integral membrane complex with high MraY activity, a complex which would not be formed in detergent. Here we report studies of MraY inhibition by full-length E protein and discuss the results in term of a different model for E action.

Methods

Media, chemicals, strains and culture methods

Growth and induction conditions for bacterial cultures have been described, including the use of Luria-Bertani (LB) broth, supplemented as appropriate with these antibiotics: ampicillin (100 μg/ml), chloramphenicol (10 μg/ml), and kanamycin (40 μg/ml) or the inducers isopropyl-β-D-thiogalactopyranoside (IPTG) and arabinose

(ZHENG *et al.* 2008b). The hosts for over-expression of *E* and *mraY* were, respectively, BL21(DE3) and BL21(DE3)plysS (Novagen). Tunicamycin (mixture of isomers A, B, C, and D), phytol and phospray, a reagent for detection of phospholipid, were purchased from Sigma. The sources of detergents were as follows: Tween 20, SDS, and Empigen BB (EBB) from Sigma; cholic acid, saponin, and Triton X-100 from EMD; Nonidet P40 from Bethesda Research Laboratories; 1,2-diheptanoyl-*sn*-glycero-3-phosphocholine (DHPC) from Avanti Polar Lipids; *n*-dodecyl-β-D-maltoside (DDM) from Anatrace.

Plasmids

Plasmids were constructed using standard PCR, digestion and ligation methods, as previously described; all final constructs were verified by sequencing at the Laboratory for Plant Genome Technology at the Texas Agricultural Experiment Station. The plasmids pETE6his and pETMY contains, respectively, the ϕX174 *E* gene extended with 6 histidine codons (BERNHARDT *et al.* 2002a) or the *E. coli mraY* gene (BERNHARDT *et al.* 2000), inserted between the NdeI and BamHI sites of pET11a vector (Novagen). The plasmid pBsMraYKan contains ^{Bs}*mraY* under the control of the P_{BAD} promoter and was derived from pBAD30-BsMraY (ZHENG *et al.* 2008a) by deleting the *bla* gene and inserting a kanamycin-resistance cassette from pZS*24-MCS-1 (LUTZ and BUJARD 1997) into the unique ClaI site.

Substrates for the in vitro *reaction*

UDP-MurNAc-pentapeptide was isolated from *B. subtilis* W23 as described (BERNHARDT *et al.* 2001a). The dansylated UDP-MurNAc-pentapeptide, UDP-MurNAc-L-γ-D-Glu-*m*-DAP(Nε-dansyl)-D-Ala-D-Ala (UDP-MurNAc-pentapeptide-DNS), was prepared by the reaction of UDP-MurNAc-pentapeptide with dansyl chloride as described (WEPPNER and NEUHAUS 1977). Phytol -phosphate (phytol-P), a C-20 analog of undecaprenol-P was used as the lipid substrate and was prepared by the chemical phosphorylation of phytol following the method by Danilov *et al.* (DANILOV *et al.* 1989). The phospholipid product is detected by thin layer chromatography (TLC) using chloroform: methanol: water (60:25:4) as the mobile phase, and confirmed as a single species using both I_2 and phospray staining. The concentration of phytol-P was determined by measuring free phosphate after hydrolysis with 4N HCl at 90°C, as described (HESS and DERR 1975). Using this lipid substrate, tunicamycin, a competitive inhibitor of the sugar-nucleotide substrate for this class of enzymes (BRANDISH *et al.* 1996b; MEIER-DIETER *et al.* 1992; STRUCK and LENNARZ 1977), exhibited efficient competitive inhibition of UDP-MurNAc-pentapeptide-DNS (data not shown).

In vitro *reaction catalyzed by MraY*

MraY activity was measured using a 10 μl reaction mixture containing 50 mM Tris pH8.0, 170 mM KCl, 10 mM $MgCl_2$ (Buffer R), 2.5 μL of MraY extract or

membranes, and the substrates or substrates plus E protein or mock E extract (see below), as indicated. Phytol-P was provided in Buffer R plus 4% DDM; the final concentration of DDM in the total reaction was 1.25%. The reaction was started by addition of the UDP-MurNAc-pentapeptide-DNS substrate. The reaction mixture was incubated at 37°C for various times. For determination of initial rates, reactions were terminated at 5 min, which was determined to be within the linear phase of the reaction. After termination by boiling for 2 min, the reaction mixture was spotted on a TLC plate (EMD Chemicals). The plate was developed for 6 to 7 hours using isobutyric acid: NH_4OH: water (66:1:33) as the mobile phase. Quantification of the product was done by scraping the spot, extracting with methanol, and measuring the fluorescent intensity. Fluorescence measurements were done in a Koala spectrofluorometer (ISS) (excitation=340nm, emission=535nm), with a volume of 100 µl in a 130-µl fluorescence micro cell (Hellma).

Preparation of MraY-enriched membranes

A total of 10 L of culture of BL21(DE3)plysS cells harboring the pETMY plasmid were induced at A_{550} = 0.6 with 1 mM IPTG for one hour and then harvested by centrifugation (rotor JA-10, Beckman) at 8K rpm (7000 x g) for 15 min in the cold. The cells were resuspended in 1/100 volume of cold French press buffer (50 mM Tris pH 8.0, 170 mM KCl, 5 mM EDTA, 1 mM PMSF, 1 mM DTT) and disrupted by French press. The lysate was cleared of whole cells by centrifugation in a JA-20 rotor (Beckman) at

5000 x g for 10 min in the cold. Membranes were collected by centrifugation at 38K rpm (130,000 x g) for 1 hour in the cold, using a Type 50.2 Ti rotor (Beckman). To extract MraY activity, membrane pellets were resuspended in Buffer R plus 1% DDM and stirred for 1 hour in the cold. Insoluble material was removed by centrifugation at 50K (100,000 x g) for 1 hour in a TLA-100.3 rotor (Beckman). The supernatant from this clearing step was used without further treatment for MraY reactions.

Over-production and purification of E

To determine optimum conditions for over-production of E_{6his}, BL21(DE3) cells harboring either the pETE6his plasmid alone or both the pBsMraYKan and pETE6his plasmids were induced at A_{550} ~ 0.6 with either 1mM IPTG or 0.2% arabinose and 1mM IPTG (added 2 minutes after arabinose), respectively. At various times, the cells from 1 ml aliquots were chilled, collected by centrifugation in the cold, resuspended in SDS-PAGE buffer in volumes chosen to normalize for constant cell mass, and analyzed by SDS-PAGE and immunoblotting, as described previously (ZHENG *et al.* 2008b).

A total of 5 L of culture of BL21(DE3) cells harboring the pBSMraYKan and pETE6his plasmids was induced at A_{550}=0.6 with 0.2% arabinose and 1mM IPTG (added 2 minutes after arabinose) for 30 min and then harvested by centrifugation (rotor JA-10, Beckman) at 8K rpm (7000 x g) for 15 min in the cold. The cells were resuspended in 1/200 volume of cold French press buffer and disrupted by French press. Whole cells and debris were removed by centrifugation at 5000 x g in a JA-20 rotor

(Beckman), and membranes were collected from the supernatant by centrifugation at 130,000xg for 1 hour in the cold, using a Type 50.2 Ti rotor (Beckman). Membrane pellets were resuspended in Buffer R plus various detergents, as indicated, and shaken gently overnight at 4°C. Insoluble material was removed by centrifugation at 130,000xg for 1 hour in the cold, using a Type 50.2 Ti rotor (Beckman).

The E_{6his} polypeptide, extracted in 2% EBB, was purified by immobilized metal affinity chromatography (IMAC) with 250 µl Talon Metal Affinity resins (Clontech) in a Poly-Prep column (Bio-Rad) using gravity elution. The elution buffer was the Buffer R supplemented with 100 mM imidazole and 0.06% EBB . Fractions containing E_{6his} protein were identified by western blot, as described (ZHENG *et al.* 2008b). A mock E extract was prepared in the same way using a strain carrying the pET11a vector. The concentration of purified E_{6his} was determined by two independent methods: A_{280}, using a molar absorption coefficient of E calculated as defined by Pace *et al.* (PACE *et al.* 1995); or by densitometry using Coomassie-blue stained SDS-PAGE gels, with egg white lysozyme (Sigma) as a standard. The two methods gave the same results.

Results

MraY preparation and fluorescence assay

Initially efforts were made to construct an allele of *mraY* encoding an N-terminal oligohistidine-tagged variant, to facilitate enrichment of MraY activity by IMAC, as

reported by Bouhss *et al.* (BOUHSS *et al.* 2004). However, the H6-MraY allele failed a stringent complementation test with a chromosomal *mraY* (ZHENG *et al.* 2008a). This result was not surprising, since the N terminus of MraY is predicted to be periplasmic, and an appended oligopeptide tag could interfere with proper folding in the membrane. Since our goal was biochemical characterization of E-mediated inhibition, which might have stringent requirements for MraY folding, we decided to use the wt *mraY* allele for over-production and enrichment.

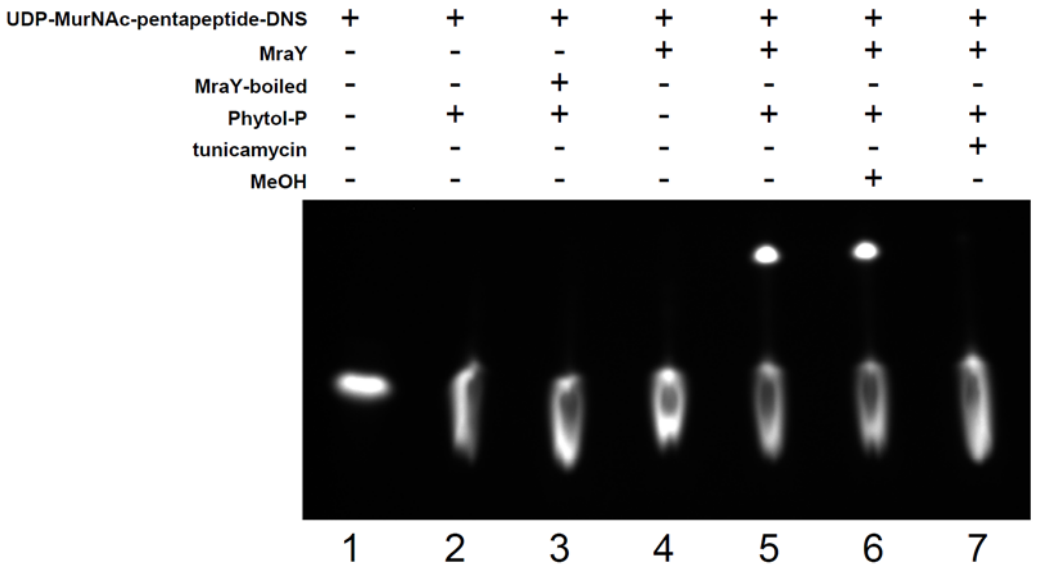

Figure 4.3. Fluorescence-based assay for MraY. Reaction mixtures containing the fluorescent substrate UDP-MurNAc-pentapeptide-DNS in Buffer R (lanes 2 - 7), supplemented with DDM-solubilized MraY (lanes 4 -7) or heat-inactivated MraY (lane 3), phytol-P (lanes 2-3, 5-7), tunicamycin in methanol (lane 7) or methanol (lane 6) were incubated at 37°C for 30 min, quenched by heating at 100°C for 2 min, and then analyzed by TLC, as described in Experimental Procedures. Lane 1 contains the UDP-MurNAc-pentapeptide-DNS alone. Reaction components are also listed above the panel, with +/- signs indicating inclusion or exclusion, respectively.

To measure MraY activity, we developed a fluorescence-based transfer assay for measuring MraY enzymatic activity in both crude membrane preparations and detergent-solubilized forms. The substrates used are UDP-MurNAc-pentapeptide-DNS, a fluorescent substrate analogue, and phytol-P, a twenty-carbon analogue of undecaprenol-P. The fluorescently-labeled product, phytol-P-P-MurNAc-pentapeptide-DNS, is separated from the reaction mixture by TLC (Fig. 4.3) and quantified by fluorescence spectroscopy. Using this assay, Michaelis-Menten kinetics was observed for both substrates. Using Ping Pong Bi Bi formalism (COOK and CLELAND 2007), the K_m for UDP-MurNAc-pentapeptide-DNS was found to be 0.2 ± .09 mM and the apparent K_m for phytol-P was 0.84 ± 0.2 mM (Fig. 4.4). We also determined the K_m parameters for the $MraY_{F288L}$ mutant protein, which has been shown to be the most extreme mutant, in terms of E-resistance, among the five (ZHENG *et al.* 2008a), and obtained the same values (Fig. 4.4). This suggests that E-resistance is not due to an altered substrate affinity.

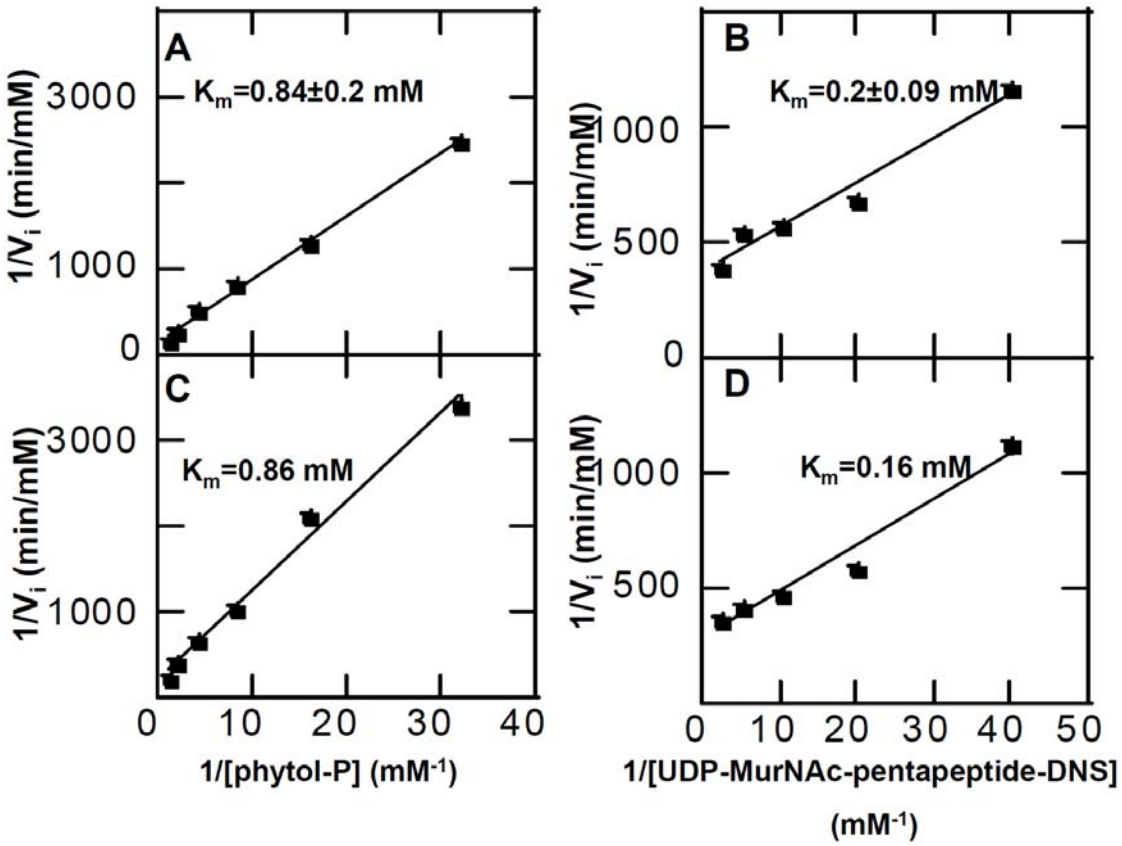

Figure 4.4. Lineweaver-Burk determinations of K_m values. Panels A and B: K_m determination of wt MraY for phytol-P and UDP-MurNAc-pentapeptide-DNS. Panel C and D, K_m determination of MraY F288L mutant for phytol-P and UDP-MurNAc-pentapeptide-DNS. Initial rates were measured as described in Experimental Procedures, first varying phytol-P concentration with UDP-MurNAc-pentapeptide present at 0.2 mM, then varying UDP-MurNAc-pentapeptide concentration with phytol-P present at 0.25 mM. K_m values were determined using equations for a Ping Pong Bi Bi system (COOK and CLELAND 2007).

Over-production and purification of E

To determine the mode of inhibition by E, purified E protein was required. Purification of E protein is especially challenging since it is lethal to *E. coli* and thus not readily over-produced. Induction of an *E* allele encoding a C-terminal oligohistidine tag

caused lysis within 15 minutes (Fig. 4.5), making recovery of membrane material from the large culture volume difficult and allowing only a small amount of E to accumulate in the membranes (Fig. 4.6A, lane 4). To overcome this obstacle, we took advantage of the insensitivity of ^{Bs}MraY to E inhibition. In the presence of the heterologous enzyme, lysis did not occur, making recovery of the membrane material much more efficient (Fig. 4.5), and E_{6his} protein accumulated to a much higher level (Fig. 4.6B). E_{6his} could be efficiently extracted from membranes of these induced cells with the zwitterionic detergent EBB or with SDS, but not with other commonly-used detergents (Table 4.1). The EBB-solubilized material was purified by IMAC, yielding a preparation 54 μM E_{6his} protein (Fig. 4.6C) that was 84% pure. A mock purification using the empty vector yielded the same background species (compare lanes 3 -8 with 9 - 15 in Fig. 4.6C) and was used as the negative control in all experiments with E.

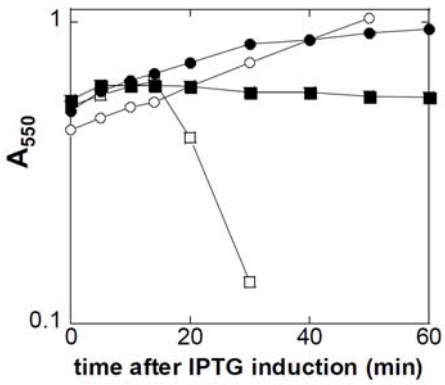

Figure 4.5. ^{Bs}MraY prevents lysis from over-expression of *E_{6his}*. Cultures of BL21(DE3) harboring the indicated plasmids were induced at A_{550}=0.5 by addition of 1mM IPTG and monitored for culture turbidity as described in Experimental Procedures. The pBsMraYKan plasmid was induced by addition of 0.2% arabinose 2 minutes before IPTG was added. Symbols: ○, pET11a; □, pETE6his; ●, pBsMraYKan and pET11a; ■, pBsMraYKan and pETE6his.

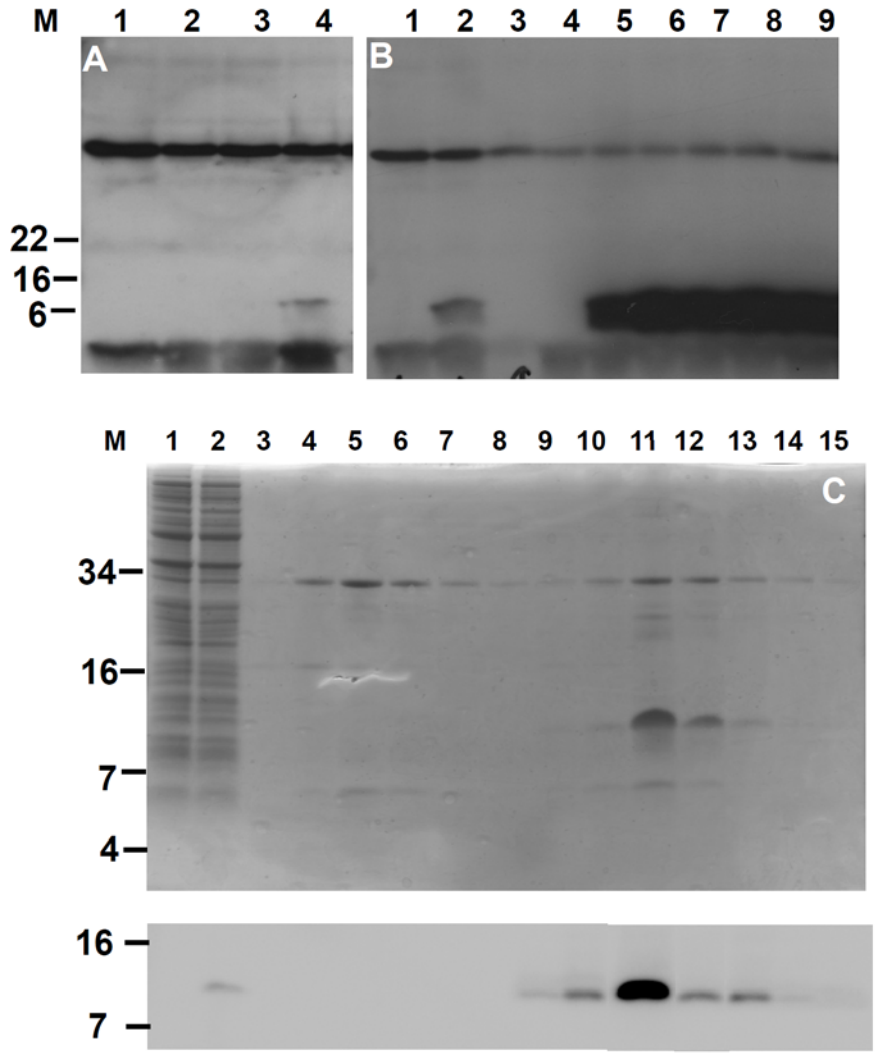

Figure 4.6. Over-production and purification of E_{6his}. Panels A and B: Accumulation of E_{6his} after induction. Cultures of BL21(DE3) cells harboring either the pETE6his plasmid or the pET11a vector, as indicated, or the pBSMraYKan plasmid, were induced at t = 0 with IPTG. The cultures were sampled at various times after induction and analyzed by immunoblot, as described in Experimental Procedures. Molecular weight (M) standards are indicated to the left of the blots.
A. Lane 1, pET11a, t = 15 min. Lanes 2 - 4: pETE6his, t = 0, 5, and 15 min.
B. Lane 1, pET11a, t = 15 min. Lane 2, pETE6his, t=15 min. Line3, pET11a + pBsMraYKan, t=60 min. Lanes 4 - 9: pETE6his + pBsMraYKan, t = 0, 5, 15, 25, 35 and 60 min. Panel C, Purification of E. Coomassie blue-stained SDS PAGE (top panel) and immunoblot (bottom panel) analysis of steps in E_{6his} purification or mock E purification (see Experimental Procedures), as indicated. M, molecular weight standards. Lanes 1 and 2: whole cell samples, pET11a vector and pETE6his. Lanes 3 and 9: 10mM imidazole wash fraction, pET11a vector and pETE6his. Lanes 4-8: elution fractions 1-5, pET11a vector. Lanes 10-15: elution fractions 1-6, pETE6his.

Table 4-1. Extraction of E_{6his} from *E. coli* membranes. Membranes containing over-produced E_{6his} were extracted with the indicated detergents, as described in Experimental Procedures. The distribution of the E_{6his} protein between soluble and insoluble fractions was estimated by immunoblot and densitometry. Concentration of detergents used is 1% for each detergent, except DHPC, which was 14 mM (~0.7%).

Detergent	**Extraction efficiency (%)**
Triton X-100	13
Tween 20	<5
Nonidet P40	18
SDS	70
Cholic acid	<5
DHPC	<5
EBB	49
Saponin	<5

E-mediated inhibition of MraY

Next, the purified detergent-solubilized $E6_{his}$ protein was examined for its ability to inhibit MraY *in vitro*. In contrast to the findings of Mendel *et al.* (MENDEL *et al.* 2006) with the synthetic peptide corresponding to the first 37 residues of E, we found the purified E_{6his} protein inhibited solubilized MraY efficiently (Fig. 4.7). We also observed inhibition by E_{6his} with membranes containing MraY, consistent with both the findings of Mendel *et al.* (MENDEL *et al.* 2006) and our original demonstration that E inhibits MraY specifically when both are present in the same membranes (BERNHARDT *et al.* 2000). For a given concentration of E, the extent of inhibition is lower than for the solubilized enzyme, which is not surprising considering that, in the assays with particulate MraY, the E protein must enter the membrane from its detergent-solubilized state. Next, the mode by which E inhibited the solubilized MraY was determined with respect to both the lipid and sugar-nucleotide substrates (Fig. 4.8) by measuring MraY

activity in the presence of varying concentrations of E_{6his}. Experiments to obtain the kinetic data were performed with crude MraY sample containing membrane detergent extracts. Kinetic analysis revealed that the K_m parameters for both UDP-MurNAc-pentapeptide-DNS and phytol-P were unchanged in the presence of E_{6his}, whereas in both cases V_{max} was decreased. Thus, E is a non-competitive inhibitor of MraY with respect to both lipid and sugar-nucleotide substrates, with an average $K_i = 0.53 \pm 0.12$ μM (Fig. 4.8).

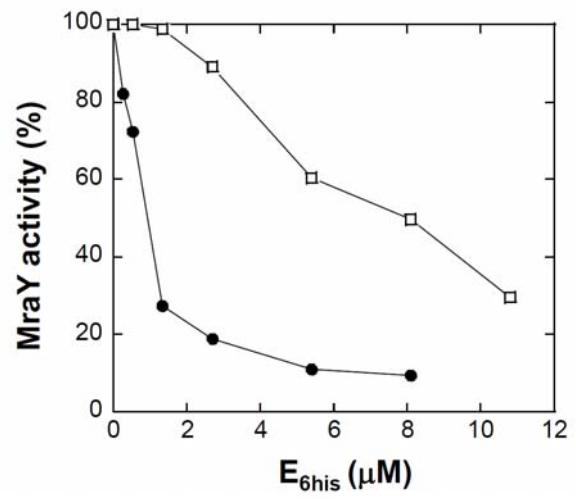

Figure 4.7. Inhibition of both particulate and detergent-solubilized MraY by E_{6his}. Activity was measured by the *in vitro* assay as described in experimental procedures. Data are expressed as percentage activity. Elution fraction #2 of a parallel purification of material from cells carrying the vector, pET11a (see Fig. 4. 6C, lane 5) served as the null E control (0 μM E_{6his}). Symbols: ●, detergent-solubilized MraY; □, membranes containing MraY.

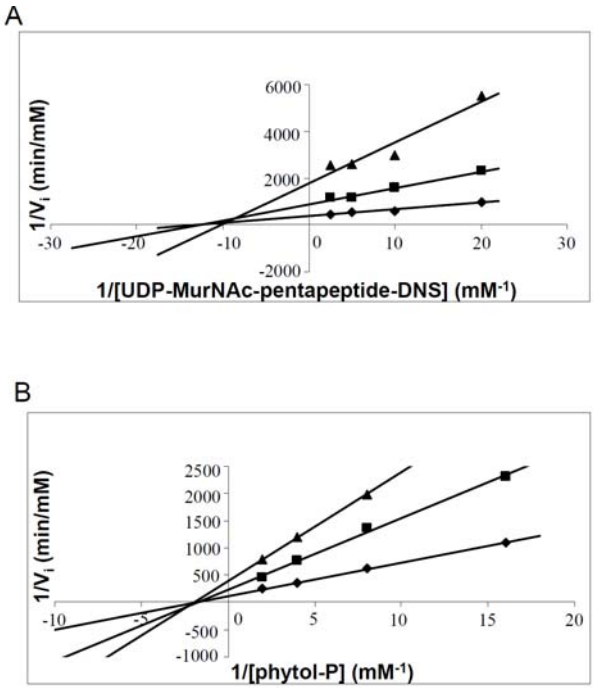

Figure 4.8. E_{6his} is a non-competitive inhibitor of MraY with respect to both soluble and lipid substrates. Assays were carried out as described in Experimental Procedures at fixed E_{6his} concentrations: ♦, 0 μM (mock E extract, figure 4.6C, lane5); ■, 0.7 μM; ▲, 1.4 μM. (A) Varying UDP-MurNAc-pentapeptide-DNS concentration in the presence of fixed concentration (0.25 mM) of phytol-P. (B) Varying phytol-P concentration in the presence of fixed concentration (0.2 mM) of UDP-MurNAc-pentapeptide-DNS.

Sensitivity of MraY mutant alleles

We then investigated the ability of E to inhibit the MraY proteins from the five mutant alleles isolated by selecting for resistance to the lysis protein. The five mutants fall into 3 classes according to the degree of inhibition at $[E_{6his}] = 2.7$ μM: $MraY_{G186S}$ and $MraY_{V291}$ are almost as sensitive to E as the wt protein; $MraY_{F288L}$ is, like $^{Bs}MraY$, not detectably inhibited; and $MraY_{P170L}$ and $MraY_{\Delta L172}$ are inhibited to a degree intermediate between the first two classes (Fig. 4.9). These results match the classes of

apparent affinities determined *in vivo* by comparing the ability of multicopy plasmids carrying these alleles to protect the wt protein from E inhibition (ZHENG *et al.* 2008a).

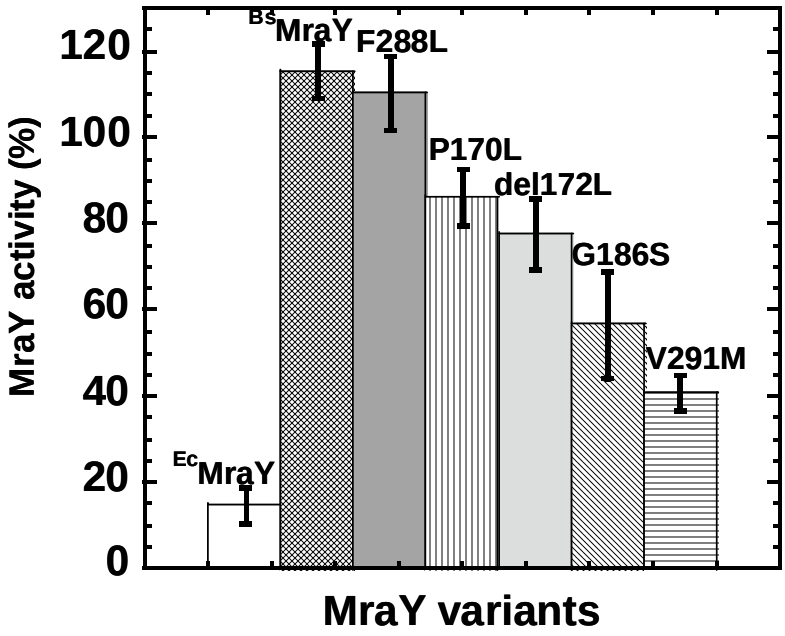

Figure 4.9. Inhibition of MraY mutants by E_{6his}. Enzymatic activities of MraY proteins produced from five *mraY* mutant alleles and ^{Bs}MraY were determined at a fixed E6his concentration (2.7 μM). Data are expressed as percentage activity.

Discussion

The *E* gene was first defined as a locus for nonsense mutants that prevented lysis in the paradigm *Microvirus* ϕX174 (HUTCHISON and SINSHEIMER 1966). Initial interest focused on its unique genomic architecture, situated entirely within another gene, in this case the essential viral morphogenesis gene *D*, but in an alternate reading frame; later it was the first gene to be altered by site-directed mutagenesis (HUTCHISON *et al.* 1978).

However, the molecular basis of its lytic function was controversial. Because no muralytic activity was detectable in lysates provoked by *E* expression, models were advanced proposing that E induced cryptic endogenous autolytic functions or, alternatively, formed multimeric pores in the bacterial envelope allowing escape of the progeny virions (BLÄSI *et al.* 1984; WITTE *et al.* 1990a; WITTE and LUBITZ 1989; WITTE *et al.* 1990b). Ultimately, genetic and physiological studies from our laboratory provided evidence for an alternative notion, that E acts as a specific inhibitor of MraY, the first membrane-embedded enzyme in the pathway of murein precursor biosynthesis. Specifically, mutations that conferred resistance to E-mediated lysis mapped to two TMDs of MraY, and MraY activity, but not the activity of an unrelated member of the translocase super-family, was inhibited in membranes containing E. Here we have presented further biochemical evidence for this mode of action. Using solubilized extracts of membranes enriched in MraY, we have demonstrated that E protein acts as a non-competitive inhibitor with respect to both the lipid and sugar-nucleotide substrates of MraY. Our results are significantly different from those of Mendel *et al.* (MENDEL *et al.* 2006), who reported that E_{pep}, a synthetic peptide corresponding to the first 37 residues of E, inhibited membrane-embedded MraY but not detergent-solubilized MraY. The discrepancy may arise from differences in the assays used. Mendel *et al.* used SDS-solubilized E_{pep} for their inhibition studies, resulting in SDS concentrations at or above its CMC (critical micelle concentration) in the final reaction mixes. Although they report that the detergent alone at this concentration had no effect on MraY activity, it is possible that it affects E-MraY interactions. In our hands, 1% SDS completely destroys

MraY activity (Y. Z., unpublished). It is also possible that the synthetic 37 residue polypeptide used was not correctly folded, perhaps due to the lack of a soluble C-terminal domain. The C-terminal hydrophilic domain of E can be replaced by some, but not all, heterologous protein folding domains (BUCKLEY and HAYASHI 1986; MARATEA *et al.* 1985), and there have been no reports that simple C-terminal truncations of E are lytic *in vivo*. Most importantly, here we have shown that the E-sensitivity of solubilized MraY proteins *in vitro* correlates with the allelic state of *mraY* and with our previous assessments of apparent E-MraY binding *in vivo* (BERNHARDT *et al.* 2000; ZHENG *et al.* 2008a). In the absence of commensurate genetic validation, results obtained with the synthetic polypeptide must be interpreted with caution.

We have now demonstrated that E inhibits MraY specifically *in vivo* (BERNHARDT *et al.* 2000), in membranes (BERNHARDT *et al.* 2001a), and in solubilized extracts. In addition, we have found that E acts in a non-competitive fashion with respect to both its lipidic and soluble substrates. Taken together, these results obviate the need to invoke the existence of a detergent-sensitive, E-sensitive hetero-multimeric complex of membrane proteins required for the biological activity of MraY (MENDEL *et al.* 2006). The existence of such a complex was difficult to reconcile with the fact that the MraY enzymes from two Gram-positive bacteria, *B. subtilis* and *S. aureus*, were found to complement *E. coli mraY* defects *in vivo* (BOUHSS *et al.* 1999; ZHENG *et al.* 2008a). The ability of these diverged proteins (43% similarity between ^{Ec}MraY and ^{Bs}MraY; 40% between ^{Ec}MraY and ^{Sa}MraY; 54% between ^{Bs}MraY and ^{Sa}MraY) to complement would not seem compatible with a model requiring intimate interactions with other *E. coli cell*

division and murein synthesis proteins. The data presented here, taken with the genetic studies and the *in vivo* protection experiments reported previously (BERNHARDT *et al.* 2000; ZHENG *et al.* 2008a), suggest a simpler model in which E binds to MraY by interactions between the single transmembrane domain (TMD) of E and TMDs 5 and 9 of MraY, and this binding results in non-competitive inhibition of the enzyme by causing a conformational change.

Protein inhibitors of biosynthetic enzymes are rare, so in this respect alone the ability of E to inhibit MraY is of interest. Moreover, MraY is universally conserved in bacteria, so understanding how E mediates non-competitive inhibition may be useful in the development of new antibacterial agents. E is particularly attractive as a probe for the mechanistic investigation of MraY because, unlike small molecule inhibitors, such as mureidomycin and tunicamycin, E is a genetic system on its own. This offers many advantages, especially since E can be tagged with any number of C-terminal protein moieties without affecting its inhibitory function. Also, the existence of the three classes of E-resistant mutants of MraY indicates that the affinity of E could be tuned by manipulation of the E sequence too. Indeed, a suppressor analysis looking for E missense changes that overcome the E-resistance mutations in TMDs 5 and 9 may allow a point-to-point interaction map to be generated, if allele-specificity can be demonstrated. Recently, the MraY of *B. subtilis* has been purified substantially on a small scale (BOUHSS *et al.* 2004). Similar progress with the *E. coli* enzyme might allow the use of the genetically tractable *E* lysis protein system to probe MraY at the structural and mechanistic level.

CHAPTER V

CONCLUSIONS AND DISCUSSION

ϕX174 effects host lysis by expressing a single gene, *E*, encoding a 91 amino acid membrane protein. *E* is not only historically significant in molecular biology but it also encodes a rare type of protein: an inhibitor of a biosynthetic enzyme, in this case the conserved enzyme, MraY that synthesizes Lipid I in the murein precursor pathway. Also, it is among the examples of proteins known to accomplish lysis without possessing a bacteriolytic activity. The experiments in this dissertation explore questions related to the evolutionary, physiological and biochemical aspects of E-mediated inhibition of MraY.

To explore the basis of the difference between the single gene lysis system and the holin-endolysin lysis system, a chimera, λE, was created in which the lysis gene *E* of the microvirus, ϕX174, replaced the entire lysis cassette of phage, λ, which includes the holin gene *S* and the endolysin gene *R*. The studies presented in Chapter II show that the chimeric phage was viable but more variability was observed both in the distribution of plaque-sizes and in the burst-sizes of single cells, compared to the isogenic S^+ parental. Using different alleles of *E*, it was found the average burst size increased with the duration of the latent period, just as observed with *S* alleles with different lysis times. Moreover, within a set of missense *E* alleles, it was found that variability in lysis timing was limited and almost exclusively derived from changes in the level of E accumulation. By contrast, missense mutations in *S* resulted in a wide variation in lysis times that was

not correlated with levels of accumulation. These results suggest that the properties of greater phenotypic plasticity and lesser phenotypic variation make the function of holin proteins more genetically malleable, facilitating rapid adaptation towards a lysis time that would be optimal for changed host and environmental conditions. The inferior malleability of single-gene systems like *E* would restrict their occurrence to phages in which coding capacity is the over-riding evolutionary constraint. The hypothesis is, then, that the apparently universal dependence of dsDNA phages on holin-endolysin lysis systems, and, conversely, the absence of simple single-gene lysis systems in these complex phages, is at least in part due to the intrinsic ability of the holin systems to evolve rapidly to a wide range different lysis times. The λE constructs should allow direct testing of this and related notions in competition and long-term selection experiments in batch and chemostat culture under varied conditions of host density and quality.

In Chapter III, a genetic system for further studying the interaction between E and MraY is reported. This system relies on the ability of *mraY* or its enzymatically inactive D267N allele to protect cells from lysis after induction of the chimeric λE prophage. Using this approach, the MraY protein from *Bacillus subtilis*, which shares 43% sequence identity with the *Escherichia coli* enzyme, was found to interact weakly, if at all, with E. A potential E binding site defined by TMDs 5 and 9 has been identified by isolating more *mraY* mutants resistant to E inhibition. Exploitation of the protection system indicated that these E-resistant alleles fall into three classes based on the apparent affinity of the encoded proteins for MraY. In many ways, this system may be a more

reliable indicator of interactions between the two membrane proteins, E and MraY, than direct biochemical assay, mainly because of the necessity for detergents in the purified system**.**

Chapter IV is dedicated to a biochemical analysis of E- mediated inhibition of MraY. Previously, studies in our lab have provided genetic and physiological evidence that E is a specific inhibitor of MraY, and showed that MraY, but not related translocases, was inhibited in membranes containing E. Recently, it was reported that a synthetic polypeptide corresponding to the N-terminal 37 residues of E could inhibit MraY in membranes, but not in solubilized extracts (MENDEL *et al.* 2006). This led to a model in which E functions by binding MraY and blocking the formation of a hetero-multimeric complex between it and other *E. coli* proteins, presumably also involved in peptidoglycan synthesis. In Chapter IV, the E-MraY interaction was re-examined using a fluorescence-based assay and purified E protein. Evidence is presented that E does inhibit solubilized MraY, and does so in a non-competitive fashion with respect to both soluble and lipidic substrates. Moreover, the E sensitivity of five MraY mutant proteins, produced from alleles selected for resistance to *E*, correlated to the apparent affinities determined by *in vivo* multicopy suppression experiments. These results suggest a new model in which E binds to MraY at a site composed of the two TMDs within which the E-resistance mutations map and that the result of this binding is a conformational change that inactivates the enzyme.

Exploitation of these conclusions

Probing MraY with E

For the past two decades, Lubitz and co-workers have published numerous papers on bacterial ghosts as vaccine candidate for therapeutic applications (JALAVA *et al.* 2002). Bacterial ghosts are produced from controlled expression of plasmid-encoded ϕX174 lysis gene *E* in Gram-negative bacteria. The resulting ghosts are cell envelopes of these bacteria devoid of cytoplasmic content, which maintain the antigenic envelope structure and thus may be effective vaccine candidates. To date, E-mediated lysis for bacterial ghost production has been obtained in various Gram-negative bacteria, including *E. coli* K12 strains, *E. coli O157:H7, Mannheimia(Pasteurella) haemolytica, Salmonella typhimurium, Salmonella enteritidis, Klebsiella pneumoniae, Bordetella bronchiseptica, Helicobacter pylori, Vibrio cholerae, Actinobacillus pleuropneumoniae, Haemophilus infuenzae, Pasteurella multocida, Pseudomonas aeruginosa, Pseudomonas putida, Ralstonia eutropha, Pectobacterium cypripedii* and *Ervinia cypripedii* (EKO *et al.* 1999; JALAVA *et al.* 2002). Taking advantage of this widespread use of E for cell lysis, the MraY proteins from these organisms were aligned together with the *E. coli* and *B. subtilis* MraY proteins. Overall, they share high sequence identity (>70%) and similar topology, with 10 predicted TMDs, with *E. coli* MraY. The catalytic residues D115, D116, and D276 in *E. coli* MraY proposed by Lloyd *et al.* (LLOYD *et al.* 2004), are present in all these MraY proteins. Importantly, the potential E binding sites, TMD5 and

TMD9 as described in Chapter III, are highly conserved, which is consistent with the wide applicability of the E lysis system (Fig. 5.1). Nevertheless, there are some surprising exceptions, most notably *M. haemolytica* and *A. pleuropneumonia*. Both have the F288L change that confers the strongest E-resistance phenotype on ^{Ec}MraY. It would be interesting to see if ^{Ec}MraY carrying the other conservative changes found in the TMDs 5 and 9 of these species restored $^{Ec}MraY_{F288L}$ to E-sensitivity. In effect, this would represent intragenic suppression of E-binding. This kind of analysis could facilitate modeling the interactions of these three putative transmembrane domains (MraY TMDs 5 and 9 and the TMD of E).

The protection system described in Chapter III could be used to select E suppressors of the E-resistant MraY mutants, by isolating plaque forming λE mutant on the lawn of host cells harboring plasmid carrying a protecting *mraY* allele, or by isolating λE phages released in the liquid culture. Mutagenesis by agents such as EMS could be employed to get more mutants that meet the requirements. Since the TMD of E contains its lytic activity, a region-specific mutagenesis by PCR could be performed to mutate the conserved residues in the TMD region, and the resulting E TMD mutants could then be tested for MraY inhibition. These studies could help obtain allele-specific suppressors in E for the MraY mutations. This would be strong evidence for specific contact between E residues and MraY residues and also could be useful for constraining models. The interaction surfaces of E and MraY could then be mapped using a collection of allele-specific E/MraY pairs.

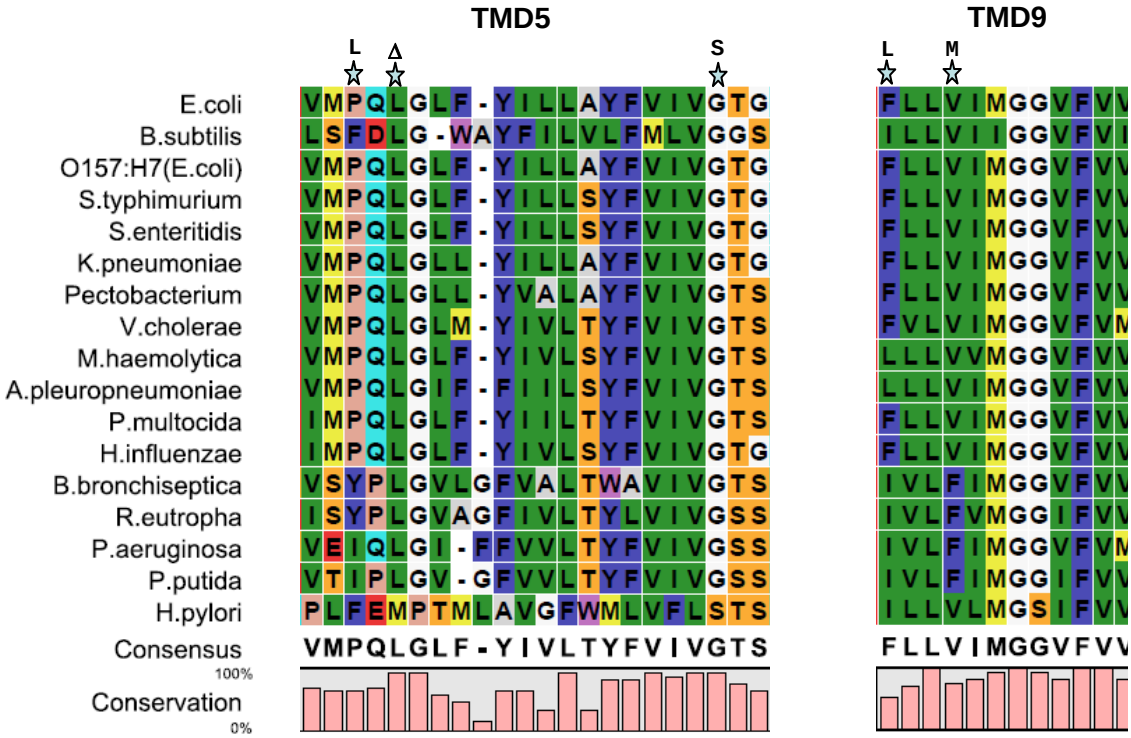

Figure 5.1. Alignment of MraY proteins from different bacteria. Shown are the regions proposed as TMDs 5 and 9 in *E. coli* MraY. The five mutations in MraY that confer E-resistance in *E. coli* are indicated with asterisks (Zheng *et al*., 2008b). The amino acid residues are colored by RasMol color scheme according to traditional amino acid properties.

Further investigation of the molecular details of the interaction between E and MraY could be performed with any of the purified MraY proteins from these organisms or purified E from these phages. Biochemical assays, along with the genetic systems could allow for determination of the minimal MraY inhibitory domain of E and critical residues required for the interaction. Recently, MraY from *B. subtilis* has been purified (BOUHSS *et al.* 2004). However, conventional purification strategies have been unsuccessful in purifying the *E. coli* enzyme.

New single-gene lysis systems in Microviridae *of intracellular bacteria*

Several phages have been isolated in recent years from *Bdellovibrio* and *Chlamydiae*, two nonenterobacterial proteobacteria with obligate intracellular parasitic life cycles. *Bdellovibrio* phage ϕMH2K, a member of *Microviridae*, has been isolated and sequenced (BRENTLINGER *et al.* 2002). ϕMH2K is distantly related to ϕX174, but extremely close to the microvirus of *Chlamydia* in genome organization and encoded proteins. The external scaffolding protein gene *D* equivalent is absent in ϕMH2K, and consequently, the potential lysis genes are short reading frames embedded in other ϕMH2K genes (YOUNG and WANG 2006). Therefore, gene *E* probably evolved inside gene *D* after ϕX174 and ϕMH2K diverged. Accordingly, it seems reasonable that that a single lysis gene evolved as an embedded gene in ϕMH2K. Indeed, inspection of the ϕMH2K genome revealed a 56 residue reading frame (nt 4132 to 4320) embedded in the +1 reading frame of the putative ϕMH2K *A* replication gene, that resembles E (Fig. 5.2).

```
ϕMH2K ORF:
                  +    +    -  +           +     +   +    +   --
MAILNLALLLWTLLATLRVTLQKNWPTETTKTTTITQFTTLQKNTPSAKNGLKNTTNKHSHEDM
        10        20        30        40        50        60


ϕX174 E:
  +    -                          ++     +    ++        + +     +
MVRWTLWDTLAFLLLLSLLLPSLLIMFIPSTFKRPVSSWKALNLRKTLLMASSVRLKPLNCSRLPCVYAQ
        10        20        30        40        50        60        70
```

Figure 5.2. The ORF in ϕMH2K that resembles ϕX174 E.

This hypothetical polypeptide has a Leu-rich hydrophobic sequence surrounding a hydroxylated amino acid (T12), as does E, and a positively charged hydrophilic C-terminal domain. *Bdellovibrio* MraY protein TMDs 5 and 9 share less than 50% identity with *E. coli* MraY, and this might be able to explain the differences in the E sequence, however, this E equivalent might inhibit MraY of *Bdellovibrio* in a different way, maybe by binding somewhere else. It would therefore be interesting to determine if MraY is still the cellular target of the independently evolved lysis gene in ϕMH2K.

Chlamydia has a unique cell wall structure possibly as a consequence of adaptation to the intracellular environment. It does not contain peptidoglycan, and instead contains glycanless wall polypeptide and cysteine-rich proteins that might function as the equivalent of peptidoglycan. Together with an outer LPS membrane, this structure might provide a cell envelope that allows for intracellular division and extracellular survival (GHUYSEN and GOFFIN 1999; KOSMA 1999). However, *Chlamydia* does have several required cell wall biosynthesis genes such as MraY and three PBPs, and is susceptible to the peptidoglycan synthesis inhibitors bacitracin, penicillin and D-cycloserine, suggesting that some cryptic cell wall structures are still there (GHUYSEN and GOFFIN 1999). So these phages might have also evolved a single-gene lysis system to encode an inhibitor of cell wall synthesis. Analysis of the genomes of *Chlamydia* phages 3, 4, Chp2, ϕCPAR39, and ϕCPG1 revealed that these phages lack obvious reading frames for a lysis gene. It would be of interest to know how these phages cause lysis in a host cell that grows without a peptidoglycan-containing cell wall in a mammalian cell cytoplasm.

LITERATURE CITED

ABEDON, S. T., P. HYMAN and C. THOMAS, 2003 Experimental examination of bacteriophage latent-period evolution as a response to bacterial availability. Appl. Environ. Microbiol. **69:** 7499-7506.

AL-DABBAGH, B., X. HENRY, M. EL GHACHI, G. AUGER, D. BLANOT *et al.*, 2008 Active site mapping of MraY, a member of the polyprenyl-phosphate N-acetylhexosamine 1-phosphate transferase superfamily, catalyzing the first membrane step of peptidoglycan biosynthesis. Biochemistry **47:** 8919-8928.

ALTMAN, E., K. YOUNG, J. GARRETT, R. ALTMAN and R. YOUNG, 1985 Subcellular localization of lethal lysis proteins of bacteriophages λ and ϕX174. J. Virol. **53:** 1008-1011.

ANDERSON, J., M. MATSUHASHI, M. HASKIN and J. STROMINGER, 1965 Lipid-phosphoacetylmuramyl-pentapeptide and lipid-phosphodisaccaride-pentapeptide: presumed membrane transport intermediates in cell wall synthesis. Proc. Natl. Acad. Sci. USA **53:** 881-889.

ATKINS, J. F., J. A. STEITZ, C. W. ANDERSON and P. MODEL, 1979 Binding of mammalian ribosomes to MS2 phage RNA reveals an overlapping gene encoding a lysis function. Cell **18:** 247-256.

BARRELL, B. G., G. M. AIR and C. A. HUTCHISON, III, 1976 Overlapping genes in bacteriophage ϕX174. Nature **264:** 34-41.

BARRETEAU, H., A. KOVAC, A. BONIFACE, M. SOVA, S. GOBEC *et al.*, 2008 Cytoplasmic steps of peptidoglycan biosynthesis. FEMS Microbiol. Rev. **32:** 168-207.

BENBOW, R. M., C. A. HUTCHISON III, J. D. FABRICANT and R. L. SINSHEIMER, 1971 Genetic map of bacteriophage ϕX174. J. Virol. **7:** 549-558.

BERNHARDT, T. G., 2001 *Breaking Free: Small Phages Inhibit Murein Synthesis to Lyse Their Host.* Ph.D dissertation, Texas A&M University, College Station.

BERNHARDT, T. G., W. D. ROOF and R. YOUNG, 2000 Genetic evidence that the bacteriophage ϕX174 lysis protein inhibits cell wall synthesis. Proc. Natl. Acad. Sci. USA **97:** 4297-4302.

BERNHARDT, T. G., W. D. ROOF and R. YOUNG, 2002a The *Escherichia coli* FKBP-type PPIase SlyD is required for the stabilization of the E lysis protein of bacteriophage ϕX174. Mol. Microbiol. **45:** 99-108.

BERNHARDT, T. G., D. K. STRUCK and R. YOUNG, 2001a The lysis protein E of ϕX174 is a specific inhibitor of the MraY-catalyzed step in peptidoglycan synthesis. J. Biol. Chem. **276:** 6093-6097.

BERNHARDT, T. G., I. N. WANG, D. K. STRUCK and R. YOUNG, 2001b A protein antibiotic in the phage Qβ virion: diversity in lysis targets. Science **292:** 2326-2329.

BERNHARDT, T. G., I. N. WANG, D. K. STRUCK and R. YOUNG, 2002b Breaking free: "protein antibiotics" and phage lysis. Res. Microbiol. **153:** 493-501.

BLÄSI, U., P. FRAISL, C. Y. CHANG, N. ZHANG and R. YOUNG, 1999 The C-terminal sequence of the lambda holin constitutes a cytoplasmic regulatory domain. J. Bacteriol. **181:** 2922-2929.

BLÄSI, U., R. GEISEN, W. LUBITZ, B. HENRICH and R. PLAPP, 1983 Localisation of the bacteriophage ϕX174 lysis gene product in the cell envelope of *Escherichia coli*, pp. 205-210 in *The Target of Penicillin*, edited by R. HAKENBECK, J. V. HOLTJE and H. LABISCHINSKI. Walter de Gruyter, Berlin.

BLÄSI, U., G. HALFMANN and W. LUBITZ, 1984 Induction of autolysis of *Escherichia coli* by ϕX174 gene E product, pp. 213-218 in *Microbial Cell Wall Synthesis and Autolysis*, edited by C. NOMBELA. Elsevier Science Publishers, New York.

BLÄSI, U., R. P. LINKE and W. LUBITZ, 1989 Evidence for membrane-bound oligomerization of bacteriophage ϕX174 lysis protein-E. J. Biol. Chem. **264:** 4552-4558.

BOUHSS, A., M. CROUVOISIER, D. BLANOT and D. MENGIN-LECREULX, 2004 Purification and characterization of the bacterial MraY translocase catalyzing the first membrane step of peptidoglycan biosynthesis. J. Biol. Chem. **279:** 29974-29980.

BOUHSS, A., D. MENGIN-LECREULX, D. LE BELLER and J. VAN HEIJENOORT, 1999 Topological analysis of the MraY protein catalysing the first membrane step of peptidoglycan synthesis. Mol. Microbiol. **34:** 576-585.

BOUHSS, A., A. E. TRUNKFIELD, T. D. BUGG and D. MENGIN-LECREULX, 2008 The biosynthesis of peptidoglycan lipid-linked intermediates. FEMS Microbiol. Rev. **32:** 208-233.

BOYLE, D., and W. DONACHIE, 1998 mraY is an essential gene for cell growth in *Escherichia coli*. J. Bacteriol. **180:** 6429-6432.

BRADLEY, D. E., C. A. DEWAR and D. ROBERTSON, 1969 Structural changes in *Escherichia coli* infected with a ϕX174 type bacteriophage. J. Gen. Virol. **5:** 113-121.

BRANDISH, P., M. BURNHAM, J. LONSDALE, R. SOUTHGATE, M. INUKAI *et al.*, 1996a Slow binding inhibition of phospho-N-acetylmuramyl-pentapeptide-translocase (*Escherichia coli*) by mureidomycin A. J. Biol. Chem. **271:** 7609-7614.

BRANDISH, P., K. KIMURA, M. INUKAI, R. SOUTHGATE, J. LONSDALE *et al.*, 1996b Modes of action of tunicamycin, liposidomycin B, and mureidomycin A: Inhibition of phospho-N-acetylmuramyl-pentapeptide translocase from *Escherichia coli.* Antimicrob. Agents Chemother. **40:** 1640-1644.

BRENTLINGER, K. L., S. HAFENSTEIN, C. R. NOVAK, B. A. FANE, R. BORGON *et al.*, 2002 Microviridae, a family divided: Isolation, characterization, and genome sequence of ϕMH2K, a bacteriophage of the obligate intracellular parasitic bacterium *Bdellovibrio bacteriovorus.* J. Bacteriol. **184:** 1089-1094.

BUCKLEY, K. J., and M. HAYASHI, 1986 Lytic activity localized to membrane-spanning region of ϕX174 E protein. Mol. Gen. Genet. **204:** 120-125.

BUGG, T. D., A. J. LLOYD and D. I. ROPER, 2006 Phospho-MurNAc-pentapeptide translocase (MraY) as a target for antibacterial agents and antibacterial proteins. Infect Disord Drug Targets **6:** 85-106.

BULL, J. J., D. W. PFENNIG and I. N. WANG, 2004 Genetic details, optimization and phage life histories. Trends Ecol. Evol. **19:** 76-82.

CHATTERJEE, A., and J. T. PARK, 1964 Biosynthesis of cell wall mucopeptide by a particulate fraction from *Staphylococcus aureus*. Proc. Natl. Acad. Sci. USA **51:** 9-16.

CHEN, M., A. UCHIYAMA and B. A. FANE, 2007 Eliminating the requirement of an essential gene product in an already very small virus: scaffolding protein B-free ϕX174, B-free. J. Mol. Biol. **373:** 308-314.

COOK, P., and W. CLELAND, 2007 *Enzyme Kinetics and Mechanism*. Garland Science, London and New York.

DANILOV, L. L., T. N. DRUZHININA, N. A. KALINCHUK, S. D. MALTSEV and V. N. SHIBAEV, 1989 Polyprenyl phosphates: synthesis and structure-activity relationship for a biosynthetic system of Salmonella anatum O-specific polysaccharide. Chem. Phys. Lipids **51:** 191-203.

DENHARDT, D. T., 1977 The isometric single-stranded DNA phages, pp. 1-104 in *Comprehensive Virology*, edited by H. FARAENKEL-CONRAT and R. R. WAYNER. Plenum, New York.

DOKLAND, T., R. A. BERNAL, A. BURCH, S. PLETNEV, B. A. FANE *et al.*, 1999 The role of scaffolding proteins in the assembly of the small, single-stranded DNA virus ϕX174. J. Mol. Biol. **288:** 595-608.

DOKLAND, T., R. MCKENNA, L. L. ILAG, B. R. BOWMAN, N. L. INCARDONA *et al.*, 1997 Structure of a viral procapsid with molecular scaffolding. Nature **389:** 308-313.

EIGNER, J., A. H. STOUTHAMER, I. VAN DER SLUYS and J. A. COHEN, 1963 A study of the 70S component of bacteriophage ϕX174. J. Mol. Biol. **6:** 61-84.

EKO, F. O., A. WITTE, V. HUTER, B. KUEN, S. FURST-LADANI *et al.*, 1999 New strategies for combination vaccines based on the extended recombinant bacterial ghost system. Vaccine **17:** 1643-1649.

ERDMANN, F., and G. FISCHER, 2007 The nickel-regulated peptidyl prolyl *cis/trans* isomerase SlyD, pp. 501-528 in *Metal Ions in Life Sciences*, edited by A. SIGEL, H. SIGEL and R. K. O. SIGEL. John Wiley & Sons, Hoboken, NJ.

FANE, B. A., K. L. BRENTLINGER, A. D. BURCH, M. CHEN, S. HAFENSTEIN *et al.*, 2006 ϕX174 et al., The *Microviridae*, pp. 129-145 in *The Bacteriophages*, edited by R. CALENDAR. Oxford University Press, Oxford.

FEIGE, U., and S. STIRM, 1976 On the structure of the *Escheichia coli* C cell wall lipopolysaccharide core and on its ϕX174 receptor region. Biochem. Biophys. Res. Commun **71:** 566-573.

GARNER, S. A., J. S. EVERSON, P. R. LAMBDEN, B. A. FANE and I. N. CLARKE, 2004 Isolation, molecular characterisation and genome sequence of a bacteriophage (Chp3) from *Chlamydophila pecorum*. Virus Genes **28:** 207-214.

GHUYSEN, J. M., and C. GOFFIN, 1999 Lack of Cell Wall Peptidoglycan versus Penicillin Sensitivity: New Insights into the Chlamydial Anomaly. Antimicrob. Agents Chemother. **43:** 2339-2344.

GMEINER, J., and S. SCHLECHT, 1980 Molecular composition of the outer membrans of *Escherichia coli* and the importance of protein-lipopolysaccharide interactions. Arch. Microbiol. **127:** 81-86.

GRÜNDLING, A., U. BLÄSI and R. YOUNG, 2000a Biochemical and genetic evidence for three transmembrane domains in the class I holin, λS. J. Biol. Chem. **275:** 769-776.

GRÜNDLING, A., M. D. MANSON and R. YOUNG, 2001 Holins kill without warning. Proc. Natl. Acad. Sci. USA **98:** 9348-9352.

GRÜNDLING, A., D. L. SMITH, U. BLÄSI and R. YOUNG, 2000b Dimerization between the holin and holin inhibitor of phage lambda. J. Bacteriol. **182:** 6075-6081.

GUZMAN, L. M., D. BELIN, M. J. CARSON and J. BECKWITH, 1995 Tight regulation, modulation, and high-level expression by vectors containing the arabinose P_{BAD} promoter. J. Bacteriol. **177:** 4121-4130.

HAMMES, W. P., and F. C. NEUHAUS, 1974 On the specificity of phospho-N-acetylmuramyl-pentapeptide translocase. The peptide subunit of uridine diphosphate-N-actylmuramyl-pentapeptide. J. Biol. Chem. **249:** 3140-3150.

HAYASHI, M., 1978 Morphogenesis of the isometric phages, pp. 531-547 in *The Single-stranded DNA Phages*, edited by D. T. DENHARDT, D. DRESSLER and D. S. RAY. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

HAYASHI, M., A. AOYAMA, D. L. RICHARDSON and M. N. HAYASHI, 1988 Biology of the bacteriophage ϕX174, pp. 1-71 in *The Bacteriophages*, edited by R. CALENDAR. Plenum Press, New York.

HAYASHI, M., F. K. FUJIMURA and M. HAYASHI, 1976 Mapping of in vivo messenger RNAs for bacteriophage phiX-174. Proc Natl Acad Sci USA **73:** 3519-3523.

HENRICH, B., W. LUBITZ and R. PLAPP, 1982 Lysis of *Escherichia coli* by induction of cloned ϕX174 genes. Mol. Gen. Genet. **185:** 493-497.

HESS, H., and J. DERR, 1975 Assay of inorganic and organic phosphorus in the 0.5-1 nanomole range. Anal. Biochem. **63:** 607-613.

HEYDANEK, M. G., JR., W. G. STRUVE and F. C. NEUHAUS, 1969 On the initial stage in peptidoglycan synthesis. 3. Kinetics and uncoupling of phospho-N-acetylmuramyl-pentapeptide translocase (uridine 5'-phosphate). Biochemistry **8:** 1214-1221.

HOLTJE, J., and E. I. TUOMANEN, 1991 The murien hydrolases of *Escherichia coli*: properties, funtions and impact on the course of infections *in vivo*. J. Gen. Microbiol. **137:** 441-454.

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

HUTCHISON, C. A., III, S. PHILLIPS, M. H. EDGELL, S. GILLAM, P. JAHNKE *et al.*, 1978 Mutagenesis at a specific position in a DNA sequence. J. Biol. Chem. **253:** 6551-6560.

HUTCHISON, C. A., III, and R. L. SINSHEIMER, 1963 Kinetics of bacteriophage release by single cells of ϕX174-infected *E. coli*. J. Mol. Biol. **7:** 206-208.

HUTCHISON, C. A., III, and R. L. SINSHEIMER, 1966 The process of infection with bacteriophage ϕX174. X. Mutations in a ϕX lysis gene. J. Mol. Biol. **18:** 429-447.

IKEDA, M., M. WACHI, H. JUNG, F. ISHINO and M. MATSUHASHI, 1991 The *Escherichia coli* mraY gene encoding UDP-N-acetylmuramoyl-pentapeptide: undecaprenyl-phosphate phospho-N-acetylmuramoyl-pentapeptide transferase. J. Bacteriol. **173:** 1021-1026.

INCARDONA, N. L., 1978 Adsorption and eclipse reactions of the isometric phages, pp. 549-555 in *The Single-stranded DNA Phages*, edited by D. T. DENHARDT, D. DRESSLER and D. S. RAY. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

INCARDONA, N. L., and L. SELVIDGE, 1973 Mechanism of adsorption and eclipse of bacteriophage ϕX174. II. Attachment and eclipse with isolated *Escherichia coli* cell wall lipopolysaccharide. J. Virol. **11:** 775-782.

JALAVA, K., A. HENSELB, M. SZOSTAKA, S. RESCHC and W. LUBITZ, 2002 Bacterial ghosts as vaccine candidates for veterinary applications. J Controlled Release **85:** 17-25.

JAZWINSKI, S. M., A. A. LINDBERG and A. KORNBERG, 1975a The gene H spike protein of bacteriophages ϕX174 and S13. I. Functions in phage-receptor recognition and in transfection. Virology **66:** 283-293.

JAZWINSKI, S. M., A. A. LINDBERG and A. KORNBERG, 1975b The lipopolysaccharide receptor for bacteriophages ϕX174 and S13. Virology **66:** 268-282.

JOHNSON-BOAZ, R., C. Y. CHANG and R. YOUNG, 1994 A dominant mutation in the bacteriophage lambda S gene causes premature lysis and an absolute defective plating phenotype. Mol. Microbiol. **13:** 495-504.

KOLISNYCHENKO, V., G. PLUNKETT, III, C. D. HERRING, T. FEHER, J. POSFAI *et al.*, 2002 Engineering a reduced *Escherichia coli* genome. Genome Res. **12:** 640-647.

KOSMA, P., 1999 Chlamydial lipopolysaccharide. Biochim. Biophys. Acta **1455:** 387-402.

LLOYD, A. J., P. E. BRANDISH, A. M. GILBEY and T. D. BUGG, 2004 Phospho-N-acetyl-muramyl-pentapeptide translocase from *Escherichia coli*: catalytic role of conserved aspartic acid residues. J. Bacteriol. **186:** 1747-1757.

LOPILATO, J., S. BORTNER and J. BECKWITH, 1986 Mutation in a new chromosonal gene of *Escherichia coli* K-12, *pcnB*, reduce plasmid copy number of pBR322 and its derivatives. Mol. Gen. Genet. **205:** 285-290.

LUBITZ, W., G. HALFMANN and R. PLAPP, 1984a Lysis of *Escherichia coli* after infection with ϕX174 depends on the regulation of the cellular autolytic system. J. Gen. Microbiol. **130:** 1079-1087.

LUBITZ, W., R. E. HARKNESS and E. E. ISHIGURO, 1984b Requirement for a functional host cell autolytic enzyme system for lysis of *Escherichia coli* by bacteriophage ϕX174. J. Bacteriol. **159:** 385-387.

LUBITZ, W., and R. PLAPP, 1980 Murein degradation in *Escherichia coli* infected with bacteriophage ϕX174. Curr. Microbiol. **4:** 301-304.

LUTZ, R., and H. BUJARD, 1997 Independent and tight regulation of transcriptional units in *Escherichia coli* via the LacR/O, the TetR/O and AraC/I1-I2 regulatory elements. Nucleic Acids Res. **25:** 1203-1210.

MACHIDA, M., K. TEKECHI and H. SATO, 2006 Genes for the peptidoglycan synthesis pathway are essential for chloroplast division in moss. Proc. Natl. Acad. Sci. USA **103:** 6753-6758.

MARATEA, D., K. YOUNG and R. YOUNG, 1985 Deletion and fusion analysis of the ϕX174 lysis gene *E.* Gene **40:** 39-46.

MARKERT, A., and W. ZILLIG, 1965 Studies on the lysis of *Escherichia coli* C by bacteriophage ϕX174. Virol. **25:** 88-97.

MCKENNA, R., B. R. BOWEN, L. L. ILAG, M. G. ROSSMANN and B. A. FANE, 1996 The atomic structure of the degraded procapsid particle of bacteriophage G4: induced structural changes in the presense of calcium ions and functional implications. J. Mol. Biol. **265:** 736-750.

MCKENNA, R., L. L. ILAG and M. G. ROSSMANN, 1994 Analysis of the sigle-stranded DNA bacteriophage ϕX174 at a resolution of 3.0 A. J. Mol. Biol. **237:** 517-543.

MCKENNA, R., D. XIA, P. WILLINGMANN, L. L. ILAG, S. KRISHNASWAMY *et al.*, 1992 Atomic structure of single-stranded DNA bacteriophage ϕX174 and its functional implications. Nature **355:** 137-143.

MEIER-DIETER, U., K. BARR, R. STARMAN, L. HATCH and P. D. RICK, 1992 Nucleotide sequence of the *Escherichia coli* rfe gene involved in the synthesis of enterobacterial common antigen. Molecular cloning of the rfe-rff gene cluster. J. Biol. Chem. **267:** 746-753.

MENDEL, S., J. M. HOLBOURN, J. A. SCHOUTEN and T. D. BUGG, 2006 Interaction of the transmembrane domain of lysis protein E from bacteriophage ϕX174 with bacterial translocase MraY and peptidyl-prolyl isomerase SlyD. Microbiology **152:** 2959-2967.

MILLER, J. H., 1972a Experiment 16: 2-aminopurine and nitrous acid mutagenesis, pp. 135-139 in *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

MILLER, J. H., 1972b *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

MONDEGO, J., J. SIMOES-ARAUJO, D. DE OLIVEIRA and M. ALVES-FERREIRA, 2003 A gene similar to bacterial translocase I (mraY) identified by cDNA-AFLP is expressed during flower bud development of *Arabidopsis thaliana*. Plant Science **164:** 323-331.

NANNINGA, N., 1998 Morphogenesis of *Escherichia coli*. Microbiol. Mol. Biol. Rev. **62:** 110-129.

OHKAWA, T., 1980 On the structure of the lipopolysaccharide core in the cell wal of *Escherichia coli* K12 W2252-llu$^-$ and its Ter-mutant cells. Biochem. Biophys. Res. Commun **95:** 938-944.

PACE, C. N., F. VAJDOS, L. FEE, G. GRIMSLEY and T. GRAY, 1995 How to measure and predict the molar absorption coefficient of a protein. Protein Sci. **4:** 2411-2423.

PARK, J. T., and T. UEHARA, 2008 How bacteria consume their own exoskeletons (turnover and recycling of cell wall peptidoglycan). Microbiol. Mol. Biol. Rev. **72:** 211-227.

PARK, T., D. K. STRUCK, C. A. DANKENBRING and R. YOUNG, 2007 The pinholin of lambdoid phage 21: control of lysis by membrane depolarization. J. Bacteriol. **189:** 9135-9139.

PARK, T., D. K. STRUCK, J. F. DEATON and R. YOUNG, 2006 Topological dynamics of holins in programmed bacterial lysis. Proc. Natl. Acad. Sci. USA **103:** 19713-19718.

POLLOCK, T. J., E. S. TESSMAN and I. TESSMAN, 1978 Identification of lysis protein E of bacteriophage ϕX174. J. Virol. **28:** 408-410.

POWELL, B. S., M. P. RIVAS, D. L. COURT, Y. NAKAMURA and C. L. TURNBOUGH, JR., 1994 Rapid confirmation of single copy lambda prophage integration by PCR. Nucleic Acids Res. **22:** 5765-5766.

PRICE, N., and F. MOMANY, 2005 Modeling bacterial UDP-HexNAc: polyprenol-P HexNAc-1-P transferases. Glycobiology **15:** 29R-42R.

RAAB, R., G. NEAL, C. SOHASKEY, J. SMITH and R. YOUNG, 1988 Dominance in lambda S mutations and evidence for translational control. J. Mol. Biol. **199:** 95-105.

RAKONJAC, J., J. N. FENG and P. MODEL, 1999 Filamentous phage are released from the bacterial membrane by a two-step mechanism involving a short C-terminal fragment of pIII. J. Mol. Biol. **289:** 1253-1265.

RAMANCULOV, E. R., and R. YOUNG, 2001a Functional analysis of the T4 *t* holin in a lambda context. Mol. Genet. Genomics **265:** 345-353.

RAMANCULOV, E. R., and R. YOUNG, 2001b Genetic analysis of the T4 holin: timing and topology. Gene **265:** 25-36.

READER, R. W., and L. SIMINOVITCH, 1971 Lysis defective mutants of bacteriophage lambda: genetics and physiology of S cistron mutants. Virology **43:** 607-622.

ROOF, W. D., S. M. HORNE, K. D. YOUNG and R. YOUNG, 1994 *slyD*, a host gene required for ϕX174 lysis, is related to the FK506-binding protein family of peptidyl-prolyl *cis-trans*-isomerases. J. Biol. Chem. **269:** 2902-2910.

ROOF, W. D., and R. YOUNG, 1995 ϕX174 lysis requires *slyD*, a host gene which is related to the FKBP family of peptidyl-prolyl cis-trans isomerases. FEMS Microbiol. Rev. **17:** 213-216.

RUIZ, N., 2008 Bioinformatics identification of MurJ (MviN) as the peptidoglycan lipid II flippase in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **105:** 15553-15557.

RYDMAN, P. S., and D. H. BAMFORD, 2003 Identification and mutational analysis of bacteriophage PRD1 holin protein P35. J. Bacteriol. **185:** 3795-3803.

SANGER, F., G. M. AIR, B. G. BARRELL, N. L. BROWN, A. R. COULSON *et al.*, 1977 Nucleotide sequence of bacteriophage ϕX174 DNA. Nature **265:** 687-695.

SANGER, F., A. R. COULSON, T. FRIEDMAN, G. M. AIR, B. G. BARRELL *et al.*, 1978 The nucleotide sequence of bacteriophage ϕX174. J. Mol. Biol. **125:** 225-246.

SAUVAGE, E., F. KERFF, M. TERRAK, J. AYALA and P. CHARLIER, 2008 The pinicillin-binding proteins: structure and role in peptidoglycan biosynthesis. FEMS Microbiol. Rev. **32:** 234-258.

SHEN, H., and J. J. CHOU, 2008 MemBrain: improving the accuracy of predicting transmembrane helices. PLoS ONE **3:** e2399.

SMITH, D. L., D. K. STRUCK, J. M. SCHOLTZ and R. YOUNG, 1998 Purification and biochemical characterization of the lambda holin. J. Bacteriol. **180:** 2531-2540.

STRUCK, D. K., and W. J. LENNARZ, 1977 Evidence for the participation of saccharide-lipids in the synthesis of the oligosaccharide chain of ovalbumin. J. Biol. Chem. **252:** 1007-1013.

STRUVE, W., and F. NEUHAUS, 1965 Evidence for an initial acceptor of UDP-Nac-muramyl-pentapeptide in the synthesis of bacterial mucopeptide. Biochem. Biophys. Res. Commun **18:** 6-12.

STRUVE, W. G., R. K. SINHA and F. C. NEUHAUS, 1966 On the initial stage in peptidoglycan synthesis. Phospho-N-acetylmuramyl-pentapeptide translocase (uridine monophosphate). Biochemistry **5:** 82-93.

TOMASZ, A., 1979 The mechanism of the irreversible antimicrobial effects of penicillins: how the beta-lactam antibiotics kill and lyse bacteria. Annu. Rev. Microbiol. **33:** 113-137.

TOMASZ, A., and S. WAKS, 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.

TRAN, T. A. T., D. K. STRUCK and R. YOUNG, 2005 Periplasmic domains define holin-antiholin interactions in T4 lysis inhibition. J. Bacteriol. **187:** 6631-6640.

VAN HEIJENOORT, J., 2001 Recent advances in the formation of the bacterial peptidoglycan monomer unit. Nat. Prod. Rep. **18:** 503-519.

VOLLMER, W., B. JORIS, P. CHARLIER and S. FOSTER, 2008 Bacterial peptidoglycan (murein) hydrolases. FEMS Microbiol. Rev. **32:** 259-286.

WANG, I. N., 2006 Lysis timing and bacteriophage fitness. Genetics **172:** 17-26.

WANG, I. N., D. E. DYKHUIZEN and L. B. SLOBODKIN, 1996 The evolution of phage lysis timing. Evol. Ecol. **10:** 545-558.

WEPPNER, W. A., and F. C. NEUHAUS, 1977 Fluorescent substrate for nascent peptidoglycan synthesis. Uridine diphosphate-N-acetylmuramyl-(N-epsilon-5-dimethylaminonaphthalene-1- sulfonyl) pentapeptide. J. Biol. Chem. **252:** 2296-2303.

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

WITTE, A., U. BLÄSI, G. HALFMANN, M. SZOSTAK, G. WANNER *et al.*, 1990a ϕX174 protein E-mediated lysis of *Escherichia coli*. Biochimie **72:** 191-200.

WITTE, A., E. BRAND, P. MAYRHOFER, F. NARANDJA and W. LUBITZ, 1998 Mutations in cell division proteins FtsZ and FtsA inhibit ϕX174 protein-E-mediated lysis of *Escherichia coli.* Arch. Microbiol. **170:** 259-268.

WITTE, A., and W. LUBITZ, 1989 Biochemical characterization of ϕX174-protein-E-mediated lysis of *Escherichia coli.* Eur. J. Biochem. **180:** 393-398.

WITTE, A., G. SCHROT, P. SCHON and W. LUBITZ, 1997 Proline 21, a residue within the alpha helical domain of ϕX174 lysis protein E, is required for its function in *Escherichia coli.* Mol. Microbiol. **26:** 337-346.

WITTE, A., G. WANNER, U. BLÄSI, G. HALFMANN, M. SZOSTAK *et al.*, 1990b Endogenous transmembrane tunnel formation mediated by ϕX174 lysis protein E. J. Bacteriol. **172:** 4109-4114.

XU, M., A. ARULANDU, D. K. STRUCK, S. SWANSON, J. C. SACCHETTINI *et al.*, 2005 Disulfide isomerization after membrane release of its SAR domain activates P1 lysozyme. Science **307:** 113-117.

XU, M., D. K. STRUCK, J. DEATON, I. N. WANG and R. YOUNG, 2004 The signal arrest-release (SAR) sequence mediates export and control of the phage P1 endolysin. Proc. Natl. Acad. Sci. USA **101:** 6415-6420.

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

YOUNG, R., 1992 Bacteriophage lysis: mechanism and regulation. Microbiol. Rev. **56:** 430-481.

YOUNG, R., and I. N. WANG, 2006 Phage lysis, pp. 104-126 in *The Bacteriophages*, edited by R. CALENDAR. Oxford University Press, Oxford.

YOUNG, R., I. N. WANG and W. D. ROOF, 2000 Phages will out: strategies of host cell lysis. Trends Microbiol. **8:** 120-128.

ZHENG, Y., D. K. STRUCK, T. G. BERNHARDT and R. YOUNG, 2008a Genetic analysis of MraY inhibition by the ϕX174 protein E. Genetics **180:** 1459-1466.

ZHENG, Y., D. K. STRUCK, C. A. DANKENBRING and R. YOUNG, 2008b Evolutionary dominance of holin lysis systems derives from superior genetic malleability. Microbiology **154:** 1710-1718.

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Zheng, Y., Struck, D.K., Bernhardt, T.G., and Young, R. (2008). Genetic analysis of MraY inhibition by the ϕX174 protein E. *Genetics* **180**: 1459-1466.