CHARACTERIZATION OF THE PHYSIOLOGICAL IMPLICATIONS OF DEFECTIVE LAMBDOID PHAGE DLP12 IN *E. coli*

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Most bacterial genomes are decorated with "non-species specific" genetic elements. These elements include defective prophages which account for up to 30% of some bacterial genomes. Defective prophages are trapped, due to mutation in or the absence of essential genes, in the chromosome in a permanent state of lysogeny. Although defective, several studies have reported that prophage genes benefit both gram positive and gram negative bacteria with respect to host fitness under a variety of conditions. DLP12 (Defective Lambdoid Prophage inserted in the 12th minute) is one of four defective lambdoid phages found in *E. coli* K12 chromosome. The DLP12 region encodes for putative holin (S^{DLP12}), a putative lysozyme (R^{DLP12}) and a putative endopeptidase (Rz^{DLP12}), which are all highly homologous to their counterparts in phage 21. Lambdoid homologs of these three genes facilitate the degradation of peptidoglycan causing cell lysis. Peptidoglycan is a heteropolymer made up of sugars and peptides. It is the main structural component of the bacterial cell wall. Although sturdy and highly resistant to turgor pressure, peptidoglycan must be a dynamic structure in order to allow cell growth and division. Thus, the regulation of peptidoglycan metabolism is critical and the enzymes involved in the process of peptidoglycan turnover must be tightly regulated in order to prevent unwanted cell lysis. Although recent work has shown that R^{DLP12} encodes an active lysozyme capable of degrading gram positive and gram negative peptidoglycan, its physiological role in *E. coli* K12 is unclear. We therefore constructed mutants of r^{DLP12} and the other

DLP12 lysis gene in PHL628, an E. coli K12 derivative that over expresses curli, and found that the mutants had altered peptidoglycan metabolism. The mutants incorporated less exogenous N-acetylglucosamine into their peptidoglycan and released little or no peptidoglycan degradation products to the supernatant (Chapter 2). We also found that the DLP12 lysis genes play a main role in σ^E mediated early stationary phase cell lysis. Consistent with effects on the cell wall, the mutants produced less curli which in turn affected cell-to-cell and cell-to-surface interactions and therefore biofilm formation (Chapter 3). We also found that normal transcription of the lysis genes appeared to be controlled by a putative antiterminator Q^{DLP12} (Chapter 4). Antiterminators are transcriptional regulators which act on RNA polymerase holoenzyme to allow it to read through terminator sequences. In lambdoid phages the antiterminator Q is required for σ^D mediated transcription of the late genes, including the lysis genes. Interestingly, we obtained preliminary evidence that Q^{DLP12} is required for σ^E mediated transcription of the lysis genes and that mutants of q^{DLP12} showed a similar phenotype to that observed in mutants for the DLP12 lysis genes (see above). In the following chapters we describe the implications of DLP12 genes for the physiology of *E. coli* and conclude that *E. coli* has co-opted these otherwise lethal genes for its own benefit.

BIOGRAPHICAL SKETCH

Faustino Andreas Toba Francis was born June 2nd 1976 in Caracas, Venezuela. He grew up in an environment of love, science and education. He attended Institutos Educacionales Asociados in Caracas and graduated with honors from high school in 1994.

After graduating, he attended Universidad Central de Venezuela (UCV) in Caracas. There he studied biology at the Science College, specializing in cellular biology. During the first years at college, Faustino participated in a semester internship at the Instituto Jardin Botánico de Caracas (Botanical Garden Institute). Later, he enrolled in teaching experience activities becoming a teaching assistant for both the Genetics and Microbiology courses for 2 full academic years. Also, during this period he joined the Dr. Alonso's Plásmidos Bacterianos (Bacterial Plasmid) lab at the Instituto de Biología Experimental (Experimental Biology Institute). In this lab he worked and completed my Undergrad Thesis on bacterial plasmids isolated from aquatic environments which was honored with an award the following year. He graduated with honors and first of the class in 2000.

After graduation Faustino was hired in the same lab as research assistance. In this position he performed as lab management and project coordination. Also, he helped in the training of new students in general lab techniques. During 2001 he was also hired as Instructor for the Cellular Biology Lab course in the Biology Department at Universidad Central de Venezuela (UCV). In 2002, Faustino was hired for a short period at the Petroleum Biology Lab at Instituto de Estudios Avanzados (Institute of Advance Studies).

In 2002 he came to Ithaca, NY to attend Cornell University and enrolled in the Microbiology PhD program. After the rotation period, Faustino joined Dr. Anthony

Hay's Environmental Microbiology and Toxicology lab as a Graduate Research Assistant. As his first project he successfully studied the use of bioreporters for the direct detection of chemicals in the soil. In 2003 he switched projects to study *E. coli* biofilms and peptidoglycan metabolism, which will become the subject of his thesis work. During this very successful research period, Faustino put together a hard working research team comprised of undergrads and research technicians. During his period at Cornell he also became a teacher assistant for the General Microbiology class for two semesters and led several small group discussions. Also, Faustino became the President of the Venezuelan Student Association for 4 years and the Vice president of Field of Microbiology Students (FOMS) for a year. He finished his PhD program and graduated in 2008.

His future plans include pursuing a Post-doc in Clinical Microbiology at Columbia University in New York.

To my family, friends and you my special one

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INTRODUCTION: INTERPLAY BETWEEN PEPTIDOGLYCAN METABOLISM AND DEFECTIVE PROPHAGE LYSIS CASSETTE

1.1 DEFECTIVE LAMBDOID PHAGES

Sometimes, due to mutations or recombination, a prophage is unable to re-enter its lytic cycle and becomes trapped in the host cell (11, 12, 14). While these defective prophages are thought to be in mutational decay, since they can not become infective again, and are expected to be eliminated by random genetic drift (11, 12, 14), a number of defective prophages have been proven beneficial for their host, increasing fitness, survival and virulence (14). For example, the *rex* gene endows the host cell with resistance to further infection from other phages (11). Two other lambdoid prophage genes, *bor* and *lom*, increase the pathogenicity of *E. coli* by changing surface proteins that interact with mammalian cells (13, 15, 98). In *P. aeruginosa*, lambda tail-like proteins, known as F-pyocins, are used as a weapon to perturb the membrane of closely related bacteria (39). In *S. dysenteriae* 1, the Shiga toxin is encoded by the remnant of a lambdoid prophage (58). These examples, coupled with the fact that prophage occupy between 10 and 30% of many bacterial genomes (14) suggest that prophages, defective or not, likely play an important role in host physiology.

Four defective lambdoid prophages have been found in the *E. coli* genome: DLP12, Rac, e14 and Qin (11, 13). DLP12 (**D**efective **L**ambdoid **P**hage inserted in the **12** minute) is found as an 18.9 KB insertion near the *argU* tRNA gene (55). DLP12 region is classified as a defective prophage because is not able to excise from the chromosome due to mutations in key genes, like *xis* (12). Interestingly, the *bor* gene

(mentioned above), which confers resistance to animal sera is one of the 22 lambdoid genes found within the DLP12 region (13). The lambda lysis genes *SRRz* are also part of DLP12 region (11, 55, 88). DLP12 *SRRz* lysis cassette is highly homologous to that of lambdoid phage 21 *SRRz* and encodes a functional lysozyme (88). In *E. coli* these genes have been named *essD*, *ybcS,* and *ybcT* respectively. Although nothing is known about the function of this lysis cassette in host cell physiology, *in-silico* sequence analysis of the *E. coli* genome predicted the presence of an RpoE regulated promoter named *essD*p immediately upstream of $SRRz^{DLP12}$ (72).

1.2 BACTERIOPHAGES AND PEPTIDOGLYCAN

After infection, with the exception of filamentous phages like M13, lytic bacteriophage need to degrade peptidoglycan in order to escape the bacterial cell and release the new viral particles for a new round of infection (115, 116). Lytic proteins that facilitate peptidoglycan degradation are encoded by *S, R* and *Rz* homologs in lambdoid and other temperate phages but are tightly regulated, permitting a lysogenic life cycle (12, 116). In lysogeny the phage chromosome integrates with the host DNA and remains in a dormant-like state (12, 14). Environmental factors, like DNA damage and capsule perturbations can induce the excision of the prophage and the re-initiation of the lytic life cycle (61, 75).

Lambdoid phages have a lysozyme (R) (or endolysin) and an endopeptidase (Rz), which can degrade peptidoglycan and a lipoprotein which binds it to the outer membrane respectively. To accomplish these tasks, lambdoid phages contain a third lysis gene, encoding a holin (*S*), whose product aids the lysozyme and endopetidase to gain access to the peptidoglycan in two distinct ways (13, 115, 116). In the canonical λ phage, the λ holin (Class I) form holes in the membrane that are large enough to

allow passage of the lysozyme and the endopeptidase which would otherwise remain sequestered in the cytoplasm (11, 106, 115, 116). In other lambdoid phages, like phage 21, the lysozyme and endopeptidase are transported by the host secretion system (63, 112, 113). In these phages the holin (Class II) functions as an ion channel that dissipates membrane potential and physically disturbs the membrane but does not allow the diffusion of proteins outside of the cell (63, 112, 113). This type of holin is has recently been termed a pinholin (63).

Both class I and II holins, including S^{λ} and S^{21}, have dual start motifs in the same open reading frame which contributes to regulation of hole formation (6, 106). Thus, *S* encodes for two proteins: the holin and its inhibitor, the antiholin (106). The timing of lysis is regulated post-translationally by the balance between holin and antiholin concentrations. In S^{λ}, the antiholin S107 (107 amino acids long) contains a Lys residue that is responsible for inhibition of S105 (105 amino acids long) polymerization (106, 115). When the relative concentration of S105 to S107 reaches 2:1 the hole is formed (106, 115). A similar situation has been described for S^{21}, where S71 is the antiholin and S68 is the holin (23, 106, 113).

λ^{21} and phage P1 do not accumulate endolysin in the host cytoplasm (112). Rather these endolysins contain a signal element, named a signal-arrest-release (SAR) domain which directs their export by the host *sec* pathway (78, 112, 113). After secretion, this SAR domain keeps the endolysin tethered to the outside of the cytoplasmic membrane in an inactive form (78, 112, 113). Since passage through a holin is not required, SAR-containing endolysins are usually found in association with pinholins (63, 64, 112, 113). Release of these endolysins occurs when pinholins collapse the membrane potential which permits activation via disulfide bond isomerization (63, 112, 113). R^{λ} and related endolysins that are paired up with class I

holins contain no signal element (23, 63, 112, 113) and are already active in the cytoplasm (7, 23, 113).

Endolysins are comprised of two domains: The N-terminus domain is the catalytic domain which is essential for cell lysis; while the C-terminus, although not essential for lysis, is the peptidoglycan binding domain (7). All endolysins cleave the β1-4 glycosidic bond between NAG and NAM in the glycan strands (4, 11, 40, 42). β1-4-N-acetylmutamidases, like R^{21}, act like true lysozymes producing disaccharides as they degrade peptidoglycan. However, not all phage endolysins are hydrolases; R^{λ} behaves like a lytic transglycosylase producing anhydroNAM-NAG disacchariders (7, 23). Endolysin activity is essential and sufficient for cell lysis; thus, lambdoid phage lysis systems are referred to as holin-endolysin systems (88, 116).

The lambda endopeptidase, however, is not essential for cell lysis, and is only required when the divalent cation concentration in the environment is high (>5mM) (91, 117). These endopeptidases are thought to attack Braun's lipid protein which anchors the peptidoglycan to the outer membrane (7, 91, 117). Rz-endopeptidase is referred to as an auxiliary protein and its activity has been described as essential only under high cationic conditions (91, 117). Microscopic observation of over expression of *Rz* showed outer membrane destabilization of liposomes (91). An interesting feature of *Rz* is that within its gene sequence another gene is found in a different open reading frame. *Rz1* is found at +1 frameshift embedded in the *Rz* sequence and encodes a lipoprotein whose C-terminus sequence interacts with Rz (91, 118). The N terminus domain of this Rz1 lipid-anchor tethers the Rz/Rz1 complex to the outer membrane.

In lambdoid phages, *SRRz* lysis region encodes for a total of five peptides that are involved in cell wall metabobolism: an S-holin, which is self-regulated by its S-antiholin; a peptidoglycan degrading R endolysin, with or without SAR element; an auxiliary Rz endopeptidase which is anchored to the outer membrane by an Rz1

lipoprotein where it can attack Brauns lipoprotein. Of these only S and R appear to be critical to cell lysis.

1.3 PEPTIDOGLYCAN

Both gram-negative and gram-positive bacteria contain peptidoglycan in their cell wall (42, 80, 81, 104). Peptidoglycan, also called murein, is the main structural component of bacterial cell wall (42, 81). While pseudopeptidoglycan is found in archea, chitin in fungi and cellulose in plants; the peptidoglycan molecule is exclusive to most all bacteria, except Mycoplasma and some other species that lack cell wall (42, 80, 81). Peptidoglycan is responsible for maintaining the bacterial cell shape, bearing the stress of the external and internal environment, and its status is essential for cell viability (59, 80). In gram positive bacteria peptidoglycan accounts for close to 70% of the cell wall and it can reach up to 80 nm in thickness (10, 111). In contrast, peptidoglycan of gram negative bacteria is only 10 nm thick and comprises only of 10% to 20% of the cell wall (10, 103, 111).

1.4 PEPTIDOGLYCAN STRUCTURE

Peptidoglycan is a covalent heteropolymer of carbohydrates and peptides (59, 81, 99). This large molecule is made up by sugars organized in glycan strands which are cross-linked by flexible peptide bridges forming a mesh structure (42, 80, 99). This peptidoglycan mesh can have a two or three dimensional structure which arises from the repeating crosslink between neighboring glycan strands by peptide bridges (99). The glycan strands provide rigidity and the peptide cross-links provide elasticity to the peptidoglycan structure. The chemical properties of both components and their arrangement allows the peptidoglycan biopolymer to be both strong and elastic so that

it can be withstand the internal osmotic pressure of several atmospheres (3-5atm) while keeping the specific cell shape (42, 59, 80, 103).

In the glycan strand the aminosugars N-acetylglucosamine (NAG) and N-acetyl muramic acid (NAM) are organized in an alternating pattern and are linked by β1-4 glycosidic bonds. NAM, a sugar only found in bacteria, posses a lactyl group which allows the linking of the crosslinking peptides (42, 81). The length of the glycan strands is highly variable among different organisms with the average close to 80 aminosugars, while in *E. coli* the average is closer to 30 residues (29, 42, 80). The average glycan strand length does not seem to correlate with cell shape (81). In *E. coli,* glycan strands containing the aminosugar 1,6 anhidromuramic acid are common (4, 42). This particular residue is the product of an intramolecular glycosyl transfer by lytic transglycosylases, which participate in metabolism, turnover and recycling of peptidoglycan in *E. coli* (4). The 1,6 anhidromuramic acid residue has both carbons (1 and 4) unavailable for elongation therefore it is only found at the end of the glycan strands.

Besides the existence of 1,6-anhydromuramic acid in *E. coli*, the structure of the glycan strand is almost completely conserved among bacteria with only a few cases where there is an O-acetylation instead of an N-acetylation of the sugar (42). On the other hand the peptide bridge is highly variable among bacteria (42, 81).

Due to the β1-4 glycosidic bond geometry the glycan strand has a helical arrangement in which sequential peptide links protrude from the glycan strand forming a 90^{o} angle with one another (42, 80). The peptide stem (or arm) connects the NAM carboxylic group linked by its alpha N terminal residue. The peptide link is formed by an alternating L and D amino acid chain (42, 81). In *E. coli* this peptide sequence is usually L-Ala-D-Gln-m-A_2pm-D-Ala-D-Ala (42). Meanwhile, gram positives like

Staphylococcus aureus usually have a sequence of L-Ala-D-Gln-L-Lys-D-Ala-D-Ala (80).

The occurrence of a dibasic amino acid in the peptide bridge is necessary for crosslink formation. In the general case of gram negatives, like *E. coli* and gram positive *Bacillus* species the meso-diaminopimelic acid (m-A_2pm) serves as the linking residue directly connecting through the non alpha amino group to the penultimate D-alanine residue from a different peptide stem, thus completing the crosslink between two glycan strands (26, 42, 81, 103). In the case of gram positive bacteria the dibasic amino acid function is carried out by an L-Lys which is connected (also through the non alpha amino group) to a side peptide stem formed by five L-Gly (80). This indirect crosslink is complete when the fifth L-Gly of the interbridge links to the penultimate D-Ala of a different peptide stem (10, 80).

In gram-negative bacteria the crosslink can also occur between glycan strands and the lipoprotein associated with the outer membrane (sometimes called Braun's lipoprotein) (8, 29, 42, 81, 94). This lipoprotein is also present in a murein free form; the number of free lipoprotein exceeds the peptidoglycan bound lipoprotein by a factor of two. Interestingly the total amount of lipoprotein in *E. coli* exceeds by 7-fold the number of individual ribosomal proteins, making it the most abundant protein in the cell (8). The lipoprotein is localized to the periplasmic side of the outer membrane in gram negative bacteria and extends to the outer side of the outer membrane (8). In *E. coli* the terminal L-Lys of the lipoprotein is connected to the m-A2pm that is normally part of the peptide bridge linking glycan strands (42).

1.5 PEPTIDOGLYCAN BIOSYNTHESIS

Peptidoglycan biosynthesis is a complex process (42, 80, 99). It starts in the cytoplasm where the monomers are synthesized and assembled, then those precursors are transported out through the membrane and, once out of the cytoplasm the glycan-peptide monomers are finally added to the growing peptidoglycan heteropolymer (42, 80, 99).

Biosynthesis starts with the activation of N-acetylglucose-6-phosphate (NAG-6P) by uracyl triphosphate (UTP) to form uracyl diphosphate-NAG (UDP-NAG) (42, 81, 99). UDP-NAG then reacts with phosphoenol pyruvate to form UDP-N-acetylmuramic acid (UDP-NAM); thus both glycan components are synthesized. But before NAG and NAM are joined together the latter is loaded with up to five amino acids linked to its lactyl group (42, 59, 81). Then, the UDP-NAM-pentapeptide molecule is attached to a long-chain (C_{55}) isoprenoid alcohol called undecaprenylphosphate, also known as bactoprenol (BP), releasing UMP (uracyl monophosphate) (42, 81, 99).

This membrane bound precursor (BP-NAM-pentapeptide) known as Lipid I is converted to Lipid II when UDP-NAG is joined through a β1-4 glycosidic bond to the NAM molecule releasing UDP (42, 80). Thus, the peptidoglycan monomer NAG-β1-4-NAM-pentapeptide is formed and still attached to BP; this Lipid II precursor is localized to the inside of the cytoplasmic membrane (42, 99). The lipophilic feature of BP allows the cells to transport the peptidoglycan hydrophilic precursors from the cytoplasm through the membrane and out into the sites to be added to the growing peptidoglycan (59, 80). This transport is carried out at a rate that matches peptidoglycan synthesis which suggests the involvement of enzymes like translocases or flippases (42, 59, 80, 99). Although there is no experimental data supporting this suggestion there are good candidates for this flipase-like activity in homologues FtsW

and RodA; two enzymes involved in shape, elongation, division and sporulation found in several bacteria but absent in *Mycoplasma* (10, 80).

The final steps of peptidoglycan biosynthesis are the transglycosylation and transpeptidation of the monomer into the preexisting glycan strands. Both processes take place outside of the cell, in the periplasmic space in gram negative bacteria, and just in the outer side of the cellular membrane in gram positive bacteria (42, 59, 80, 99).

Transglycosylation involves the joining of a precursor NAG-NAM(pentapeptide)-BP to a growing peptidoglycan strand. The NAG residue in this precursor is joined through a β1-4 glycosidic bond to the BP terminal of a growing peptidoglycan strand; the "old" bactoprenol is released and dephosphorylated and the new elongated strand $[NAM\text{-}NAG(pentapeptide)]_{n+1}$-BP is available for the next -NAG-NAM(pentapeptide)-BP addition (42, 80, 99). It is not clear what determines the length of the peptidoglycan strands but the strands end with a 1,6-anhydromuramic acid. In *E. coli* lytic transglycosylases are responsible for the 1,6-anhydromuramic acid formation. These enzymes, like lysozyme, cleave the β1-4 glycosidic bond but instead of a hydrolysis, lytic transglycosylases transfer the bond onto the carbon 6 of the same muramic acid yielding 1,6-anhydromuramic acid. An example of a soluble lytic transglycosylase is the 70Kda periplasmic protein Slt70 (40, 41).

Transpeptidation takes place outside the cytoplasm and without the assistance of any energy molecule, such as ATP (80), this occurs in two steps. In *E. coli*, the first step involves the removal of the last D-Ala residue from one of the peptide bridges which in itself creates the intermediate tetrapeptide substrate for the second step, where the energy released from this amino acid removal drives the bond formation between the newly exposed D-Ala in the first peptide chain (or donor) and the non-

alpha amino group of the dibasic amino acid (m-A_2pm in *E. coli*) of the second (or acceptor) peptide chain (42, 80, 99).

1.6 PENICILLIN BINDING PROTEINS (PBPS)

The enzymes involved in transglycosylation and transpeptidation are known as penicillin-binding proteins (PBP) (42, 80, 99). These enzymes are inhibited by penicillin which, due to its structural similarities to the transpeptidation substrate D-Ala-D-Ala, covalently binds to the PBPs active site; hence their name penicillin-binding protein (5, 27, 42). Penicillin acts as suicide substrate forming an intermediate called penicilloyl which is not cleared from the PBPs active site causing steric inhibition; other β-lactams work in a similar fashion (27, 42).

PBPs are part of the acyl transferase family, which are enzymes that cleave peptide bonds through a serine mediated acyl-enzyme intermediate with the catalytic center located near the N-terminus (28, 42, 80). PBPs are classified in two groups: High molecular weight (HMW PBPs) and low molecular weight (LMW PBPs); also β-lactamases such as *E. coli's* ampC are considered penicillin binding proteins (10, 42, 80, 99).

HMW PBPs are found in the periplasim and are anchored to the outside of the cytoplasmic membrane through a non-cleavable signal peptide; they contain two domains located in the outer side of the membrane (28, 80). The C-terminal domain contains the serine active site that catalyzes the cross linking of the peptide stems in the peptidoglycan, this is also the penicillin binding site (27, 42, 80). The activity of the N-terminal domain divides HMW PBPs in two classes: A and B. Class A HMW PBPs N terminal domain catalyzes the transglycosylation of the glycan strands; thus, these PBPs (PBP1a/1b/1c in *E. coli*) are bifunctional enzymes capable of extending

the strand (transglycosylation) and forming the cross-links (transpeptidation) of peptidoglycan (5, 10, 28, 42, 80).

Class B PBPs (PBP2, PBP3 in *E. coli*) have a non-penicillin binding N-terminal domain with a yet to be determined fuction, although it is thought to be involved in cell morphology (10, 80). In. *E. coli* PBP2 and PBP3 are, as of yet, thought to be monofunctional transpeptidases involved in cell shape/elongation and cell dvision, respectively (10, 87). β-lactams that alter the cell shape, like mecillinam, are bound more effectively by PBP2 than any other PBPs, also PBP2 has been found to interact with the *E. coli* cytoskeleton protein MreB (a cell elongation related protein) and inner membrane proteins RodA, MreC and MreD (cell shape related proteins) (10, 87). In contrast PBP3, also known as FtsI, is thought to interact with FtsZ and the other Fts proteins in the divisional septum. The cytoskeleton role in peptidoglycan synthesis will be discussed below.

LMW PBPs, in *E. coli* PBP4/5/6/6b/7/8, are usually monofuctional peptidases (5, 80); they either have endopeptidase or carboxylase activity with the exception of PBP4, which can carry out both. The DD-carboxylase activity controls the level of peptidoglycan cross-linking by cleaving the carboxy-terminal D-Ala-D-Ala, a step required for cross-linking (28, 80, 104). Mutations in PBP5 suggest this LMW PBP has a primary role among LMW PBPs in cell shape, although it has a cooperative activity with other LMW PBPs as additional mutations are required before a phenotype can be associated with PBP5 (10, 60).

β-lactamases, which are also included in PBPs, are secreted enzymes; in gram positives β-lactamases are secreted into the environment, in gram negatives these enzymes are accumulated in the periplasm (28). β-lactamases form the same acyl-enzyme intermediate with penicillin (and other β-lactams) but have a higher kinetic turnover rate hydrolyzing the β-lactam and rendering it biologically inert (28, 47, 57).

Their only known function is this hydrolysis of β-lactam antibiotics. β-lactamases do not compete with peptidoglycan metabolism because they are not able to carry out DD-peptidase activity (28, 47, 57). β-lactamases and LMW PBPs are structurally quite similar and only differ in key amino acids near the N-terminal catalytic center; increasing evidence suggests that β-lactamases arose from PBPs that were part of the cell wall synthesis machinery (47, 57).

1.7 OTHER ENZYMES INVOLVED IN PEPTIDOGLYCAN METABOLISM

Lytic transglycosylases (LTs) are a class of autolysins involved in peptidoglycan biosynthesis and cell division (4, 40-42, 104). They act like lysozyme, attacking the β1-4 glycosidic bond between NAG and NAM in the glycan strands of the cell wall (4, 42, 104). But unlike lysozyme, LTs do not hydrolyse the glycosidic bond. Instead, LTs catalyze an intramolecular transglycosylation which yields 1,6-anhydromuramic acid (anhNAM) (4, 42, 104). In the case of *E. coli* there are up to six LTs; some are soluble and move freely in the periplasm, such as Slt (Slt35 and Slt70, e.g), while others are anchored (bound) to the membrane, such as Mlt (MltA and MltB, e.g) (4, 42, 104). The majority of LTs are exoenzymes, which release NAG-NAM-peptide from either the reducing or the non-reducing end of the glycan strand (4, 41, 42). There is one membrane bound endo-LT, EmtA, which cleaves glycosidic bonds in the middle of glycan strands (4, 51, 93).

Another group of enzymes involved in peptidoglycan metabolism are the amidases. Ubiquitous among bacteria, these amidases hydrolyze of the bond between NAG and the L-alanine (N-acetylmuramyl-L-alanine amidase) of the peptide stem (36-38, 42, 70, 77). In *E. coli* these amidases play an essential role in cell division by cleaving the septum allowing the separation of the daughter cells (42, 70). Evidence of

this importance is the fact that deletion mutants of *ami*A/B/C shows a phenotype where up to 30% of the cells form chains of three to six unseparated cells (37, 70). Twin-arginine protein transport (Tat) pathway mutants also exhibit this chained cells phenotype; this observation could be explained by the fact that AmiA and AmiC are exported through Tat (43, 108). A similar phenotype has been observed in gram positive amidase mutants (108).

These *E. coli* amidases also play the part of autolysins, contributing to peptidoglycan degradation and cell lysis when peptidoglycan synthesis is inhibited (36, 37) Another role played by these amidases is the further breakdown of peptidoglycan turnover products for cell wall recycling (see below) (30, 42). LTs, endopeptidases, and amidases all participate in peptidoglycan degradation when insertion of new peptidoglycan monomers is blocked by β-lactams, resulting in cell lysis (36, 42).

Although *E. coli* contains LTs, it has been reported to be one of the few bacteria which have no true lysozymes (40, 41). Lysozymes are found in almost all peptidoglycan containing bacteria (40). Lysozymes are glycosidases which hydrolyze the β1-4 glycosidic bond between NAG and NAM in the peptidoglycan (40, 77). The enzyme binds the reducing end of the glycan strand and releases disaccharide peptides (NAG-NAM(peptide)) monomers as it cleaves the glycosidic bond. The release product differentiates Lysozymes from LTs, which release the specific NAG-anhNAM(peptide) monomer (41, 42). In most bacteria lysozymes, like the other enzymes which target the peptidoglycan, play an essential role in the bacteria life cycle due to their role in peptidoglycan maturation (84).

Given their aggressive enzymatic activity, lysozymes must be under strict regulation to avoid undesired autolysis of the bacteria (42). Regulation can be exerted by transcriptional and posttranscriptional regulation, substrate modification or

specificity (84). Transcriptional control of *lytD*, a glucosamidase, in *B. subtilis* is dependant on the vegetative sigma factor σ^D, which activates the transcription of *lytD* upon entering stationary phase (84). This regulation ensures that the autolysin will only be transcribed when needed, at the moment of cell division (84). In Lambdoid phages, transcription of phage lysozymes is control by the late promoter $P_{R'}$ and the antiterminator Q, this ensures the transcription of the lysozyme, and concomitant host cell lysis, only occurs after the assembly of the new phage particles (77, 116). Studies with membrane depolarizing agents suggest a posttranscriptional regulation of autolysins based on membrane perturbation and proton motive force in *B. subtilis* since depolarization of the membrane enhances hydrolytic activity of autolysins (45, 48, 84).

Substrate modification and specificity ensure that the lysozyme producing bacteria is not affected by its own lysozyme, which is thought to be secreted as a bacterial warfare agent with the purpose of degrading other microorganism petidoglycan; muramidase-2 in *Enterococcus hirae* is able to degrade peptidoglycan from *Micrococcus luteus* but is ineffective against *E. hirea's* own peptidoglycan (40). Deacetylation of the petidoglycan in *S. pneumoniae* makes its own peptidoglycan resistant to lysozyme (105).

1.8 MULTIENZYME COMPLEX MODEL

The peptidoglycan is a completely closed and rigid structure; this suggests a problem when cells need to grow in size or divide (41, 42, 59). In order to enlarge the cell wall, existing bonds have to be cleaved to allow the addition of new monomers (i.e glycan strands); the same situation is found when cells need to divide, bonds need to be broken to allow the constriction of the membrane and the formation of the septum (41, 42, 80). To accomplish enlargement and constriction of the cell wall bacteria require the concerted action of peptidoglycan hydrolases and synthases (5, 42). This process must be well regulated to avoid the risk of lysis which could arise from uncontrolled breakage of the peptidoglycan network. Experimental evidence suggest that hydrolases and synthases work by adding new monomers or strands before breaking preexisting bonds, this strategy is called "make-before-break" (17, 41, 42, 80).

A peptidoglycan growth mechanism has been proposed for gram positive bacteria; this mechanism suggests that new layers are added in an "inside-to-outside manner" (41, 42, 50, 59, 80). As the gram positive cell elongates, the new glycan strands are added just outside of the cell membrane and below the cell wall stress bearing layers; at the same time the old strands are hydrolyzed from the outermost stressed layer of the cell wall (41, 42, 50, 80). As new strands are added they subsequently move outwards where they bear the stretching force; finally this outer layer is hydrolyzed by autolytic enzymes (41, 42, 80). This allows the peptidoglycan to increase its size while avoiding the risk of lysis (42, 80).

A more elaborate mechanism was suggested for gram negatives, particularly *E. coli* (41, 42, 80). This model complies with the "make-before-break" and "inside-to-outside" strategies and also accounts for turnover rates found in *E. coli* (about 50%)

(41, 42, 50, 59, 80). Known as the three for one model, in *E. coli* peptidoglycan grows by the addition of three glycan strands and the subsequent removal of one glycan strand, called the docking strand (41, 42, 50, 80). This model requires very delicate coordination, in both time and space, between several peptidoglycan synthases and hydrolases to avoid cell lysis (42, 80).

Protein interaction studies based on affinity chromatography have demonstrated protein protein contacts between peptidoglycan synthases and hydrolases in *E. coli*, *B. subtilis* and *C. cresentus* (41, 42, 80). Thus, a multienzyme complex consisting of a Lytic transglycosylase and several PBPs has been proposed as responsible for peptidoglycan growth in *E. coli* (10, 42, 80). This complex is suggested to have a processive feature similar to nucleic acid polymerases (42, 104). The "three for one" model states that a building block of three cross-linked glycan strands are added to the preexisting peptidoglycan network; this building block is cross-linked by the multienzyme complex to two available m-A_2pm residues flanking the docking strand in the peptidoglycan (41, 42, 80, 104). The multienzyme complex also carries out the degradation or removal of the docking strand which automatically pulls the newly added strands into the petidoglycan mesh (41, 42, 80, 104).

The multienzyme complex model also explains how cells control the activity of LTs (40-42). Studies have shown that over production of either soluble or membrane bound LTs does not result in increased lysis. For Slt70, a soluble LT, the interaction with other components is expected in the multienzyme complex to enhance its catalytic activity (40-42). For membrane bound LTs, like MltA, it has been suggested that their membrane anchor prevents the enzyme from reaching the peptidoglycan unless it is interacting with enzyme in the complex (40, 41). Interestingly, MltB overproduction does cause rapid lysis but only after it is cleaved and released from the

membrane. It is this activated form, called Slt35 which actually reaches and degrades the peptidoglycan, causing lysis (40-42).

The rod-shape of certain bacteria suggests the existence of two multienzyme complexes competing for elongation and cell division. Some cocci-shaped cells seem to only synthesize peptidoglycan at the divisional septum. In *E. coli*, two multienzyme complexes are found; one, which contains PBP2, is localized in the lateral wall and at the poles in a spot pattern distribution, PBP2 has been associated with cell elongation and cell elongation enzymes like MreB/C/D and RodA (10, 42, 80, 87). The second, where PBP3 substitutes for PBP2, is found at the division septum (42, 80). As part of the division septum machinery, PBP3 interacts with other division enzymes like FtsA, FtsL, FtsN, FtsQ, FtsW and FtsZ (10, 42, 56, 80). The Mre proteins and the Fts proteins are part of the prokaryote cytoskeleton discussed below.

1.9 CYTOSKELETON AND PEPTIDOGLYCAN SYNTHESIS

In bacteria the cytoskeleton is a group of structural proteins which run as filaments just underneath the cytoplasmic membrane and are responsible for cell shape in both gram positive and gram negative bacteria (10, 82). Their main role in cell shape also includes directing peptidoglycan synthesis, both in elongation and division (10, 114).

MreB is an actin homolog found in rod shaped bacteria (53, 80, 82). These bacteria turn spherical when there is a mutation in *mreB*. Consistent with this role, MreB homologs are not found in most cocci shaped bacteria, like Streptococci or Staphylococci (53). Although MreB only shares partial homology with actin, it folds in the characteristic form of the actin superfamily (10, 53, 82). MreB also contains the canonical actin ATPase domain (53, 82). *E. coli* and other bacteria contain only one

form of MreB, but other bacteria like *B. subtilis* contain multiple homologs to MreB, such as Mbl and MreBH (10, 53, 82).

In several bacteria MreB is found in the mreBCD operon, which has been shown to be essential for cell viability (53, 82). In rod shaped cells, mutations in this mreBCD operon can be recovered with slight over-expression of the divisional septum proteins (i.e. Fts proteins) (53, 82). MreC and MreD are membrane proteins which, based on protein interaction experiments, associate with MreB, forming the MreBCD complex (53, 80, 82). GFP tagging experiments show that these proteins are organized in a helical pattern located all throughout the cell and just underneath the cytoplasmic membrane (10, 53, 80). Co-localization experiments have demonstrated an association between the MreBCD complex and elongation specific PBPs, like PBP2 in *E. coli* (10, 53, 82). Also, it has been hypothesized that the MreBCD complex interacts with RodA, the peptidoglycan precursor translocase (10, 53, 80). All this evidence suggests that the MreBCD complex is responsible for cell width by directing peptidoglycan elongation (10, 80, 82). In support of this idea is the finding that the MreBCD complex does not participate in peptidoglycan constriction, since mutations in mreBCD operon have no effect on the localization of the division septum or division specific PBPs (PBP3 e.g.) in the cell (10, 80).

The other main role played by the bacterial cytoskeleton is the formation of the division septum, for this process the main player is FtsZ (10, 56, 80, 114). FtsZ is a protein that resembles tubulin in eukaryotic cells (10, 56, 80, 82). As in the case of the actin-like MreB, most bacterial FtsZ homologs have weak sequence identity with eukaryotic tubulin but share a similar 3D structure and the canonical GTP hydrolyzing motif found in tubulin (10, 82, 102).

FtsZ homologs are found in almost all bacteria and archea, its sequence is highly conserved supporting the notion of FtsZ‘s importance for cell viability (10, 56, 114). It

has been found that FtsZ, like tubulin in eukaryotes, is an essential gene and is required for cell division (80, 82). FtsZ forms protofilaments which polymerize in a synergistic manner, forming a ring at the equator of the cell that spans the circumference of the cell and is localized just underneath the cytoplasmic membrane (56, 80). The constriction of this structure, called the Z-ring, brings about the division of the bacterial cell, yielding two daughter cells (59, 82, 102). The Z-ring requires other proteins to carry out its function (56, 80, 102). In *E. coli*, the formation of the divisome requires the regulated recruiting by the Z-ring of at least 10 other interacting proteins (FtsA, FtsB, FtsI, FtsK, FtsL, FtsN, FtsQ, FtsW, ZipA and AmiC) (56, 59, 80, 82, 102, 114). Co-localization experiments have shown in *E. coli* that the division specific transpeptidase FtsI or PBP3 is part of this divisome (59, 80, 82). Inactivation of PBP3 has shown that PBP3 enzymatic activity is required for the constriction of the Z-ring (82). This evidence suggests that the peptidoglycan multienzyme complex containing PBP3 is responsible for the synthesis of peptidoglycan at the divisional septum (59, 82). As described previously, an amidase (AmiC) is required to separate the new daughter cells (80, 82).

1.10 PEPTIDOGLYCAN TURNOVER

Although peptidoglycan is responsible for the cell shape and bears the cytosolic turgor pressure, it is more than just a rigid structure (42, 80). Studies of peptidoglycan growth and metabolism have made clear the fact that it is a very dynamic structure; but this metabolism has to be highly regulated to avoid cell lysis (42, 80, 93). Peptidoglycan turnover, as a consequence of growth, is about 50% per generation in *E. coli* (41, 42, 62, 104). In *E. coli*, the products of peptidoglycan turnover are mainly 1,6-anhydromuramic acid containing residues, which suggests that turnover is a

consequence of LTs activity on peptidoglycan (42). These products accumulate in the periplasm and can be broken down further by the action of carboxypeptidases and amidases (AmiA and AmiC) (41-43). These breakdown products: NAG-anhNAM-peptide monomers, NAG-anhNAM disaccharide monomers, and peptides can be transported back into the cell by the muropeptide transporter (AmpG), the oligopeptide transport system (Opp) or the murein peptide permease (Mpp) (17, 41, 42, 62, 93). AmpG is an anhydro disaccharide monomer (NAG-anhNAM) specific permease that can also transport the anhydro disaccharide peptide (17).

In *E. coli* more than 90% of the turnover material is recycled back in to the peptidoglycan every generation, resulting in a loss of only 5% to 8% per generation. This is a stark contrast to *Bacillus* which losses up to 30% of its peptidoglycan every generation (17, 30, 41, 42, 62). In *E. coli* once the turnover products are transported into the cytoplasm they are further processed by anhNAM-specific amidase (AmpD), an N- cetylglucosaminidase (NagZ), and a L,D-carboxypeptidase (LdcA) to regenerate the peptidoglycan precursors (i.e NAG, NAM, pentapetide) which in turn will be recorporated into the cell wall (17, 41, 42, 93, 104). The existence of a specific ligase (Mpl) avoids the need for total degradation of the peptide bridges which can be reattached to newly synthesized UDP-NAM and then be recycled back to the peptidoglycan (42).

An interesting fact, which is often overlooked in gram negatives, is the obstacle that the peptidoglycan meshwork poses for the translocation of certain proteins to the outer membrane (22, 84). It has been calculated that the peptidoglycan structure has pores of about 5nm in diameter, which will allow the free passage of proteins up to 55 KDa (22, 42). Thus, it has been proposed that the peptidoglycan will have to undergo modifications or rearrangements that allow the translocation of larger proteins (22). In support of this, the operons encoding many surface organelles include type IV pili,

conjugative pili, and the pullulanase export machinery, whose biogenesis requires translocation of globular proteins that exceed 5 nm in diameter, encode peptidoglycan metabolism enzymes called specialized lytic transglycosylases (22, 103).

As a result of growth, turnover, recycling and other modifications the peptidoglycan profile of a cell changes as the cell ages. Peptidoglycan matures gradually as the cell enters non-growing or slow-growing phases, like stationary phase (65, 83, 96). HPLC derived peptidoglycan profiles show that cells undergo several changes throughout their life cycle. In *E. coli*, peptidoglycan cross-links increase nearly five fold as cells transition from exponential to stationary phase. This increase is most noticeable as an increase in the number of tripeptide to tripeptide bridges through m-A_2pm-m-A_2pm (29, 65, 83, 96). Also the number of cross links to Braun's lipoprotein increases while the average length of the glycan strand is reduced (29, 65, 83, 96). Based on the structure and function of the cell wall, these changes suggest a strengthening of the mechanical features of peptidoglycan and, in some bacteria, a change in cell shape, as the cells enter stationary phase (29, 65, 83, 96). As peptidoglycan matures cells become less sensitive to antimicrobial agents like β-lactams, this adaptation is known as phenotypic tolerance (29, 74, 96, 97).

1.11 PEPTIDOGLYCAN LYSIS PRODUCTS

Peptidoglycan breakdown products, due to lysis or normal metabolism, are a mixture of disaccharides, anhydrodisacharides and pentapeptides (42). Studies have suggested that these products play a role as secondary messengers in several bacterial processes (42, 44, 46). In gram negative bacteria, the products of lytic transglycosylases (anhNAM) are imported into the cytoplasm where they trigger the production of β-lactamases (42, 44). Studies on resuscitation-promoting factors

(Rfps), which bring bacteria back from a non-dividing state, have shown that peptidoglycan breakdown products are an essential signal for bacteria to leave this state of dormancy (46, 76).

The transcription of genes known to encode cell surface organelles has also been shown to respond to peptidoglycan breakdown products (1, 85). NAG-P has been shown to inhibit expression of *fimB*, part of the switch which controls expression of type fimbriae or type I pili (85, 86). These fimbriae are a proinflammatory adhesin that allow bacteria to anchor to specific host receptors and invade epithelial cells (85, 86). One host cell defense mechanism against pathogens elaborating fimbriae appears to be the release of NAG and other aminosugars that inhibit the expression of *fimB* gene (85, 86).

Curli, another adhesion fiber implicated in pathogenesis, has also been reported to be inhibited by intracellular NAG-P accumulation (1, 2). Curli are β-sheet, non-branching amyloid fibers produced by enteric bacteria (1, 2). Two operons are essential for curli formation *csgDEFG* and *csgBA*. *csgDEFG* encodes the outer membrane secretion protein CsgG. CsgE and CsgF are two accessory proteins which assist in CsgA and CsgB secretion and assembly; while CsgD is a positive regulator for the divergently transcribed *csgBA* operon (1, 2, 9, 67, 100). *csgA* encodes for the major subunit of the fiber while *csgB* encodes for the nucleator peptide responsible for polymerizing CsgA (1, 34, 35, 100). Both, CsgA and CsgB are secreted outside the cell. CsgB remains anchored to the outer membrane and directs the polymerization of CsgA which forms amyloid fibers (1, 34, 35, 100). Curli bind fibronectin found on the surface of epithelial cells and promote internalization of the bacterial cells (31, 32, 49). Curli are considered part of the pathogen-associated molecule pattern that activates the innate immune system since they are recognized by Toll-like receptors (1, 95, 100).

Curli are not only involved in cell-to-cell interaction, they have been shown to participate in cell-to-surface attachment and biofilm formation (16, 49, 101). Overproduction of curli endows *E. coli* with enhanced attachment allowing it to form robust biofilms, enhancing its capacity to colonize urethral catheters and contributing to its role as urinary tract pathogen (49, 68, 101).

1.12 BIOFILMS

Bacteria can attach to biotic and abiotic surfaces and grow in encased complex communities called biofilms (18, 19, 24, 33). A great number of bacterial species, if not the majority are prone to grow in biofilms (73, 109).

Biofilm cells are entrapped in a heterogeneous extracelluar polymeric substance (EPS) made of a complex mixture of polysaccharides, proteins and nucleic acids secreted by biofilm bacteria or persisting as remnants of previous cell lysis (24, 92, 109). The EPS assists in cell-to-cell adherence, protects bacteria from unfavorable environment conditions, and it also acts as a barrier against the diffusion of antimicrobial molecules (18, 19, 25). In *E. coli*, it has been shown that EPS is required for biofilm architecture (20, 109).

Planktonic cells are said to be in a different metabolic state than their biofilm counterparts. Even within a single biofilm, cells are in different metabolic states depending on their proximity to the surface (33, 52, 89). In general, the metabolic status of biofilms cells resembles that of non-growing, stationary phase or slow growing bacteria (19, 33, 73). Although some antimicrobial molecules are able to penetrate the EPS, these metabolic differences endow biofilm bacteria with an additional level of protection to antimicrobials, many of which only exert their effect on actively growing cells (19, 25, 90).

DNA microarrays have shown that biofilm development is a complex process, and gene expression is tightly regulated during biofilm formation and development (3, 33, 89, 110). The first step in biofilm formation is attachment (69). Extracellular appendages, like pili, flagella, fimbriae and curli mediate cell attachment to artificial or biological surfaces (33, 54, 66, 71, 101). Attachment is dependent on surface characteristics like electrostatic charge, hydrophobicity, and the presence of specific biological receptors (54, 71).

After initial cell-to-surface attachment, bacteria aggregate in microcolonies (33). Cell division and cell-to-cell interactions between microcolonies give rise to macrocolonies (33). Mature biofilms arise from a complex association of macrocolonies and are decorated with pillars and mushroom-like structures (33, 79). Between these structures nutrients, cell-to-cell signals and byproducts flow through water filled channels and tunnels (21, 33, 79). The last step in biofilm development is cell dispersion, where cells are released from the biofilm to resume their planktonic life style and are able to re-colonize new or adjacent surfaces (33, 73, 107).

1.13 CONCLUSION

In the following chapters we explore the implications of the defective lambdoid phage DLP12 in the physiology of its *E. coli* host. We studied how the DLP12 lysis genes seem to have been co-opted by *E. coli*, as they are actively transcribed from an *rpoE* dependent promoter. The regulation of this transcription also responds to a DLP12 Q antiterminator. We showed that although these genes are not essential for laboratory growth, they are active in early stationary phase and play a big role in RpoE-mediated cell lysis. In addition to lysis, we observed that the activity of these DLP12 genes affects peptidoglycan recycling and turnover during normal cell

division. Furthermore, the absence of these genes hinders *E. coli* biofilm formation via reduction of curli production.

Our results show that these adopted genes enhance *E. coli* fitness in two important aspects: One, they modify peptidoglycan to increase survival from antimicrobial agents, like detergents. And two, they play a part in the regulation of important virulence factors, like curli fibers. Importantly, both of these aspects require an accurate regulation of these otherwise lethal genes.

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CHAPTER 2

PHYSIOLOGICAL IMPLICATIONS OF DLP12 LYSIS OPERON IN *E. coli*

2.1 ABSTRACT

Part of the DLP12 region of *E. coli* encodes putative lambdoid phage lysis genes. In functional phage the products of similar genes would normally target peptidoglycan, being required for cell lysis and the release of viral progeny. Although the DLP12 prophage is defective, we show in this chapter that the genes encoding the DLP12 holin (S), lysozyme (R) and endopeptidase (Rz) are coordinately transcribed from their native promoter *essDp*. Overproduction of DLP12 genes caused increase cell lysis, suggesting that they encode functional proteins. GFP-promoter fusion assays suggest that the DLP12 promoter *essDp* responds to RpoE regulation and that the DLP12 lysis genes are required for RpoE mediated early stationary cell death. Deletion mutants for the DLP12 lysis genes showed an increased sensitivity to detergents when challenged with SDS, suggesting differences in peptidoglycan status in these mutants. When peptidoglycan metabolism was studied further we found that the DLP12 mutants incorporated less N-acetyl glucosamine into their peptidoglycan than the wild type (5-8 % less per generation than the wild type). Interestingly, the mutants released little or no peptidoglycan degradation products to the supernatant even though wild type *E. coli* normally loses between 5 and 8% of its peptidoglycan per generation. This reduction in precursor incorporation coupled with the lack degradation product release suggests that the mutants may be recycling more of their peptidoglycan than the wild type. Normally *E. coli* recycles 90% of it peptidoglycan degradation products, but it can only recycle anhydro-muropeptides formed by lytic transglycosyalses. We hypothesize that the DLP12 lyoszyme is responsible for the

production of nonrecyclable muropeptides although the physiological benefit of such an activity remains unclear.

2.2 INTRODUCTION

Bacterial genomes are adorned with "non species-specific" genetic elements, like transposons, insertion sequences and prophages (6, 37). Prophages have been found in more than 60% of sequenced bacteria genomes, and comprise between 10% and 20% of some bacterial genomes (8, 37). Functional prophages are temperate bacteriophages which have adopted a lysogenic life style. In lysogeny, phage DNA recombines with the host chromosome and adopts a dormant state (6). As a prophage, phage gene expression is minimal and the phage replicates along with the host chromosome (6). However, the majority of the prophage elements found in bacterial genomes are defective prophages; these prophages have lost the ability to re-enter the lytic life cycle and are trapped in the host cell (6, 8).

Due to the inability to reenter the lytic cycle, it has been suggested that defective prophages are in mutational decay and are expected to eventually be eliminated by random genetic drift (6, 8). Despite this assertion, some defective phage remnants are also found in close related bacteria which suggest a single evolutionary origin. Such is the case for the *E. coli* lambdoid prophage element DLP12 which is found in other enterics including *Salmonella* which diverged more than 100 million years ago (25). This long evolution history suggests that some phage remnants may confer an evolutionary advantage on their hosts such as increasing host fitness, survival, and virulence (8, 37). For example, the prophage lambda *rex* gene endows host cells with resistance to further infection from other phages (5) whereas two DLP12 genes, *bor* and *lom*, increase the pathogenicity of *E. coli* by encoding surface proteins which

interact with mammalian cells (7, 9, 41). In *P. aeruginosa*, lambda tail-like proteins, known as F-pyocins, are use as a weapon to perturb the membrane of closely related bacteria (17). These examples and the persistence of similar elements in bacterial genomes suggest that prophages, defective or not, play an important role in host physiology.

Four defective lambdoid prophages have been found in the *E. coli* genome: DLP12, Rac, e14 and Qin (5, 7). DLP12 (**D**efective **L**ambdoid **P**hage inserted in the **12** minute) is found as an 18.9 KB insertion near the *argU* tRNA gene (26) and is classified as a defective prophage because it is not able to excise from the chromosome due to mutations in key genes, like *xis* (5, 7). Lambdoid lysis genes similar to *SRRz* are also encoded by DLP12 (5, 26, 37). In functional lambda phage, *SRRz* encode a holin (*S, essD* or *ybcR*), an endolysin or lysozyme (*R* or *ybcS*), and endopeptidase (*Rz* or *ybcT*) (5, 6). Recent work has shown that the DLP12 lysis genes are actively transcribed from an RpoE dependent promoter (*essD*p) (34) and that *R* encodes a functional lysozyme (37).

Endolysins, like the lambdoid phage *R* protein, are essential for completing the lytic cycle and allow newly made phage to escape the cell by hydrolyzing the glycosidic bond between NAG-NAM; while endopeptidases, like *Rz*, cleave the peptide bridge which forms the crosslinks to the outer membrane (5, 6, 18, 39, 48). Although the glycosylase activity of the R endolysin is sufficient to cause cell lysis it has been suggested that the Rz endopeptidase activity is required in environments with an excess of Mg^{2+} cations (39, 48, 50). In canonical Lambda the holin S allows passage of R to the periplasm (5, 6, 46-48) whereas in Lambdoid phage 21 the holin depolarizes the membrane (14, 32, 33, 46) releasing R which has been tethered to the periplasmic side of the cytoplasmic membrane, and allowing it to attack the peptidoglycan (32, 44, 45). Given the similarity of DLP12 lysis genes to those from

λ^{21} and the fact that the lysozyme R encodes a functional protein; one wonders, why *E. coli* would keep such killer lysis genes around?

Holins similar to S have been implicated in programmed cell death and are known to be regulated in response to a variety of cell stresses (2). In *E. coli* RpoE directs the transcription of several genes in response to membrane stress and elevated levels of RpoE cause *E. coli* cell lysis in early stationary phase, although the mechanism behind this has never been established (23, 30, 34).

Given the transcriptional dependence of the DLP12 lysis cassette on RpoE, we hypothesized that these phage remnants might play a role in cell lysis. In the present study we report that over-expression of the DLP12 lysis genes increased cell lysis and that these genes are essential for RpoE mediated cell death. Our results also suggest that S^{DLP12} behaves like a pinholin and that R^{DLP12} mediated lysis is dependent on membrane potential status. Finally we show that that loss of the lysis genes affects cell wall integrity and alters normal peptidoglycan turnover in *E. coli*.

2.3 RESULTS AND DISCUSSION

Although purified R^{DLP12} was recently shown to encode a functional lysozyme *in-vitro* (37) nothing is known about its role during normal cell growth or about the role of the other proteins encoded by the lysis operon. When we knocked out either *S, R,* or Rz^{DLP12} we saw no significant effects on growth for the latter two genes, however, the entrance of the *S* mutant had delayed entrance into stationary phase (Figure 2.1**)**. The absence or limited affect on cell growth was not entirely surprising given that several studies have shown that loss of individual peptidoglycan hydrolases often has no direct effect on cell growth or peptidoglycan metabolism due to functional redundancy of a large number of related proteins (3, 20, 21, 36).

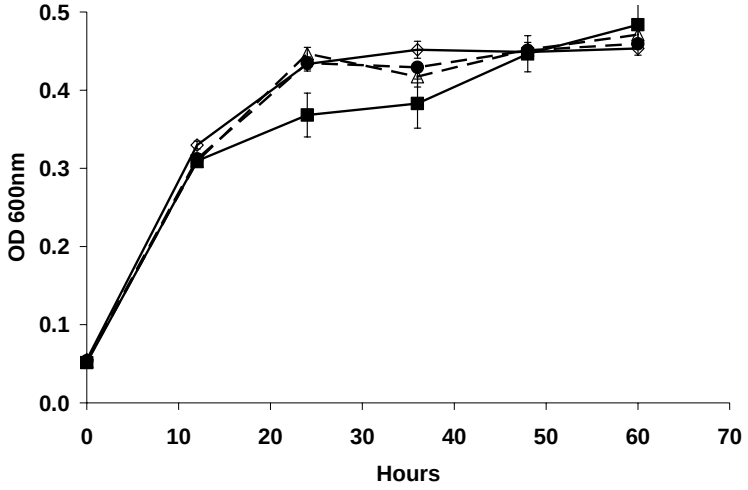

Figure 2.1: Growth curve: Overnight cultures were diluted in fresh LB and allowed to grow at 37°C. OD600 was monitored in a μQuant spectrophotometer. Experiments were done in triplicates. Wild type control PHL628 (◇), PHL628 ΔS^{DLP12} (■), PHL628 ΔR^{DLP12} (△), and PHL628 ΔRz^{DLP12} (●) show very similar growth pattern (α=0.05). DLP12 mutants are not affected for growth.

Despite no or limited effect on growth rate, deletion of the lysis genes did appear to affect cell wall status as evidenced by the response of the mutants to SDS, a detergent that has been frequently used to interrogate cell wall status (21, 43). Figure 2.2 shows that when exposed to LB supplemented with 5% SDS, the mutants lysed more than the wild type control strain. Complementation with wild type alleles restored the mutants to wild type levels (Figure 2.2).

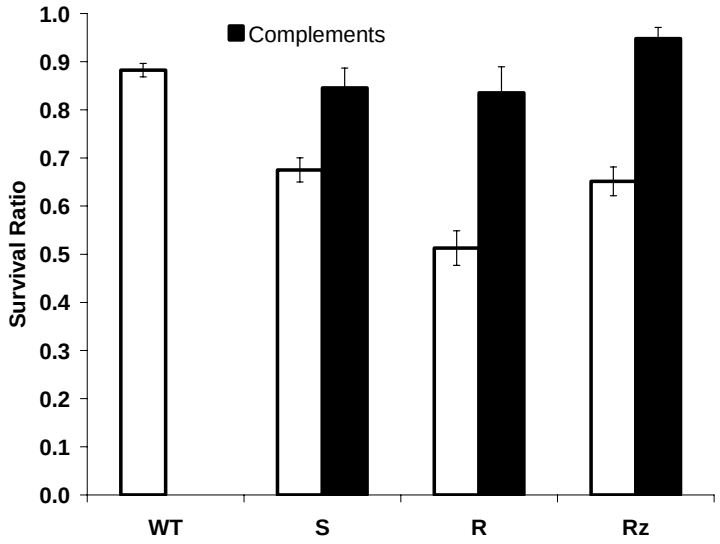

Figure 2.2: Membrane susceptibility to SDS: Overnight cultures were diluted 1:100 and challenged with 5% SDS for 3h at 37C. OD600 was measured and compared to unchallenged cultures. Experiments were done in quadruplicates. All mutants lost tolerance to SDS when compared to wild type control (α=0.05). Complements rescue the tolerance phenotype.

To explore cell wall status further we studied peptidoglycan metabolism and found that the mutants were compromised in their ability to incorporate ^{14}C N-acetylglucosamine (NAG) into peptidoglycan. In general, mutants only incorporated around 50% as much labeled NAG as the wild type *E. coli* (Figure 2.3). This was not due to either reduced NAG uptake or enhanced NAG catabolism as the R^{DLP12} mutant actually transported NAG into the cell at a faster rate than the wild-type (Figure 2.4), but metabolized it at approximately the same rate (Figure 2.5)

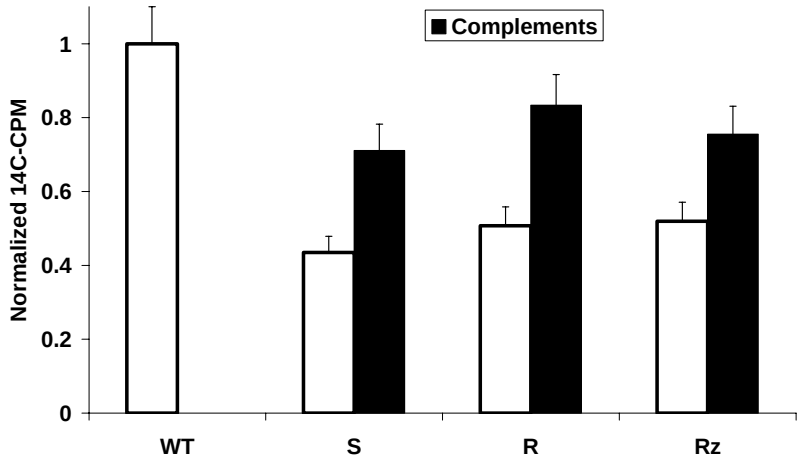

Figure 2.3: ^{14}C labeled NAG incorporation: Cells were grown in the presence of ^{14}C label NAG. After 12 hours, cells were boiled, filtered, and assessed for counts per minutes in a Beckman Coulter® LS 6500 scintillation counter. Experiments were done in triplicates. All mutants; PHL628 ΔS^{DLP12} (S),, PHL628 ΔS^{DLP12} (R) and PHL628 ΔS^{DLP12} (Rz) were significantly affected in incorporation when compared to PHL628 wild type control (WT) (α=0.05). Complements (black bars) partially recover the incorporation phenotype.

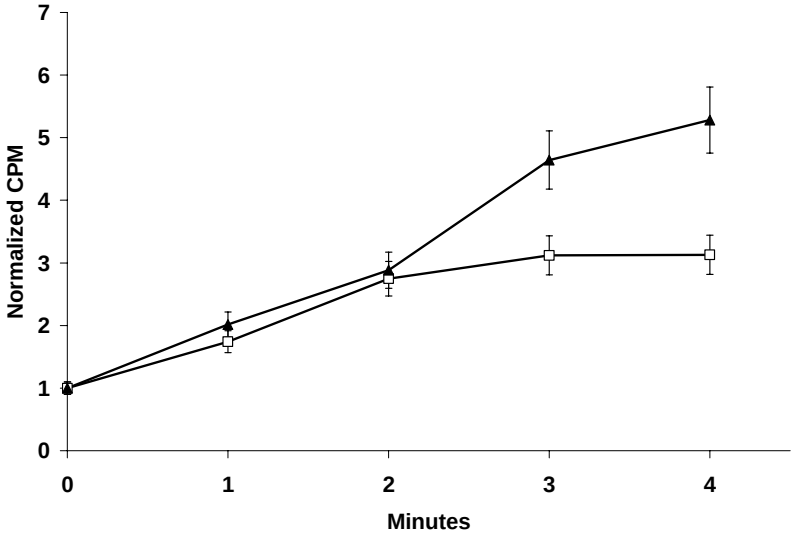

Figure 2.4: ^{14}C labeled NAG uptake: Cells were grown overnight at 30^{o}C in Media A. Cells were diluted in fresh Media A supplemented with ^{14}C label NAG. During 4 minutes, cells were filtered and washed with fresh media supplemented with unlabeled NAG. Filters were assessed for counts per minutes in a Beckman Coulter® LS 6500 scintillation counter. Experiments were done in triplicates. R^{DLP12} mutant (▲) showed significant increase in NAG uptake when compared to wild type control (□) (α=0.05). Data normalized against CPM at time zero.

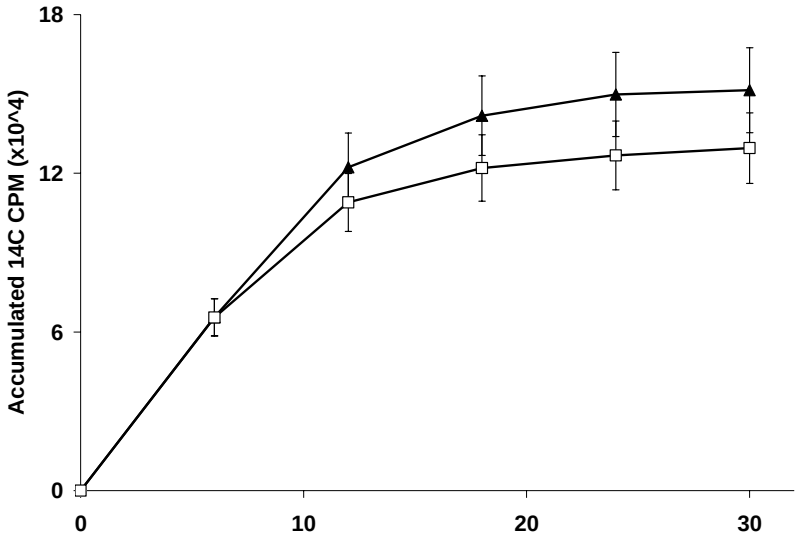

Figure 2.5: ^{14}C labeled NAG mineralization: Cells were grown overnight at 30^{o}C in Media A. Cells were diluted in fresh Media A supplemented with ^{14}C label NAG and a NaOH trap was set up. After 30 hours, NaOH traps were assessed for counts per minutes in a Beckman Coulter® LS 6500 scintillation counter. Experiments were done in triplicates. R^{DLP12} mutant (▲) showed no statistical difference in NAG mineralization when compared to wild type control (□) (α=0.05).

Since the incorporation assay could distinguish between deficits in the synthesis of new peptidoglycan or failure to release soluble peptidoglycan degradation products, we also assessed label release from the peptidoglycan. Surprisingly, neither the S^{DLP12} nor the R^{DLP12} mutant lost any ^{14}C label from their peptidoglycan over 15 h (Figure 2.6). This contrasted markedly with the wild-type which lost more that 60% of its labeled NAG in the same time frame, averaging approximately 5% of the ^{14}C label loss per generation (data not shown), which is consistent with previous reports of peptidoglycan turnover in *E. coli* (15, 20). Although the Rz^{DlP12} mutant did show some peptidoglycan turnover, it was still statistically less than that of the wild type ($P<0.05$). In the case of both incorporation and release, complementation restored the mutants to near wild type levels.

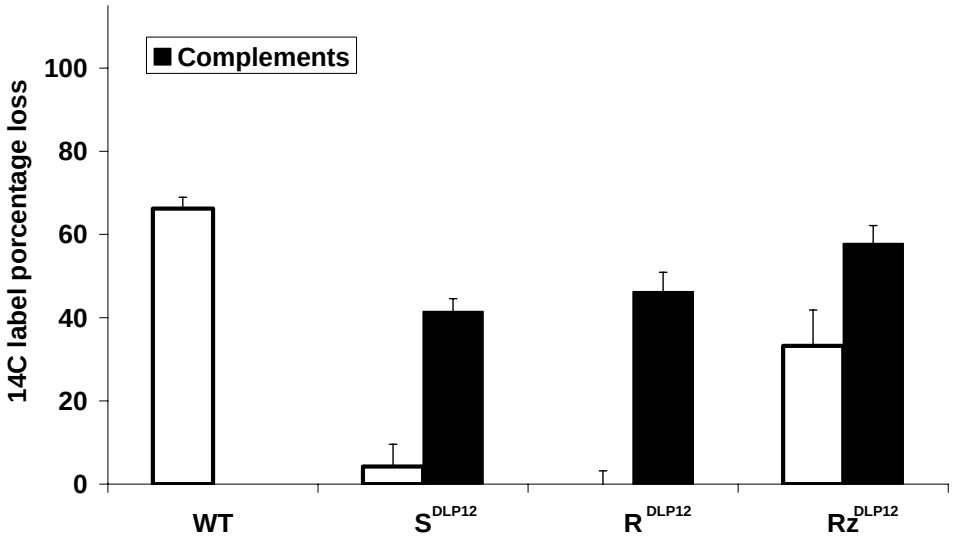

Figure 2.6: Peptidoglycan turnover: Cells were grown in the presence of ^{14}C labeled NAG. After 12 hours, cells were washed and diluted in fresh media with unlabeled NAG and allowed to grow. 15 hours after re-incubation, cells were boiled, filtered, and assessed for counts per minutes in a Beckman Coulter® LS 6500 scintillation counter. Experiments were done in triplicate. All mutants were affected for turnover when compared to wild type control. ΔS^{DLP12} and ΔR^{DLP12} showed no loss of label; although ΔRz^{DLP12} showed loss of label this was still significantly less than wild type control (α=0.05). Complements partially recover the turnover phenotype.

Retention of label within the peptidoglycan could mean that either peptidoglycan recycling was not occurring or that peptidoglycan by-products were being recycled with enhanced efficiency (Figure 2.7). Given that microscopic observation showed the mutants to have normal morphology (data not shown) and that inhibition of peptidoglycan turnover alters cell morphology (4, 20, 36), it seems likely that peptidoglycan recycling was enhanced in the mutants. This would also explain the reduced label incorporation by the mutants, since less new peptidoglycan precursors would have to be imported if recycling was more efficient (Figure 2.7).

E. coli normally turns over approximately 50% of its peptidoglycan per generation, but it is very efficient at recycling the released muropeptides, reincorporating 90% of what was released (Figure 2.7) (10, 15, 19, 20, 31).

Anhydromuropeptides (NAG-anhNAM(peptide)) are produced by periplasmic lytic transglycosylases and are transported back into the cell by AmpG (22) where they are recycled and incorporated back to the peptidoglycan (Figure 2.7) (10, 19, 20, 31, 40). There is no known mechanism for recycling non-anhydromuropeptides (NAG-NAM(peptide)) (20, 36), the predicted peptidoglycan hydrolysis products of R^{DLP12}. Therefore these would normally diffuse into the extracellular milieu (Figure 2.7). The most parsimonious explanation for our observations is that the DLP12 lytic proteins liberate muropeptides from the peptidoglycan that cannot be recycled and are lost (or diffused) to the environment, although the physiological importance of this activity remains unclear.

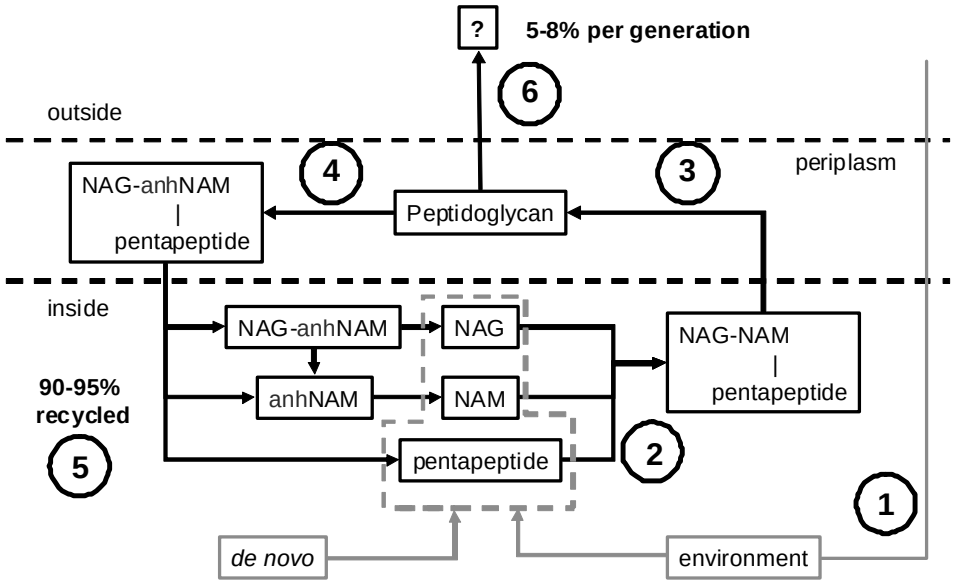

Figure 2.7: Peptidoglycan metabolism: Peptidoglycan building blocks (in dashed box) are synthesized de novo or are taken from the environment (1). Building blocks are joined together to form the peptidoglycan monomers (2). Translocated monomers are incorporated into the peptidoglycan mesh (3). Peptidoglycan growth, modifications and maturation releases monomers (4). Released monomers (90-95%) are recycled back to replenish the pool of building blocks (5). Per generation, during peptidoglycan metabolism, a fraction of the monomers (5-8%) are not recycled or are lost (released to the environment) (6).

In contrast to the subtle effects observed in the deletion mutants, over-expression of each gene individually had dramatic effects that resulted in cell lysis. Cell debris accumulation was observed in the bottom of the well when R^{DLP12} was over-expressed and was quantified as an apparent increase in absorbance by our bottom reading spectrophotometer (Figure 2.8A and Figure 2.9). Using a side reader, to avoid the precipitating cell debris, we confirmed the previous interpretation that over expression of DLP12 lysis genes results in increased cell lysis (Figure 2.8B). Consistent with the changes in absorbance, over-expression of any of the individual lysis genes caused a decrease in colony forming units (CFUs) (Figure 2.10) and a 10-100 fold increase in the release of cytosolic β-galactosidase activity into the supernatant (Figure 2.11).

R^{DLP12} over-expression caused a two thirds reduction in CFUs at 12 hours (Figure 2.10**)**. As observed in Figure 2.10, the CFU reduction was even more dramatic in the strain over-expressing S^{DLP12}. While Rz^{DLP12} over-expression was also significantly reduced in CFUs (α=0.05) over-expression of this gene affected CFUs the least (Figure 2.10). This is consistent with the suggestion that lambda Rz homologs are not essential for on cell lysis except when high concentrations of cations are present in the environment (48-50).

Over-expression of R^{DLP12} in a ΔS^{DLP12} background did not cause increased lysis (Figure 2.9). However, the lysis effect of R^{DLP12} over-expression could be rescued by exposing cultures to 2,4-Dinitrophenol (DNP) (Figure 2.11). Although the lysis shown in Figure 2.11 is not as saltatory as that reported for other Holin-Endolysin systems encoded by lambdoid phages (46) probably because of lower expression levels, the DNP dependence is consistent with S^{DLP12} encoding a "pinholin" like the one found in the lambdoid phage 21 (32).

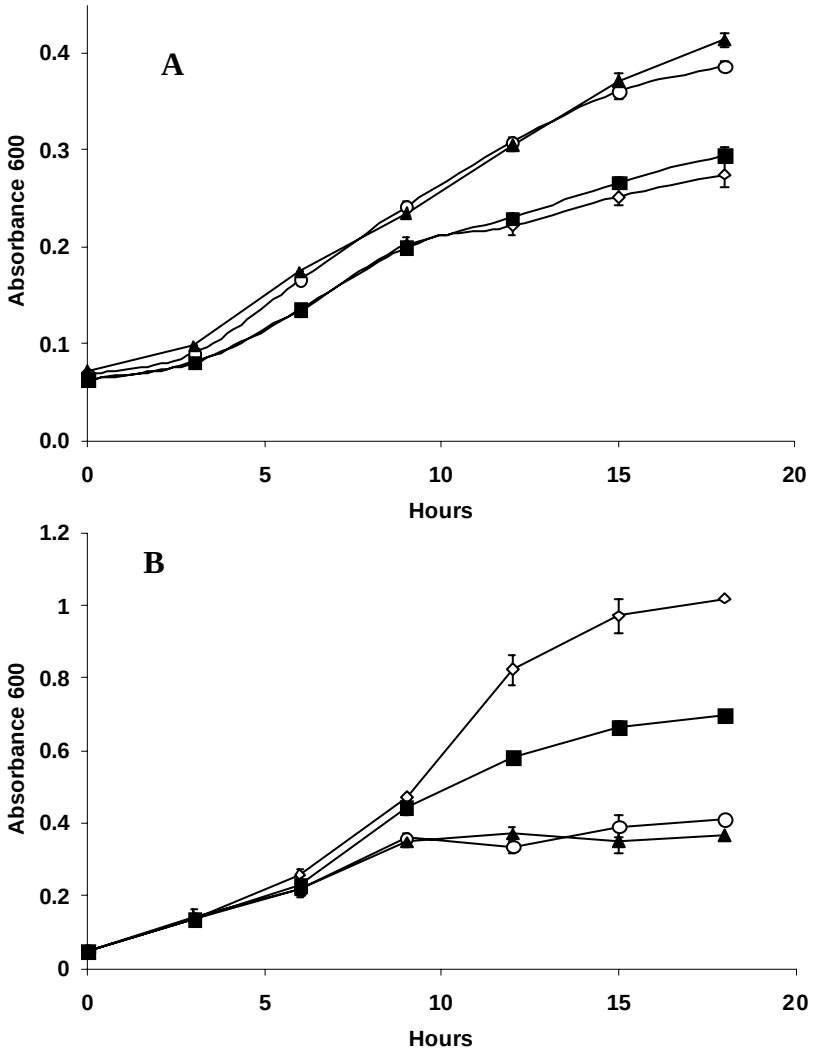

Figure 2.8: Over-expression of S^{DLP12}, R^{DLP12} and Rz^{DLP12}: Overnight cultures were diluted in fresh LB and allowed to grow at 37°C. OD600 was monitored in a μQuant bottom reader spectrophotometer (A) and a Bechman side reader (B). Experiments were done in triplicates. Interestingly, PHL628 pBBR1MCS:S^{DLP12} (▲) and pBBR1MCS:R^{DLP12} (●) showed an abnormal OD600 increase when compared to the wild type control PHL628 (◆) (α=0.05). pBBR1MCS:Rz^{DLP12} (■) had only a miniscule increase compared to wild type control PHL628 (◆) (α=0.05). This increase in OD600 was interpreted as $SRRz^{DLP12}$ induced cell lysis based on subsequent results (See below).

During phage mediated lysis, holins permeabilize the cytoplasmic membrane causing collapse of the membrane potential and in case of S^{λ}, also allowing endolysins to reach the periplasm (32, 44, 45). Other holins, however, like those found in phage

21, do not permit transport of proteins into the periplasm (32), but, due to their small size (less than 8KDa), induce lysis by perturbing the membrane potential which releases and activates the anchored R-endolysin, ultimately causing cell lysis (32, 46). DLP12 and phage 21 lysis genes share identical operon organization and high homology. The predicted amino acid sequence of S^{DLP12} is 85% identical to that of S^{21}. This high degree of homology, coupled with the dependence on DNP for lysis in the S^{DLP12} knockout suggests that S^{DLP12} behaves like a pinholin.

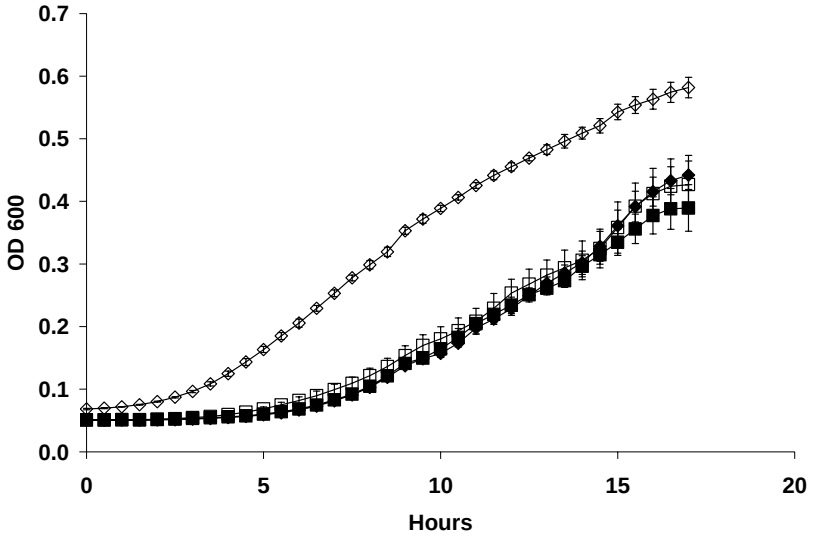

Figure 2.9: Over-expression of R^{DLP12}: Overnight cultures were diluted in fresh LB and allowed to grow at 37°C. OD600 was monitored in a μQuant spectrophotometer. Experiments were done in triplicates. Interestingly, PHL628 pBBR1MCS:R^{DLP12} (◇) showed an abnormal OD600 increase while wild type control PHL628 (◆), PHL628 ΔS^{DLP12} (■), and PHL628 ΔS^{DLP12} pBBR1MCS:R^{DLP12} (□), showed a very similar growth patter (α=0.05). This increase in OD600 was interpreted as R^{DLP12} induced cell lysis based on subsequent results.

Indirect evidence of such a pinholin function comes from analysis of the cognate endolysin of DLP12 whose predicted amino acid sequence is 97% identical to that of R^{21}. Further *in-silico* analysis has revealed that the predicted amino acid sequence of R^{DLP12} encodes a putative signal activity release (SAR) domain (37, 44, 45). In phage

21, a similar SAR sequence targets the nascent peptide for export by the *sec* translocon, however, unlike canonical leader peptides, the SAR sequence is not cleaved and the endolysin remains anchored to the inner-membrane until membrane potential perturbations release it, allowing it to be become active and gain access to the peptidoglycan (35, 44, 45). Our evidence that R^{DLP12} over-expression in an S^{DLP12} mutant causes lysis only after exposure to DNP demonstrates that R^{DPL12} transport is not dependent upon S^{DLP12} and, consistent with a SAR regulated endolysin, R^{DLP12} release and activation requires membrane perturbation (Figure 2.12).

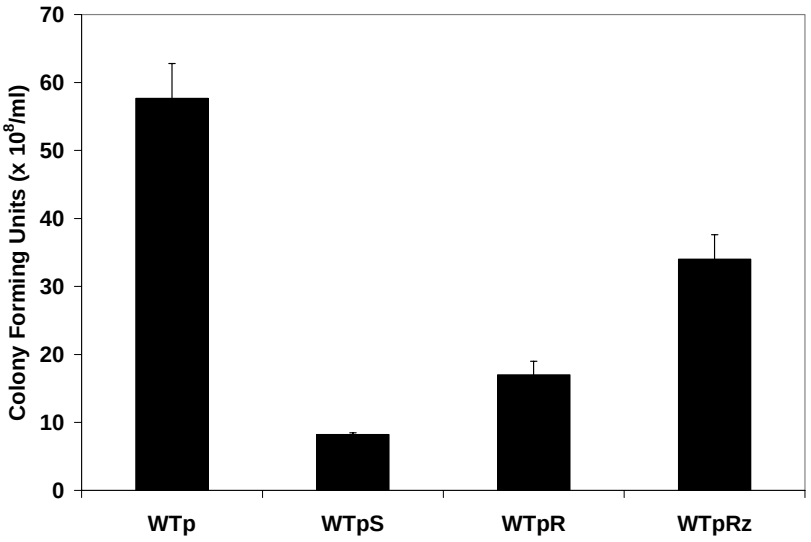

Figure 2.10: Colony Forming Units: Overnight cultures were diluted in fresh LB and allowed to grow at 37°C. At 12 hours, samples of each culture were plated on LB for colony counts. Experiments were done in triplicates. Over-expressing strains: PHL628 pBBR1MCS:S^{DLP12} (WTpS), PHL628 pBBR1MCS:R^{DLP12} (WTpR), and PHL628 pBBR1MCS:Rz^{DLP12} (WTpRz) showed a significant decrease in CFUs when compared to the wild type control strain (WTp) (α=0.05).

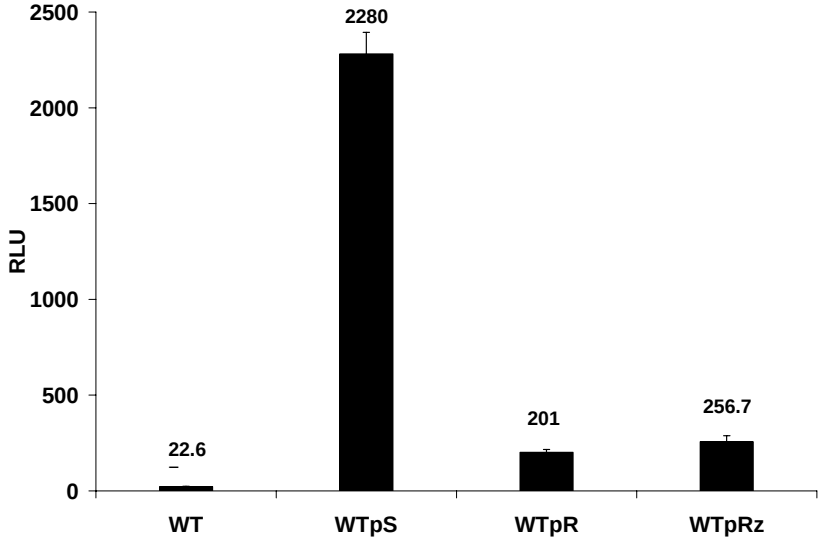

Figure 2.11: β-galactosidase activity: Overnight cultures were diluted in fresh LB and allowed to grow at 37°C. At 36 hours, samples of cell free supernatant of each culture were assayed for β-galactosidase. Galacton plus produces light when cleaved by β-galactosidase. Relative light units (RLU) were measured with a Packard Luminometer. Over-expressing strains: PHL628 pBBR1MCS:S^{DLP12} (WTpS), PHL628 pBBR1MCS:R^{DLP12} (WTpR), and PHL628 pBBR1MCS:Rz^{DLP12} (WTpRz) showed a significant increase in RLUs when compared to the wild type control strain (WTp) (α=0.05).

So what evolutionary advantage is to be gained by *E. coli* from keeping these potentially lethal lysis proteins? The impacts on peptidoglycan modification described above yield no clear insights into this matter. Is it possible that these genes encode some other specialized function that enhances *E. coli* fitness? Holin/endolysin combinations have recently been shown to be important in bacterial programmed cell death, this apparently important evolutionarily conserved trait is only beginning to be understood (2). Given the predicted function of DLP12 lysis proteins, we hypothesized that the products of $SRRz^{DLP12}$ might help mediate programmed cell death.

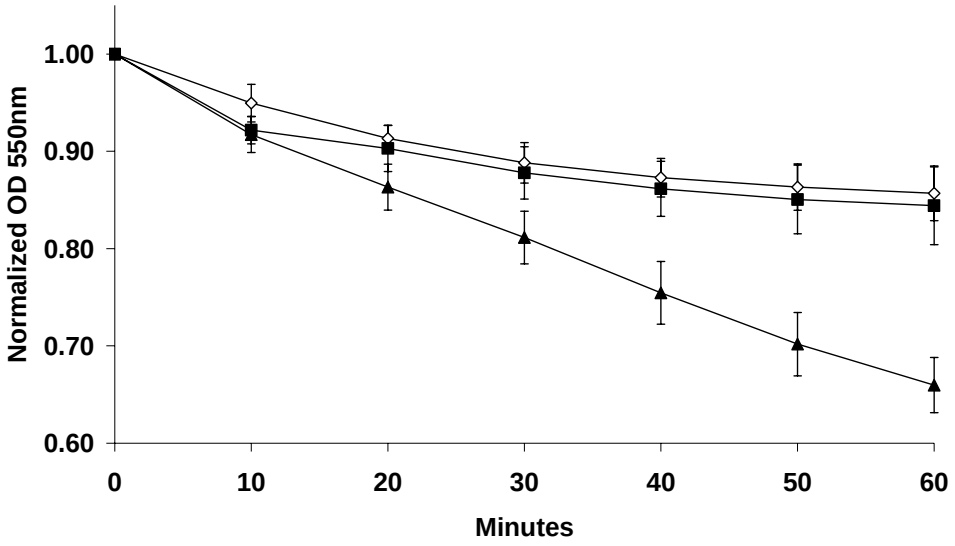

Figure 2.12: Membrane Depolarization: Exposure to the uncoupler 2,4-dinitrophenol (DNP, an uncoupler) was required for lysis in PHL628 ΔS^{DLP12} background. Experiments were done in triplicates. While the wild type control PHL628 (◇) and PHL628 ΔS^{DLP12} (■) were affected by DNP in a similar way, PHL628 ΔS^{DLP12} pBBR1MCS:R^{DLP12} (▲) was the most affected by DNP (α=0.05). R^{DLP12} mediated lysis is dependent on the membrane potential.

Such a function must be tightly controlled in order to ensure survival of the cell under normal conditions. Rhodius et al. previously predicted that the $SRRz^{DLP12}$ operon was regulated by the cell envelope stress sigma factor RpoE (34). We confirmed those results *in-vivo* by monitoring green fluorescence from a transcription fusion between *essDp* and GFP in *E. coli* harboring *rpoE* under the control of an arabinose dependent promoter (23). Figure 2.13 demonstrates that arabinose mediated *rpoE* induction resulted in significantly increased transcription from *essDp.*

We then sought to determine if over expression of the DLP12 lysis operon could account for the significant increase in cell lysis reported to occur when RpoE is over-expressed (23). Arabinose induced overexpression of RpoE resulted in early stationary phase lysis in wild type *E. coli* as expected, but not in either the R^{DLP12} or S^{DLP12} mutant (Figure 2.14). Lysis was only minimally affected in the Rz^{DLP12} mutant.

Complementation of the mutants restored the early stationary cell lysis phenotype to near wild type levels (Figure 2.14). Our results clearly demonstrate that S^{DLP12} and R^{DLP12} are required for early stationary phase lysis at high levels of RpoE. Given these observations and the importance of RpoE in regulating reponses to cell envelope stress, it is possible that the DLP12 lytic proteins may play a role in programmed cell death.

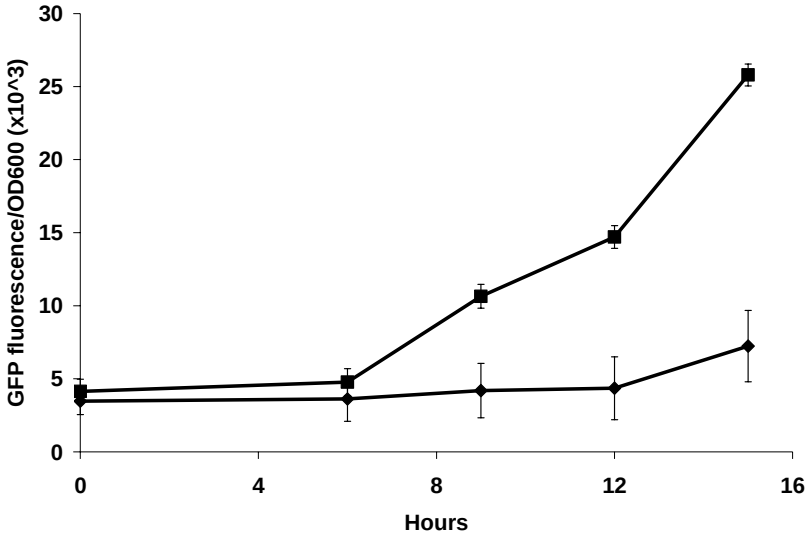

Figure 2.13: RpoE-mediated *essD*p transcription: pFAT1 (pJBA110:*essD*p) was used to assess the RpoE-mediated *essD*p transcription. Overnight cultures were diluted in fresh low salt LB, supplemented with arabinose for *rpoE* induction, and allowed to grow at 30°C. Fluorescence was read using a Bio-Tek Synergy HT spectrophotometer with a 485/20 excitation filter and a 530/25 emission filter and corrected with the OD600 of the corresponding culture. Experiments were done in quadruplicates. A steady increasing GFP signal was observed starting at 6 hours after *rpoE* induction (■), while no induction of *rpoE* only register a minor change at 12h (◆).

Results presented here strongly suggest that the lysozyme (R^{DLP12}) and holin (S^{DLP12}) encoded by DLP12 have been co-opted by *E. coli* and play a significant role in peptidoglycan integrity and metabolism. S^{DLP12} has all the features of a pinholin (32), which appears to destabilize the cytoplasmic membrane thereby facilitating release and activation of SAR (45) bound R^{DLP12}, a lysozyme with demonstrated

ability to degrade peptidoglycan (37). Our transcriptional reporter experiments confirmed predictions that the *SRRz*^{DLP12} operon is under the transcriptional control of the cell envelope stress sigma factor RpoE (34). Over expression of RpoE in *R*^{DLP12} and *S*^{DLP12} mutant backgrounds demonstrated that previously reported RpoE-induced cell lysis is mediated by *SRRz*^{DLP12} in *E. coli* (23). All this evidence suggests that the *E. coli* host has taken control of the *SRRz*^{DLP12} operon and the regulated expression of these lysis genes has a physiological role in *E. coli*.

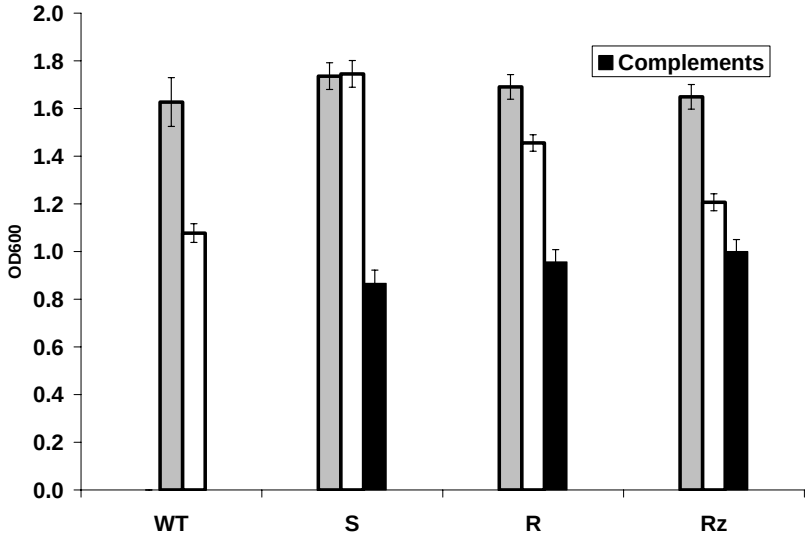

Figure 2.14: RpoE induced lysis: Overnight cultures were diluted in fresh low salt LB, allowed to grow at 30^{o}C, and *rpoE* was induced as previously described (23). At 36 hours cultures were sampled for OD600. Experiments were done in triplicate. Mutants over-expressing *rpoE* showed less lysis than expected. Δ*S*^{DLP12} and Δ*R*^{DLP12} were resistant to RpoE-induced lysis (white bars), and showed comparable growth (α=0.05) to wild type control strain not over-expressing *rpoE* (grey bars). In contrast, Δ*Rz*^{DLP12} only showed minimal difference when compared to wild type control (white bars). When compared with wild type over-expressing *rpoE* (white bar), the expected lysis phenotype was recovered in all the complements (black bars).

2.4 METHODS

Bacterial strains and growth conditions

E. coli strain PHL628 is a MG1655 derivative with an ompR234 mutation (42). *E. coli* PHL628 and mutants were routinely grown on Luria Bertani (LB) media at 37^{o}C overnight with shaking (150 rpm) and supplemented with 50 ul/ml Kanamycin (Kan) unless otherwise stated. For early stationary phase lysis assay and promoter studies low salt LB (5 g/l NaCl) media was used and incubated at 37^{o}C. In ^{14}C labeled N-acetylglucosamine (NAG) incorporation assays *E. coli* PHL628 and mutants were grown on minimal media (MSM) (28) supplemented with casamino acid (0.2%) and 50μl/ml Kan unless otherwise stated. When required, plates and media were supplemented with Ampicillin (Amp 150μl/ml) and/or Chloramphenicol (25μg/ml). For induction, media was supplemented with 1% lactose and 1% arabinose according to the experimental needs.

Mutants and plasmids construction

Deletion mutants were constructed by allele replacement using Lambda RED strategy as described elsewhere (12). Briefly, Chloramphenicol (Cm) interrupted version of the genes were created using PCR-mediated ligation (11). In general, each construct consisted of a 5'and a 3' 500-bp homology region to the gene of interest link by a 1kb Cm cassette flanked by flip recombinase target (FTP) sites. This linear DNA was transformed into wild type *E. coli* PHL628 cell expressing the Lambda RED recombinase. The 5' end and 3'end homology regions allowed RED system to replace the wild type allele with our interrupted version. Clone screening was carried out on

Table 2.1: Primers, plasmids and bacterial strains.

Name	Sequence	Reference
Primers		
S^{DLP12} Forward1	5'-GGGCCCACTTCGTCACACAGCTTAAACCCG-3'	This study
S^{DLP12} Reverse1	5'-GGATCCTGGCGACAGTGGCAAGGTCTTTAT-3'	This study
R^{DLP12} Forward1	5'-GGGCCCTGCGAGAGGTGAATAATGCCTCCA-3'	This study
R^{DLP12} Reverse1	5'-GGATCCTTCGCTAGCTTCAGTTCTCTGGCA-3'	This study
Rz^{DLP12} Forward1	5'-GGGCCCACTCGTCGTGAGATTGAGCGTGAA-3'	This study
Rz^{DLP12} Reverse1	5'-GGATCCGGAAGCGCGTGTGTATTGCTCAAA-3'	This study
essD p Forward1	5'-GGTACCTGCAGCTACAGCAGAAATTGCCAC-3'	This study
essD p Reverse1	5'-TCTAGAGCGACACCCGTTGTTAACTTATCC-3'	This study
S^{DLP12} internal Reverse	5'-GCGACACCCGTTGTTAACTTATCC-3'	This study
S^{DLP12} internal Forward	5'-TGCGAGAGGTGAATAATGCCTCCA-3'	This study
R^{DLP12} internal Reverse	5'-ACACCTTCCAGACCATCGTTACCA-3'	This study
R^{DLP12} internal Forward	5'-ACTCGTCGTGAGATTGAGCGTGAA-3'	This study
Rz^{DLP12} internal Reverse	5'-ATGACAGGCTGACGATGATGCAGA-3'	This study
Rz^{DLP12} internal Forward	5'-AGCAGTGCAGATAGAGCTGACCAT-3'	This study
P1S	5'-GGATAAGTTAACAACGGGTGTCGCGTGTAGGCTGGAGCTGCTTCG-3'	This study
P2S	5'-TGGAGGCATTATTCACCTCTCGCACATATGAATATCCTCCTTA-3'	This study
P1R	5'-TGGTAACGATGGTCTGGAAGGTGTGTGTAGGCTGGAGCTGCTTCG-3'	This study
P2R	5'-TTCACGCTCAATCTCACGACGAGTCATATGAATATCCTCCTTA-3'	This study
P1Rz	5'-TCTGCATCATCGTCAGCCTGTCATGTGTAGGCTGGAGCTGCTTCG-3'	This study
P2Rz	5'-ATGGTCAGCTCTATCTGCACTGCTCATATGAATATCCTCCTTA-3'	This study
Plasmids		
pBAD24		Guzman, 1995
pBAD24:*rpoE*		Kahbir, 2005
pBBAD18T		Sukchawalit, 1999
pBBAD18T:*rpoE*		This study
pBBRMCS		Kovach, 1995
pBBRMCS:S^{DLP12}		This study
pBBRMCS:R^{DLP13}		This study
pBBRMCS:Rz^{DLP14}		This study
pJBA110		Andersen, 1998
pJBA110:*essD* p		This study
pCP20		Datsenko, 2000
pKD3		Datsenko, 2000
pKD46		Datsenko, 2000
Strains		
E. coli PHL628		Vidal, 1998
E. coli PHL628 ΔS^{DLP12}		This study
E. coli PHL628 ΔR^{DLP12}		This study
E. coli PHL628 ΔRz^{DLP13}		This study
E. coli PHL628 ΔS^{DLP12} pBBR1MCS:S^{DLP12}		This study
E. coli PHL628 ΔR^{DLP12} pBBR1MCS:R^{DLP12}		This study
E. coli PHL628 ΔRz^{DLP12} pBBR1MCS:Rz^{DLP12}		This study
E. coli PHL628 pBAD24:*rpoE*		This study
E. coli PHL628 ΔS^{DLP12} pBAD24:rpoE		This study
E. coli PHL628 ΔR^{DLP12} pBAD24:rpoE		This study
E. coli PHL628 ΔRz^{DLP12} pBAD24:rpoE		This study
E. coli PHL628 ΔS^{DLP12} pBBR1MCS:$S^{DLP12\ pBAD24:rpoE}$		This study
E. coli PHL628 ΔR^{DLP12} pBBR1MCS:$R^{DLP12\ pBAD24:rpoE}$		This study
E. coli PHL628 ΔRz^{DLP12} pBBR1MCS:$Rz^{DLP12\ pBAD24:rpoE}$		This study
E. coli PHL628 ΔS^{DLP12} pBBR1MCS:R		This study
E. coli PHL628 ΔS^{DLP12} pJBA110:*essD* p		This study
E. coli PHL628 ΔS^{DLP12} pBBAD18T:rpoE pJBA110:*essD* p		This study
E. coli PHL628 pBBR1MCS:S^{DLP12}		This study
E. coli PHL628 pBBR1MCS:R^{DLP12}		This study
E. coli PHL628 pBBR1MCS:Rz^{DLP12}		This study

plates supplemented with the appropriate antibiotic. After confirming recombination, the Cm marker was removed using FLP-recombinase from plasmid pCP20 (12). All plasmid vectors are temperature sensitive and were cured from the cells by growth at 43ºC. The markerless knockout mutants were confirmed by PCR and sequencing.

Complements were constructed using full wild type version of the genes cloned into pBBR1MCS (lactose inducible) (24).

For *rpoE* over-expression, pBAD24:*rpoE* plasmid was obtained from Dr. Yamada (23). In this plasmid, *rpoE* expression is under control of an arabinose inducible promoter. An empty version of pBAD24 was used as control vector (16). To circumvent same antibiotic selection markers in the promoter experiments (see below), *rpoE* was also cloned into the expression vector pBBAD18T (38), also under the control of the pBAD promoter.

For promoter expression, the promoter-less *gfp* vector pJBA110 was used (1). Plasmid pFAT1 was constructed by cloning the DLP12 lysis gene promoter, *essDp*, upstream of the *gfp* gene in pJBA110 using KpnI and XbaI restriction sites (1).

Wild type control strains and mutant control strains were constructed by transformation of empty vectors as required per experiment.

Growth curve, colony counts, β-galactosidase activity and membrane perturbation

Overnight LB cultures were diluted 1:100 into fresh media. The new cultures were grown at 30^{o}C for 24 hours in 96-well plates. OD600 was measured every 30 minutes in a μQant spectrophotometer (Bio-Tek Company Info). To assess the actual growth of the different strains, media samples were taken during growth curve to perform colony counts and assess β-galactosidase activity in the supernatant.

Culture samples were obtained at 12h for Colony Forming Units (CFUs) determination. CFUs were counted on LB plates supplemented with the correct antibiotic. At the same time, β-galactosidase activity was determined in the cell-free supernatant using the luminescent substrate Galacton-Plus (Tropix Applied Biosystems) following manufacturers instructions. The light emitted in each sample was read in a Packard lummicount (Packard part of GMI, Inc.).

To perturb the membrane potential, a final concentration of 10mM 2,4-dinitrophenol was added to the media after 12 hours of growth, cell lysis was monitored by OD600 in a μQant spectrophotometer (Bio-Tek Company Info). All experiments were conducted in triplicate.

Membrane susceptibility to SDS

Cells were challenged with an inhibitory concentration of SDS to study their membrane susceptibility (21). Overnight LB cultures of the mutants were diluted 1:100 (OD600=0.05) and exposed to 5% SDS. Then, cultures were incubated with shaking for 3h at 37C and OD600 was measured. Absorbance was compared to unchallenged cultures incubated under the same conditions.

^{14}C-NAG Protocol for turnover

Peptidoglycan turnover was measured using a modification of a previously described method (13). Briefly, overnight LB cultures were diluted 1:100 on Media A. ^{14}C labeled NAG (0.2μCi) and unlabeled NAG (0.05%) were added to the growth media. To allow peptidoglycan labeling, cell cultures were grown at 30^{o}C for 12 hours. Then, labeled cells were washed 3 times with fresh Media A; and diluted 1:4 in media supplemented with unlabeled NAG (0.05%). Cells were incubated for an

additional 15 hours. Then, samples were boiled in 4% SDS and filtered through 0.2 μm Durapore® filter (Millipore®), Filters were washed once with LiCl buffer and three times with milliQ water. Then, filters were placed in scintillation cocktail, ScentiSafe® Econo 1 (Fisher Scientific®) and read in Beckman Coulter® LS 6500 scintillation counter.

^{14}C labeled NAG uptake

Substrate uptake protocol was based on a previously described method (29). Briefly, overnight LB cultures were diluted 1:10 on Media A and allowed to grow overnight at 30^{o}C. Cells were washed with phosphate buffer and diluted 1:1 in fresh Media A supplemented with ^{14}C labeled NAG. Cell cultures were allowed to uptake ^{14}C labeled NAG for 4 minutes. Then, cells were filtered through 0.2 μm Durapore® filter (Millipore®) filter and were washed 3 times with fresh Media A supplemented with unlabeled NAG to remove uninternalized ^{14}C labeled NAG. Filters were placed in scintillation cocktail, ScentiSafe® Econo 1 (Fisher Scientific®) and read in Beckman Coulter® LS 6500 scintillation counter. Experiments were done in triplicates.

^{14}C labeled NAG incorporation

^{14}C labeled NAG incorporation protocol was modified from a previously described method (13). Briefly, overnight LB cultures were diluted 1:100 on Media A. ^{14}C labeled NAG and unlabeled NAG were added to the growth media. To allow ^{14}C labeled NAG incorporation, cell cultures were grown at 30^{o}C for 12 hours. Then, samples were boiled in 4% SDS and filtered through 0.2 μm Durapore® filter (Millipore®) filter. Filters were washed once with LiCl buffer and three times with

milliQ water. Then, filters were placed in scintillation cocktail, ScentiSafe® Econo 1 (Fisher Scientific®) and read in Beckman Coulter® LS 6500 scintillation counter. Experiments were done in triplicates.

^{14}C labeled NAG mineralization

^{14}C labeled NAG mineralization protocol was modified from a previously described method (27). Briefly, overnight LB cultures were diluted 1:100 on Media A. ^{14}C labeled NAG was added to the growth media. A NaOH (0.5M) trap was set up inside the media vessel. To allow ^{14}C labeled NAG mineralization, cell cultures were grown at 30^{o}C for 30 hours, shaking. Then, an aliquot of the NaOH trap was added to scintillation cocktail, ScentiSafe® Econo 1 (Fisher Scientific®) and read in Beckman Coulter® LS 6500 scintillation counter. Experiment was done in triplicates.

RpoE overexpression

Plasmid pBAD24:*rpoE* (23) was transformed into cells and selected on LB Amp. Over-expression was induced as described previously (23) to study the effect of over-expression of *rpoE* in DLP12 mutants. Briefly, overnight cultures were diluted 1:100 in low salt LB (5g/l NaCl) and aliquoted in 96-well plates (COASTAR). Cultures were incubated at 37C and their absorbance (OD600) monitored for 72h. Arabinose was added at 12h to induce the over-expression of *rpoE* from the pBAD promoter. The experiments were done in quadruplicates.

Promoter fusion studies

Reporter plasmid pFAT1, a pJBA110 (1) derivative constructed in our lab, was transformed into cells and selected for on LB Amp plates. Cells were allowed to grow in low salt LB at 37^{o}C. Arabinose was added at 6h to induce expression from vector pBBAD18T, which contained a copy of *rpoE*. At various time points fluorescence was measured using a Bio-Tek Synergy HT spectrophotometer with a 485/20 excitation filter and a 530/25 emission filter and corrected with the OD600 of the corresponding culture. Measurements were done in quadruplicate.

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CHAPTER 3

ROLE OF DLP12 LYSIS GENES IN *E. coli* BIOFILM FORMATION

3.1 ABSTRACT

Phages have recently been implicated as important in biofilm development, although the mechanism whereby phage impact biofilms remains unclear. *E. coli* K-12 carries a defective lambdoid phage in its genome named DLP12. Among the genes found in DLP12 are homologs of the Lambda phage genes encoding enzymes involved in cell lysis (S, R, Rz). The role that these DLP12 lysis genes play in biofilm formation was examined in deletion mutants of *E. coli* PHL628, a curli overproducing, biofilm-forming K-12 derivative. Strains lacking S, R, and Rz were unable to form wild type biofilms. Interestingly, the effect of the S knockout on biofilm formation was less dramatic than the other deletions. All mutants exhibited a compromised attachment to abiotic surfaces and aggregated less well than the wild type, although once again, deletion of S had less of an effect than deleting either R or Rz. These results were consistent with the electron micrographs of the mutants which showed a decreased number of curli fibers on the cells' surface. Consistent with this finding, we also observed that expression from the promoter of *csgB*, which encodes the curli subunits, was down regulated in the mutants. As curli production is transcriptionally down regulated in response to cell wall stress, we challenged the mutants with SDS and found them to be more sensitive to the detergent than the wild type. This suggested that deletion of the lysis genes appeared to affect cell wall status, which likely accounts for the reduction in curli production. These observations suggest that S, R, and Rz genes encoded by DLP12 are not merely genetic interlopers, but have

been domesticated by *E. coli* for normal cellular processes such as cell wall maintenance.

3.2 INTRODUCTION

E. coli PHL628 is an *E. coli* MG1655 (F-) derivative with an *ompR234* mutation. One of the consequences of this mutation is over-expression of the *csgBAC* operon which encodes the structural subunits of curli (21, 35, 38). Curli overproduction in turn increases the ability of the cells to develop into dense biofilms (31, 38). Cells in a biofilm are different at the transcriptional levels from their planktonic counterparts (17, 35, 43). One consistent difference across a number of bacterial genera is the upregulation of prophage genes in biofilm cells (35, 43). Phage exposure and prophage activity have been implicated in biofilm dispersion, enhanced biofilm traits such as attachment and phage tolerance, and in metabolite recycling (41, 42).

Nearly seventy percent of all sequenced bacterial chromosomes contain defective prophages, with some chromosomes being composed of up to twenty percent bacteriophage genetic material (7). *E. coli* MG1655 contains a number of defective lambdoid phage remants including DLP12, (Defective Lambda Prophage) inserted at the 12th minute of the chromosome (5, 26). In DLP12 four open reading frames have been identified, *essD, ybcS, rzpD,* and *rzoD,* that are homologous with the lysis genes found in the lambdoid phage P21 and which are encoded by *s,r,*r_z, and r_{z1} respectively (5, 26). Although studies have shown that other phage genes have been domesticated by the host cell for its own benefit (5, 6, 37), the role of the DLP12 lysis genes in *E. coli* has never been reported.

In the canonical lambdoid virus, S is a holin which allows the endolysin (R) and endopeptidase (Rz) to escape into the periplasmic space. R and Rz are then able to

degrade the cell wall and destabilize the outer membrane, causing host cell lysis (5, 45, 46). More recently another lysis paradigm has been described in which R is exported to the periplasm in a sec-dependent manner (44). There it is tethered to outside of the cytoplasmic membrane until the membrane is perturbed by the holin. This event releases and activates R, causing host cell lysis (44).

Regardless of the exact mechanism, the question arises as to why *E. coli* has held on to these genes? Given their known function (cell death via lysis), one might assume the retention of these genes by *E. coli* to be an evolutionary accident, that these "killer" lysis genes are just genetic interlopers that are either not expressed or are in a state of mutational decay and encode non-functional proteins (reviewed in (7)). However, recent genome wide expression studies have shown that the DLP12 lysis genes are actively transcribed in a σ^E dependent manner (29). Another recent study has also shown that the product of ybcS (R^{DLP12}) is an active endolysin capable of degrading gram-positive and gram-negative peptidoglycan (34), although its physiological relevance in the *E. coli* cell cycle was never examined. It is not clear how *E. coli* could benefit by expressing genes that are normally meant to kill it via lysis.

E. coli, however, does employ a specialized suite of lytic transglycosylases, amidases and endopeptidases to modify peptidoglycan (PG) during normal cell growth and even in stationary phase (4). Peptidoglycan modifications are very important to proper cellular function, with new strands added and old ones removed continuously (40). Controlled expression of these enzymes is critical to the survival of the cell. Interestingly, peptidoglycan breakdown products serve as signals that regulate beta-lactam resistance (19, 20) and are recognized by eukaryotic cells during bacterial pathogenesis (Keep 2006,).

Although the role of DLP12 lysis gene products has never been examined, given the association between phage and biofilms, we hypothesized that the remnants of this defective phage might affect *E. coli* biofilm development. In this work we constructed individual mutants in all three DLP12 lysis genes (S, R, Rz) in of *E. coli* PHL628. Mutants were then tested for biofilm traits like aggregation, attachment, and biofilm architecture, and the production of curli was also examined, as was the status of the cell wall. We report here on these results which implicate the products of the lysis genes of DLP12 as having a role in cell wall maintenance and an indirect role in biofilm development.

3.3 RESULTS AND DISCUSSION

To study the biofilm architecture of the DLP12 mutants, 3D renderings of the biofilms were made from image stacks generated by Leica Confocal Imaging Software. While the wild type biofilm was characterized by numerous large, robust tower-like structures, the mutants biofilms were largely undifferentiated (Figure 3.1). We also observed that that the poor biofilms which did form, detached readily from the substratum (data not shown). Quantitative assessment of the biofilms using COMSTAT software (18) revealed that all the mutants were significantly (α=0.05) compromised in total biomass and average thickness (Figure 3.2 A and B). These effects were most dramatic in the putative lysozyme (R) and transpeptidase (Rz) mutants whose biofilms were reduced from wild type levels by almost 7 fold with respect to biomass and 10 fold with respect to average thickness. Interestingly, the biofilm of the putative holin (S) mutant was less attenuated and only manifested a 3 fold reduction in biomass and 5 fold reduction in average thickness (Figure 3.2 A and B). The mutants were attenuated with respect to their ability to attach to PVC (Figure

3.3) and aggregated less than wild type (Figure 3.4). Once again, while S was compromised in its ability to autoaggregate, it was not as deficient as the putative lysozyme (R) and transpeptidase (Rz) mutants. Complementation restored all phenotypes to near wild type levels except for the aggregation phenotype of the S which was little altered by expression of an ecotopic copy of the gene.

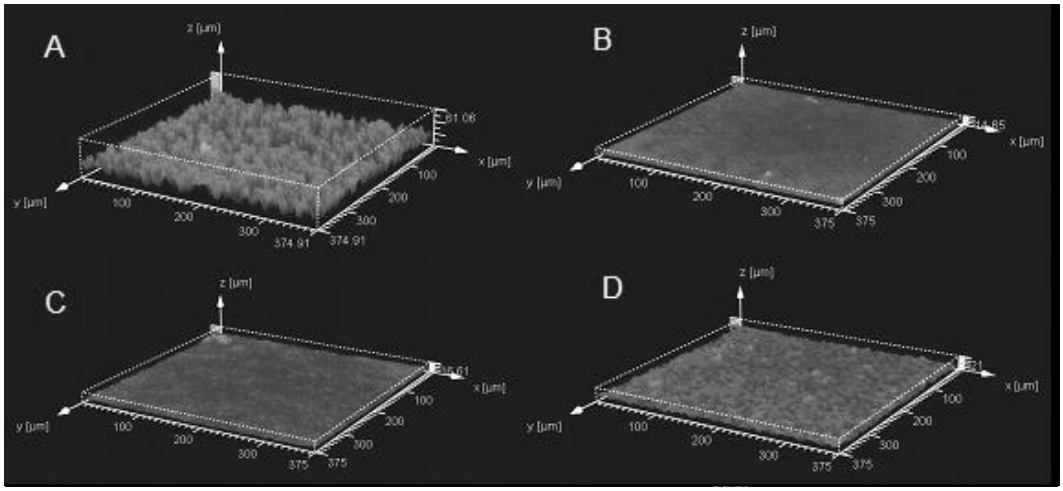

Figure 3.1: Biofilm Architecture: Biofilm were grown in MatTek microwell dishes in minimal media with casamino acids for 72h at 37C with moderate shaking (50rpm). Media was changed every 24h. Cells were stained with acridine orange and visualized under a Leica Confocal Microscope. 4 image stacks were obtained from each dish and 3D image renditions were obtain with the Leica software. The experiments were done in duplicates for a total of 8 image stacks per strain. (A) PHL628 wild type. (B) ΔS mutant. (C) ΔR mutant. (D) ΔRz mutant.

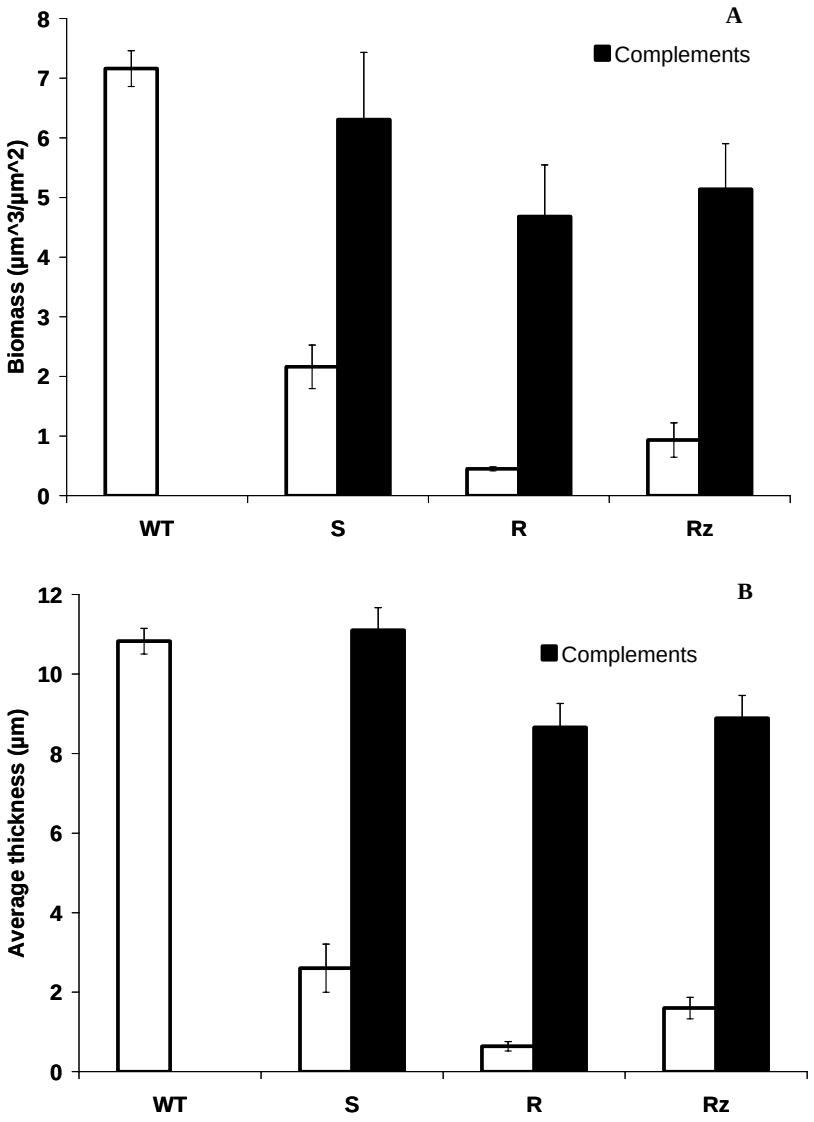

Figure 3.2: Biofilm Biomass and Average Thickness: Biofilm were grown in MatTek microwell dishes in minimal media with casamino acids for 72h at 37C with moderate shaking (50rpm). Media was changed every 24h. Cells were stained with acridine orange and visualized under a Leica Confocal Microscope. A total of 8 image stack per strains were analyze to determine biomass (A) and average thickness (B) using the image analysis software COMSTAT.

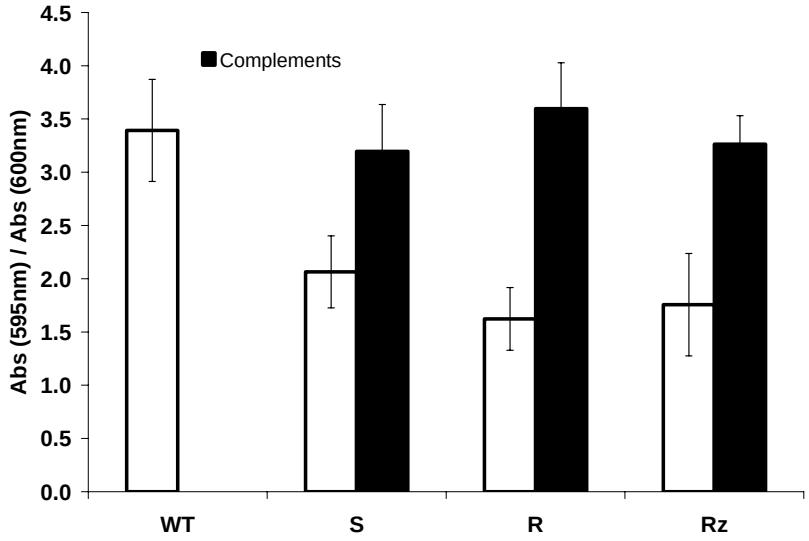

Figure 3.3: Attachment assay: Overnight cultures were diluted and allowed to attach to PVC wells for 16h at 30C with moderate shaking (50rpm). Then, OD600 was measured and crystal violet was added to the wells and incubated for 15 minutes. Wells were washed with distilled water. Wells were allowed to dry, CV was recovered with EtOH and OD595 was measured. OD600 was compared to OD595 to assess for attachment. The experiment was done in triplicates.

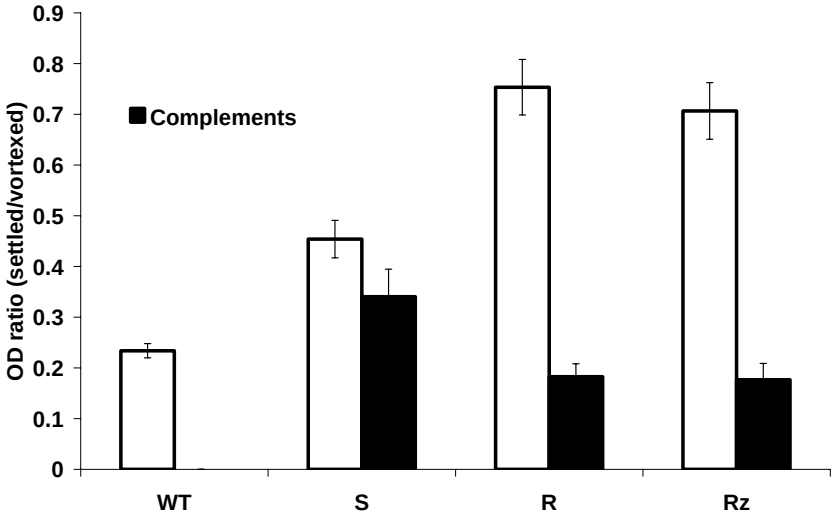

Figure 3.4: Autoaggregation assay: Overnight cultures were stacked vertically and allowed to settle for 12h at 37C without shaking. OD600 was measured on samples taken from the top 5mm of each culture. Cultures were then vortexed and sampled again. OD600 of the unvortexed samples was compared to their vortexed counterparts. The experiment was carried out in duplicate.

With the attachment and autoaggregation assays showing that the deletion mutants manifest phenotypes known to result from changes in extracellular structures (15, 38), transmission electron microscopy was performed in order to visualize possible differences between mutants and PHL628. Electron micrographs of the wild type *E. coli* PHL628 revealed a robust layer of curli on and around cell (Figure 3.5a). In contrast, electron micrographs of the mutants revealed little to no curli around the cells (Figure 3.5b, 3.5c, 3.5d). In order to get a more quantitative assessment of the apparent curli deficiency, mutant strains were subjected to a Congo Red pull down assay (16). Congo Red binds curli fibers and the amount of Congo Red bound by cells is proportional to the amount of curli on the surface (16). As a negative control a deletion of *csgA*, the gene that encodes the curlin subunit, was constructed in the PHL628 background. Although the lysis gene mutants were not as compromised as the *csgA* mutant with respect to Congo Red binding, the assay revealed that all the mutants had significantly (α=0.05) less curli than wild type (Figure 3.6). Interestingly, despite the differences seen in this assay and the via EM, the mutants still turned red on Congo Red indicator plates.

Given the putative activities encoded by the DLP12 lysis gene (i.e. holin, lysozyme, and endopeptidase) it seemed reasonable to hypothesize that alterations in peptidoglycan modification were responsible for the observed defects in curli elaboration. Peptidoglycan metabolism (i.e. maturation, turnover) has been shown to impact biofilm formation (12) and to be involved in type II, III, and IV secretion systems (25, 39). Translocation of proteins larger than 55 kD is inhibited by the size of the holes in peptidoglycan and so remodeling would be required to ensure that the necessary transport system components made it to the outer membrane (10, 25). Many biofilm traits like aggregation, attachment, swimming, and swarming motility depend on the translocation of subunits used to make cellular appendages like flagella,

fimbriae and curli, and thus depend on functional transport and secretion systems (15, 25, 28, 38). However, all of the proteins encoded by the *csg* operons are less than 30kD and would not be expected to require peptidoglycan remodeling for export.

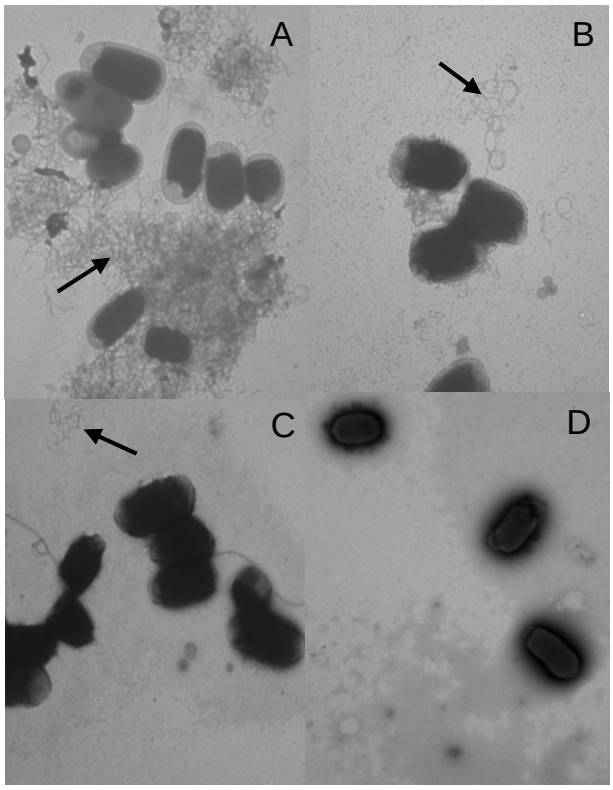

Figure 3.5: Electron Microscopy: 300 mesh Formvar grids were floated on cell cultures, washed and then negatively stained with 3% AMB. Grids were studied under a Philips Electron Microscope 201. Arrows point at curli fibers. (A) PHL628 wild type. (B) ΔS mutant. (C) ΔR mutant. (D) ΔRz mutant.

To investigate if the lack of curli was caused by a reduction in transcription or a deficit in either export or assembly, expression from the *csgB* promoter (P_{csgB}) was monitored using a short-lived P_{csgB}-GFP reporter (23). After 24 hours of growth on MSM CA, each of the mutants showed a marked decrease in GFP fluorescence normalized to cell density compared to that of the wild type. The S mutant exhibited a 5 fold decrease in P_{csgB} derived fluorescence whereas the R and Rz mutants had a

more dramatic effect with a 17-fold and more than 20 fold decrease respectively (Figure 3.7). This suggested that the observed decrease in curli was not merely due to a lack of transport. Rather, this was evidence that the products of the DLP12 lysis genes seem to somehow affect the regulation of curli expression.

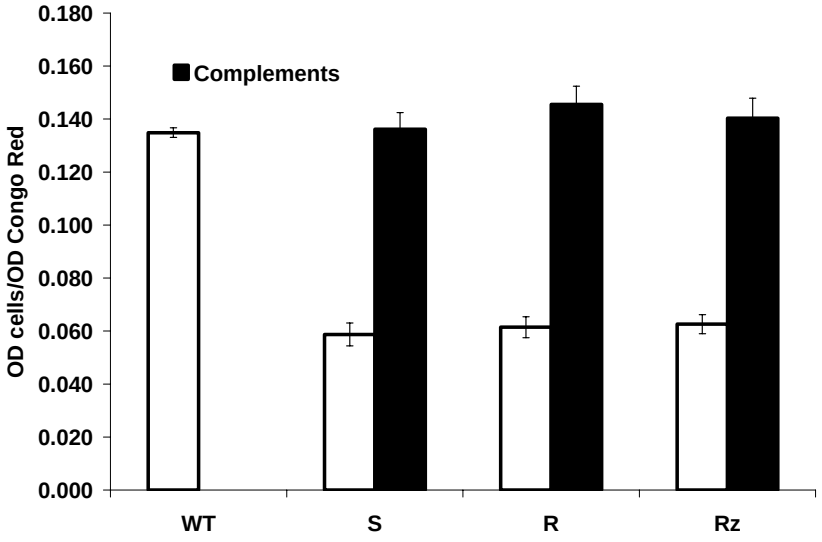

Figure 3.6: Quantitative Congo Red binding assay: Cells were grown on low salt LB plates for 72h at 30C. Cells were scraped from the plate, resuspended in phosphate buffer and OD600 was measured. Congo Red was added and incubated for 10 min at room temperature. Cells were pelleted, supernatant was sampled and OD500 was measured. OD600 was compared to OD500 to determine Congo Red binding. Experiments were done in triplicate. A curli deficient mutant (csgA) was included for comparison. Dre, I think you should subtract the csgA mutant and make that the "zero"

Why would loss of the DLP12 lysis gene products affect curli expression? Cell wall status has been shown to be a signal for curli fiber synthesis, transport, secretion and extracellular assembly (3, 14, 21, 36). Transcription of *csgBAC* is positively regulated by CsgD, which is part of the *csgDEFG* operon and is divergently transcribed from csgBAC. The regulation of *csgDEFG* transcription is controlled by at least three different phosphorelay systems: EnvZ/OmpR, CpxRA, and RcsBCD (1,

2). All three systems respond to a wide range of envelope stresses and have significant overlap with one another.

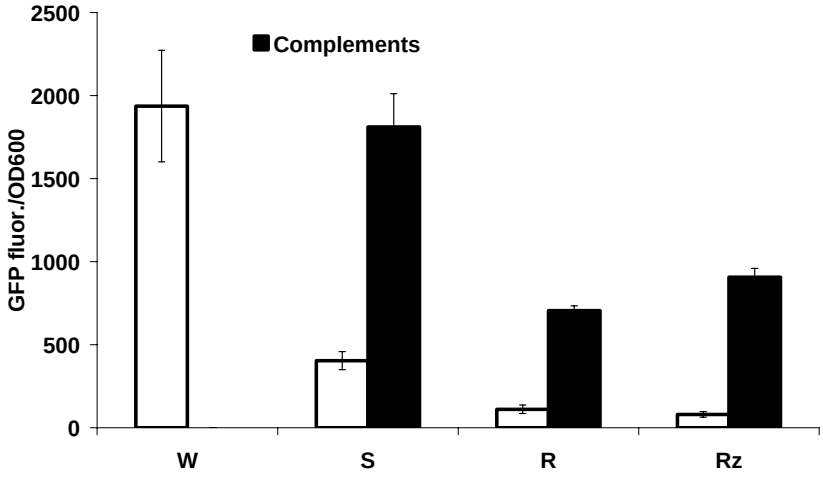

Figure 3.7: Curli operon expression: Cells bearing the reporter vector pJBA110 carrying the csgB promoter (P_{csgB}) fused to a short lived GFP were studied for gene expression. Fluorescence was read using a Bio-Tek Synergy HT spectrophotometer with a 485/20 excitation filter and a 530/25 emission filter and corrected with the OD600 of the corresponding culture.

Consistent with this an indirect effect due to cell wall perturbation, we found that peptidoglycan metabolism was altered in *SRRz*^{DLP12} mutants (Chapter 1). This unbalanced metabolism might cause an accumulation of peptidoglycan breakdown products in these mutants. Peptidoglycan breakdown products, due to lysis or normal metabolism, are a mixture of disaccharides, anhydrosacharides and pentapeptides, which play a role as secondary messengers for several bacterial processes (19, 20, 24). In gram negative bacteria, the products of lytic transglycosylases (anhNAM) are imported into the cytoplasm where they trigger the production of β-lactamases (19, 20). Appendages have also been shown to respond to peptidoglycan breakdown products (1, 2, 32, 33). NAG and its phosphorylated form (NAG-P) have been shown

to inhibit expression of adhesion factor *fimB* (32, 33). Curli have also been reported to be inhibited by intracellular NAG-P (2).

EnvZ/OmpR is a positive regulator of *csgDEFG* expression and affects curli production in response to low osmolarity by upregulating curli gene expression through csgD (1, 22). CpxRA responds to envelope stress and misfolded proteins in the periplasm (1, 3), while RcsRA also surveys membrane stress and directs capsular polysaccharide synthesis through the *cps* operon (1, 36). RcsAB have been associated with RecA independent prophage induction (30). Importantly, both CpxR/A and RcsB/C/D are known to downregulate the expression of the csgBAC operon (1, 2). At this point we still do not know anything about the nature of the signal that arises when the lysis genes products are lost nor how that signal might be transduced to the curli operon, however, there is ample evidence in the literature to suggest a link between cell wall status and curli production.

How could peptidoglycan status be measured by the cell? It is not clear if or how the CpxR/A and RcsB/C/D systems which down regulate curli production, perceive alterations in peptidoglycan status, however, Barnhart and Chapman recently demonstrated that transcription of *csgBAC* is down regulated when *N*-acetylglucosamine-phosphate (NAG-P) accumulates as a consequence of either defects in endogenous NAG-P turnover, or due to exogenous NAG additions (2). As both a building block and a breakdown product of peptidoglycan, NAG abundance would seem like one possible signal molecule that might be used to inform the cell of peptidoglycan status. More work needs to be done to determine if intracellular NAG-P or some other peptidgolgycan breakdown product plays a role in signaling the apparent stress caused by loss of the DLP12 lysis genes.

Results from our earlier 14C labeled NAG experiments suggest that loss of the lysozyme and endopeptidase decrease peptidoglycan turnover as well as NAG uptake

and incorporation. This perturbance could lead to an increased accumulation of muropeptide precursors. Salvage of these would be expected to result in increased intracellular levels of NAG-P and based of the work of Barnhart and Chapman, lead to reduced curli production (2). Folkesson et al. have shown that alterations in peptidoglycan salvage can decrease the virulence of *Salmonella enterica* although the exact mechanism whereby that occurs remains unclear (11).

Clearly more work is required to understand the full role of these phage remnants have in *E. coli*, however, this report demonstrates that the DLP12 lysis genes are important for curli production and biofilm formation and are not just genetic interlopers in a state of mutation decay.

3.4 METHODS

Bacterial strains and growth conditions

E. coli strain PHL628 is a MG1655 derivative with an ompR234 mutation (38). *E. coli* PHL628 and mutants were routinely grown on Luria Bertani (LB) media at 37^{o}C overnight with shaking (150 rpm) and supplemented with 50 ul/ml Kanamycin (Kan) unless otherwise stated. To obtain biofilms, *E. coli* PHL628 and mutants were grown on minimal media (MSM) (27) supplemented with casamino acid (0.2%) and 50 ul/ml Kan unless otherwise stated. When required, plates and media were supplemented with Ampicillin (Amp 150 ul/ml) and/or Chloramphenicol (25 ug/ml).

Mutants

Deletion mutants were constructed by allele replacement using Lambda RED strategy as described elsewhere (9). Briefly, Chloramphenicol (Cm) interrupted

version of the genes were created using PCR-mediated ligation (8). In general, each construct consisted of a 5'and a 3' 500-bp homology region to the gene of interest link by a 1kb Cm cassette flanked by flip recombinase target (FTP) sites. This linear DNA was transformed into wild type *E. coli* PHL628 cell expressing the Lambda RED recombinase. The 5' end and 3'end homology regions allowed RED system to replace the wild type allele with our interrupted version. Clone screening was carried out on plates supplemented with the adequate antibiotic. After confirmed recombination the Cm marker was removed using FLP-recombinase from plasmid pCP20 (9). All plasmid vectors are temperature sensitive and were cured from the cells by growing cells at 43ºC. The markerless knockout mutants were confirmed by PCR and sequencing.

Complements were constructed using full wild type version of the genes cloned into pBBR1MCS (lactose inducible).

Biofilm architecture

Biofilms were grown on MatTek Cultureware glass bottom dishes (Ashland, MA) for imaging. Overnight cultures were inoculated (1% v/v) into 3ml of minimal media (MSM) supplemented with 0.2% casamino acid (CA). Dishes were incubated at 30ºC for 72h with shaking on an orbital shaker at 50rpm. Media was exchanged every 24h with new fresh media. After 72h cells biofilms were stained with acridine orange and studied under a Leica confocal microscope. To assess biofilm formation and architectural features a total of 8 Z-image stacks (0.25μm steps) were obtain for each mutant and then analyzed with COMSTAT (18).

Table 3.1: Primers, plasmids and bacterial strains.

Primers		
Name	**Sequence**	**Reference**
S^{DLP12} Forward1	5'-GGGCCCACTTCGTCACACAGCTTAAACCCG-3'	This study
S^{DLP12} Reverse1	5'-GGATCCTGGCGACAGTGGCAAGGTCTTTAT-3'	This study
R^{DLP12} Forward1	5'-GGGCCCTGCGAGAGGTGAATAATGCCTCCA-3'	This study
R^{DLP12} Reverse1	5'-GGATCCTTCGCTAGCTTCAGTTCTCTGGCA-3'	This study
Rz^{DLP12} Forward1	5'-GGGCCCACTCGTCGTGAGATTGAGCGTGAA-3'	This study
Rz^{DLP12} Reverse1	5'-GGATCCGGAAGCGCGTGTGTATTGCTCAAA-3'	This study
essD p Forward1	5'-GGTACCTGCAGCTACAGCAGAAATTGCCAC-3'	This study
essD p Reverse1	5'-TCTAGAGCGACACCCGTTGTTAACTTATCC-3'	This study
S^{DLP12} internal Reverse	5'-GCGACACCCGTTGTTAACTTATCC-3'	This study
S^{DLP12} internal Forward	5'-TGCGAGAGGTGAATAATGCCTCCA-3'	This study
R^{DLP12} internal Reverse	5'-ACACCTTCCAGACCATCGTTACCA-3'	This study
R^{DLP12} internal Forward	5'-ACTCGTCGTGAGATTGAGCGTGAA-3'	This study
Rz^{DLP12} internal Reverse	5'-ATGACAGGCTGACGATGATGCAGA-3'	This study
Rz^{DLP12} internal Forward	5'-AGCAGTGCAGATAGAGCTGACCAT-3'	This study
P1S	5'-GGATAAGTTAACAACGGGTGTCGCGTGTAGGCTGGAGCTGCTTCG-3'	This study
P2S	5'-TGGAGGCATTATTCACCTCTCGCACATATGAATATCCTCCTTA-3'	This study
P1R	5'-TGGTAACGATGGTCTGGAAGGTGTGTGTAGGCTGGAGCTGCTTCG-3'	This study
P2R	5'-TTCACGCTCAATCTCACGACGAGTCATATGAATATCCTCCTTA-3'	This study
P1Rz	5'-TCTGCATCATCGTCAGCCTGTCATGTGTAGGCTGGAGCTGCTTCG-3'	This study
P2Rz	5'-ATGGTCAGCTCTATCTGCACTGCTCATATGAATATCCTCCTTA-3'	This study
Plasmids		
pBBRMCS		Kovach, 1995
pBBRMCS:S^{DLP12}		This study
pBBRMCS:R^{DLP13}		This study
pBBRMCS:Rz^{DLP14}		This study
pJBA110		Andersen, 1998
pJBA110:*csgB* p		Junker, 2004
pCP20		Datsenko, 2000
pKD3		Datsenko, 2000
pKD46		Datsenko, 2000
Strains		
E. coli PHL628		Vidal, 1998
E. coli PHL628 ΔS^{DLP12}		This study
E. coli PHL628 ΔR^{DLP12}		This study
E. coli PHL628 ΔRz^{DLP13}		This study
E. coli PHL628 ΔS^{DLP12} pBBR1MCS:S^{DLP12}		This study
E. coli PHL628 ΔR^{DLP12} pBBR1MCS:R^{DLP12}		This study
E. coli PHL628 ΔRz^{DLP12} pBBR1MCS:Rz^{DLP12}		This study
E. coli PHL628 pJBA110:csgBp		This study
E. coli PHL628 ΔS^{DLP12} pJBA110:csgBp		This study
E. coli PHL628 ΔR^{DLP12} pJBA110:csgBp		This study
E. coli PHL628 ΔRz^{DLP12} pJBA110:csgBp		This study
E. coli PHL628 ΔS^{DLP12} pBBR1MCS:S^{DLP12} pJBA110:csgBp		This study
E. coli PHL628 ΔP^{DLP12} pBBR1MCS:R^{DLP12} pJBA110:csgBp		This study
E. coli PHL628 ΔRz^{DLP12} pBBR1MCS:Rz^{DLP12} pJBA110:csgBp		This study

Attachment

Attachment to PVC surface was studied as described previously (13). Overnight cultures were diluted 1:5 to a total volume of 120 ul on a 96-well PVC plates and incubated at 30C shaking (50 rpm on an orbital shaker) for 16 hours. After 16 h turbidity (OD600) were measured in each well. Then, 100 ul of a solution of 1% Crystal Violet (CV) was added to each well. After 15 minutes of incubation at room temperature wells were thoroughly washed with water. Plates were allowed to dry for 15 minutes at room temperature. After the plate was dry 100 ul of 95% EtOH was added to each well and incubated for 10 minutes at room temperature. Then, CV absorbances (OD 500) were measured in each well. The original well turbidity (OD600) was compared to the CV absorbance (OD500). Experiments were done in triplicate.

Autoaggregation

Cell autoaggregation was measured as described previously (15). Briefly, 4ml of overnight LB cultures of each knockout mutants and each of their complements were incubated static for 12h at 37C. OD 600 was measured from samples taken from the top 5mm of each culture with minimal disturbance. Then, cultures were vortex and sampled again. OD600 values (undisturbed and vortex) for each mutant were compared as a measurement of cell autoaggregation. Experiments were done in quadruplicate. To determine if fimbriae played a role in autoaggregation 1% a-Methyl-D-Mannopyranoside was added to the media in a separate experiment that was carried out as described above.

Electron microscopy

Cell were grown on LB at 37C shaking overnight. 300 mesh Formvar copper grids (EMS, Hatfield, PA) were floated on 25ul of the overnight cultures for 1 minute. Then the grids were transferred to a solution containing 3% ammonium molybdate (3% AMB) pH=7.0 or a solution containing 1% potassium phosphotungstic acid (1% KPTA) for 2 minutes. Grids were then rinsed in MilliQ water for 1 minute and allow to dry before observation. Cells were studied on a Philips Electron Microscope at different magnification ranging from 7000x to 45000x. Snap shots were taken using a MicroFire digital camera and software by Optronics.

Quantitative Congo Red binding assay

Cells were plated on low salt LB (5g/l NaCl) and incubated at 30C for 72h (16). After three days cells were scraped from the plates and resuspended in 500ul phosphate buffer pH 7.0. Serial dilutions were prepared in phosphate buffer and turbidity of each dilution was measured (OD600). Then, cells were centrifuged at 14000 g for 3 minutes and resuspended on 1ml of a 0.002% solution of Congo Red. Tubes were incubated at room temperature for 10 min to allow Congo Red binding. After the incubation tubes were centrifuged at 14000 g for 10 min, and the Congo Red remaining in the supernatant was measured (OD500) and compared to a cell-free Congo Red tube that was treated as a control tube. Dilution turbidity (OD600) and Congo Red absorbance (OD500) were plotted in graphs. Experiments were done in triplicate.

Promoter fusion studies

Reporter plasmid pJBA110 carrying the csgB (curli) promoter was transformed in each of the PHL628 strains (23). Cells were allowed to grow in MSM CA. At various time points fluorescence was measured using a Bio-Tek Synergy HT spectrophotometer with a 485/20 excitation filter and a 530/25 emission filter and corrected with the OD600 of the corresponding culture. Measurements were done in quadruplicate.

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CHAPTER 4

ANTITERMINATION IN THE RPOE-REGULATED DLP12 LYSIS OPERON

4.1 ABSTRACT

Transcriptional regulators usually interact with the RNA polymerase holoenzyme to enhance or prevent transcription from specific promoters. Among transcription regulators, antiterminators are unique in that they remain bound to the holoenzyme after transcription initiation and allow paused holoenzyme to read through terminator sequences. In lambdoid phages, the Q antiterminator is required for transcription of the late genes, including the lambdoid lysis genes. The products of these lysis genes target the peptidoglycan wall causing cell lysis. Homologs of these genes are found in the defect lamdoid phage DLP12 which is found in *E. coli* K12. Previously we showed that loss of the DLP12 lysis genes affected peptidoglycan metabolism, SDS resistance and biofilm formation by *E. coli*. We also found that transcription from the lysis gene promoter (*essDp*) was enhanced by over expression of σ^E. Here we studied the regulation of DLP12 lysis genes by the putative antiterminator Q^{DLP12}, which is found upstream of the lysis genes in DLP12. Although not essential for growth, Q^{DLP12} mutants and over-expressing strain showed phenotypes previously observed in DLP12 lysis gene mutant and over-expressing strains respectively. For the mutants these included altered peptidoglycan metabolism, SDS resistance, bifilm formation, and curli expression. When overexpressed Q^{DLP12}, caused increased cell lysis that was dependent on the presence of functional copies of R and S, although Q mediated cell lysis could be induced in an S mutant with the addition of dinitrophenol. Over expression of σE enhanced cell lysis in a Q^{DLP12}

dependent fashion and over expression of both σE and Q^{DLP12} had an additive effect on cell lysis. Results from *an essD*-GFP promoter fusion were consistent with the lysis data, showing that σE-mediated over expression was Q^{DLP12} dependent and that overexpression of both proteins resulted in additive affects on transcription. These data suggest interplay between σ^E and Q^{DLP12} for the regulation of transcription of the DLP12 lysis genes and are the first example of a phage antiterminator playing an active role in phage independent host physiology. They are also the first pieces of evidence suggesting antiterminator interactions with σ^E.

4.2 INTRODUCTION

RNA polymerase (RNAP) is highly conserved in all bacteria. The core enzyme, formed by $\alpha_2\beta\beta'\omega$ subunits, requires a σ (sigma) factor to complete the holoenzyme (8, 10, 30, 31). This holoenzyme is capable of initiating transcription from specific σ factor–recognized promoters (10, 30, 41). Although the majority of transcription regulators act in the first steps of transcription by activating or repressing promoters; other regulators act after transcription has already started (10). These regulators interact and assist the holoenzyme to overcome temporary or permanent barriers, such as pause sites or terminators (10, 18). Anti-terminators proteins are among this type of regulators; they can affect the elongation properties of the holoenzyme by allowing it to read through specific terminator sequences (10).

The BglG/SacY family of antiterminators allows the holoenzyme to read through Rho-independent terminators (16). In *E. coli*, BglG regulates the *bgl* operon, involved in the uptake of β-glucosides (1, 16). In *B. subtilis*, SacY regulates the expression of *sacB*, which encodes for levansucrase (37, 40). A well characterized example of an anti-terminator is λQ, this anti-terminator acts on the transcription from

the lambda late promoter $P_{R'}$ (10, 30, 31, 44). The activity of λQ depends on the existence of a Q-binding element (QBE) or Q-utilization site (qut site) in the DNA; these qut sites are species-specific among lambda relatives (λ82, P22, phage 21, e.g), which means that a Q protein will only recognize its specific qut site (10, 17, 18, 34). The other requirement for Q activity is a pausing sequence found in the initial transcript (10, 17, 18, 30, 34, 44).

Pausing of the transcription complex is due to the interaction between the σ factor, as part of the holoenzyme, and the pause element on the transcript (10, 18, 30, 34). The qut site-bound λQ can only modify the elongation complex when the holoenzyme is paused at the σ-specific pause element (10, 30, 34, 44). Q contacts the holoenzyme where region 4 of the σ factor and the β-flap of RNAP interact (10, 30, 36). This is thought to displace σ as the holoenzyme escapes the pause element (10, 18, 31, 34). Once the Q-modified holoenzyme overcomes this pause it can read through other terminators found downstream (10, 30, 34). *In vitro* studies suggest that λQ remains as a stable component interacting with a region close to the b-flap in the Q-modified holoenzyme (10, 18, 30, 34).

In lambdoid phages Q intervenes in the regulation of lysis genes; bacteriophages need to degrade peptidoglycan in order to escape the host cell (43). Q-mediated regulation ensures that the transcription of these genes, and concomitant host cell lysis, occurs after the assembly of the new phage particles (32, 35, 44)\.

Lambdoid phages switch between lytic and lysogenic states (5). Occasionally, due to mutations or faulty recombination, the lysogen or prophage is unable to re-enter its lytic cycle and becomes trapped in the host cell (5, 6). The role these defective prophages play in host physiology is unclear and they are thought to be in mutational decay. Although unable to produce viable phage, several prophage genes have been

shown to be beneficial for their host strain, increasing the host fitness, survival and virulence (5, 6).

E. coli harbors a defective lambdoid prophage found in the 12th minute of the genome which has imaginatively been call DLP12 (25). DLP12 encodes a putative Q antiterminator (*ybcQ*) and lysis proteins (*essD* , *ybcS*, and *ybcT*) which are highly homologous to the antiterminator (Q), holin (S), lysozyme (R), and endopeptidase (Rz) of phage 21 (26, 38). Recently this R homolog was shown to encode a functional lysozyme (38) and we show in Chapter 2 that all of the DLP12 lysis genes encode proteins with a physiological role in *E. coli* peptidoglycan metabolism. In chapter 3 we showed that deletion of the lysis genes also affected curli expression and biofilm formation.

In lambdoid phage, transcription of phage lysozymes is controlled by the late promoter $P_{R'}$ and the antiterminator Q, this ensures the transcription of the lysozyme, and concomitant host cell lysis, only occurs after the assembly of the new phage particles (35, 43). Thus, we hypothesized that *ybcQ* might play a part in regulating transcription of $SRRz^{DLP12}$ and therefore be relevant to the phenotypes we discovered in chapters 2 and 3. Very little is known about the regulation of $SRRZ^{DLP12}$. Our promoter studies in chapter 2 and previous work by others (33) strongly suggest that the $SRRZ^{DLP12}$ promoter (*essD*p) is under the regulation of RpoE, which directs the transcription of numerous operons in response to membrane stress (23, 33).

In the present work we study the effect of deleting Q^{DLP12} on $SRRZ^{DLP12}$ expression and related phenotypes. We show that it affects RpoE-mediated early stationary phase lysis, peptidoglycan metabolism, curli expression, and biofilm formation in *E. coli*.

4.3 RESULTS AND DISCUSSION

As expected, based on the results from the DLP12 lysis gene mutants (Chapter 2), deletion of *Q* did not affect growth under laboratory conditions (Figure 4.1). However, consistent with the hypothesis that Q's main function is regulating transcription of $SRRz^{DLP12}$, we found that the *Q* mutant was compromised in all of the other phenotypes shown to affect the lysis gene mutants in Chapter 2, including SDS susceptibility (Figure 4.2), autoaggregation, biofilm formation (Figure 4.3 and Table 4.1), *csgB* transcription (Figure 4.4) and curli production (Figure 4.5). As observed in lysis gene mutant cells (Chapter 2), Q^{DLP12} mutant micrographs showed reduced curli (Figure 4.6), The Q^{DLP12} mutant also showed reduced petidoglycan incorporation and turnover (Figure 4.7 and 4.8). In most of these experiments the magnitude of the effect was similar to that seen for the *S* mutant but not as dramatic as seen for the *R* mutant (Chapter 2). Complementation restored most of the phenotypes to near wild type levels.

Although loss of Q resulted in decreased transcription of the genes encoding curli (*csgBAC*), it is the latter which likely accounts for the deficits in attachment, autoaggregation, and biofilm formation (3, 14, 42). It is unlikely that Q plays a direct role in regulating curli production. It seems more probable that the decrease in transcription of the *csg* operon is due to cell wall stress which is known to down regulate curli production (3, 4), and which is evidenced by SDS susceptibility (Figure 4.2) and peptidoglycan alterations (Figure 4.7 and 4.8). More work needs to be done in order to understand how cell wall stress signal is transduced to the curli operon.

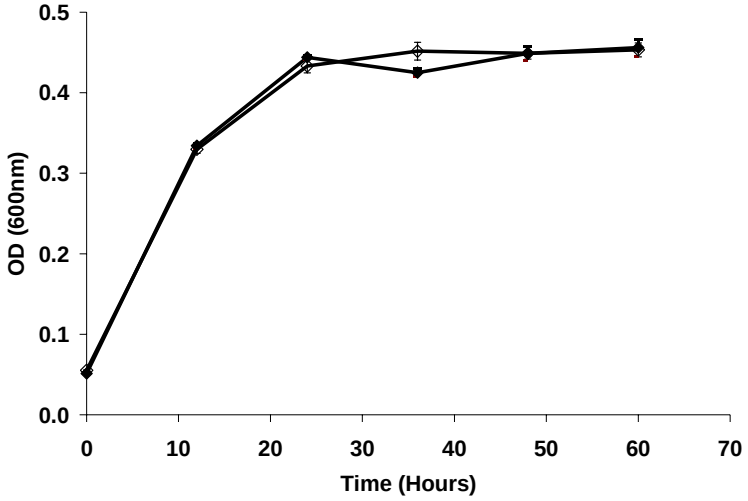

Figure 4.1: Growth curve: Overnight cultures were diluted in fresh LB and allowed to grow at 37^{o}C. OD600 was monitored in a μQuant spectrophotometer. Experiments were done in triplicates. Wild type control PHL628 (◇), PHL628 ΔQ^{DLP12} (◆), show very similar growth pattern (α=0.05). ΔQ^{DLP12} mutants are not affected for growth.

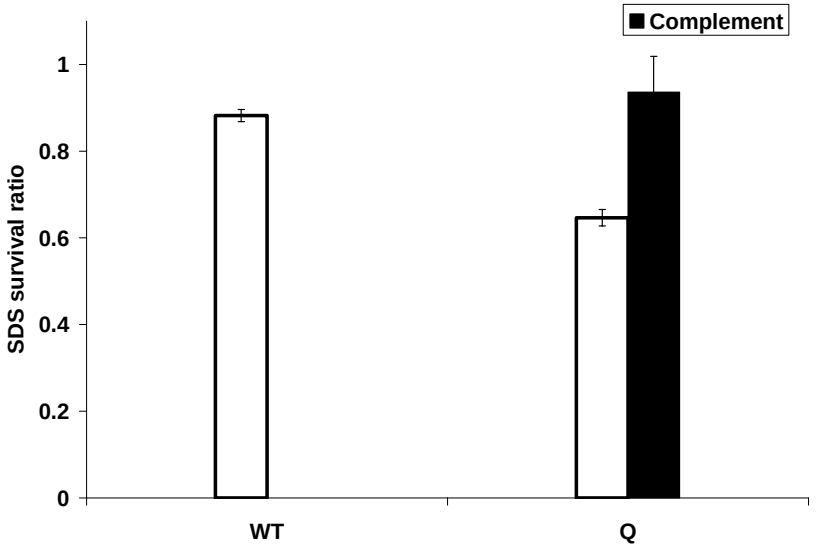

Figure 4.2: Susceptibility to SDS: Overnight cultures were diluted 1:100 and challenged with 5% SDS for 3h at 37C. OD600 was measured and compared to unchallenged cultures. Experiments were done in quadruplicates. ΔQ^{DLP12} mutants lost tolerance to SDS when compared to wild type control (α=0.05). Complement rescued the tolerance phenotype.

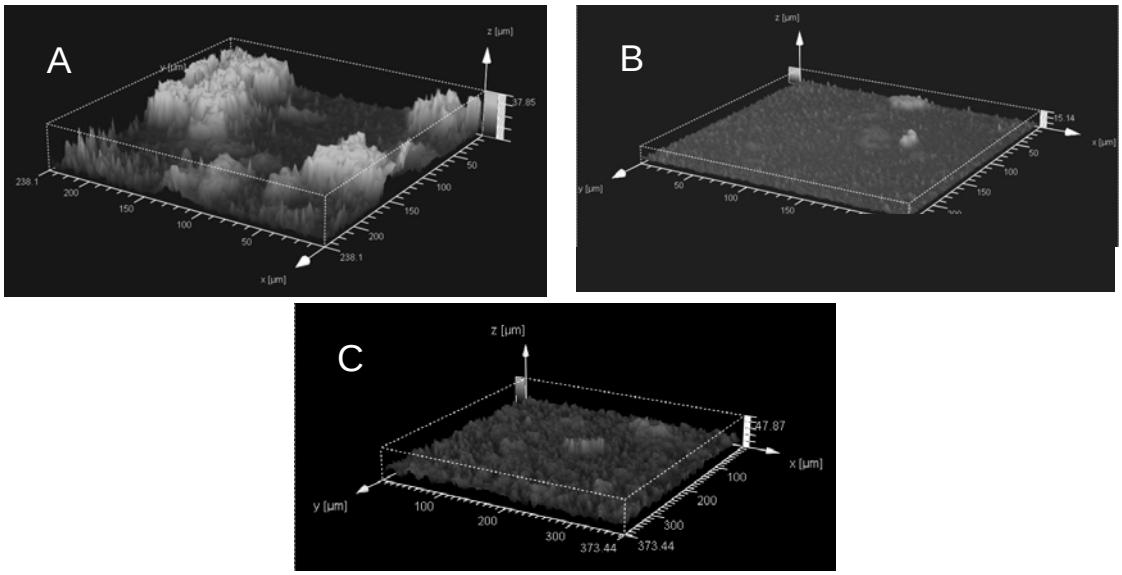

Figure 4.3: Biofilm Architecture: Biofilm were grown in MatTek microwell dishes in minimal media with casamino acids for 72h at 37C with moderate shaking (50rpm). Media was changed every 24h. Cells were stained with acridine orange and visualized under a Leica Confocal Microscope. 4 image stacks were obtained from each dish and 3D image renditions were obtained with the Leica software. The experiments were done in duplicates for a total of 8 image stacks per strain. (A) PHL628 wild type. (B) ΔQ^{DLP12} mutant. (C) ΔQ^{DLP12} mutant complemented with a full version of Q^{DLP12}.

Table 4.1: Biofilm features: Cells were allowed to grow biofilms. Biofilm features were determined as previously (Chapter 2). We observed that ΔQ^{DLP12} mutant was affected for biomass, thickness, attachment and autoaggregation (α=0.05). Every phenotype was rescued in the ΔQ^{DLP12} complemented mutant. Experiments were done in quadruplicates. Parentheses indicate standard errors.

	Biomass (µm^3/µm^2)	Average thickness (µm)	Attachment	Autoaggregation
PHL628	6.289 (1.117)	6.544 (0.996)	3.393 (0.479)	0.234 (0.014)
PHL628 ⌣Q	1.669 (0.722)	1.654 (0.706)	2.298 (0.241)	0.772 (0.044)
PHL628 ⌣Q pBBR1MCS:Q	4.721 (0.838)	4.912 (0.747)	3.384 (0.515)	0.274 (0.054)

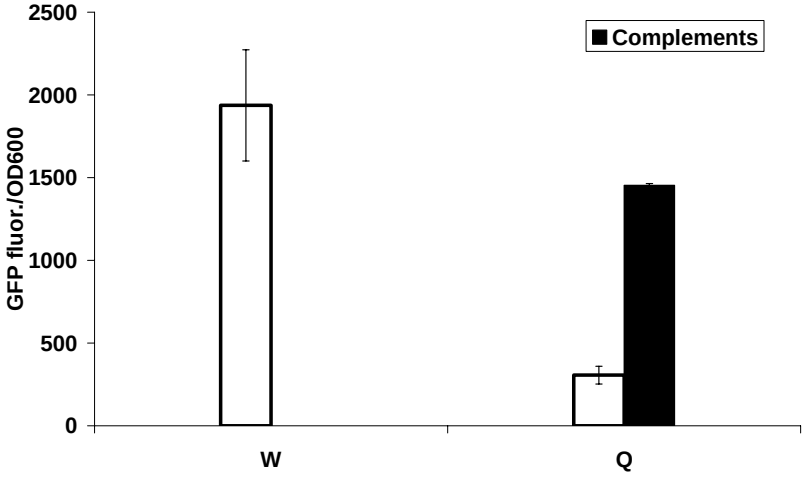

Figure 4.4: Curli operon expression: Cells bearing the reporter vector pJBA110 carrying the *csgB* promoter (P_{csgB}) fused to a short lived GFP were studied for gene expression. Fluorescence was read using a Bio-Tek Synergy HT spectrophotometer with a 485/20 excitation filter and a 530/25 emission filter and corrected with the OD600 of the corresponding culture. ΔQ^{DLP12} mutant showed a reduced curli operon expression, which was recovered in the complement

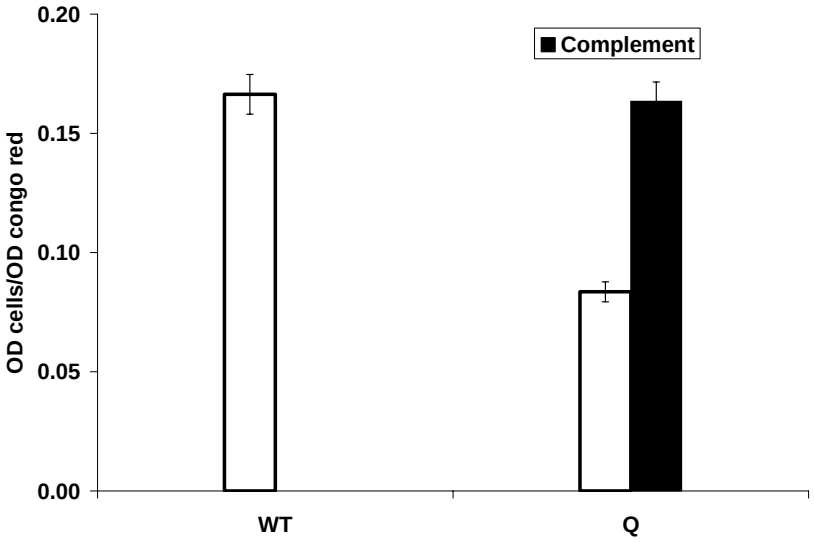

Figure 4.5: Quantitative Congo Red binding assay: Cells were grown on low salt LB plates for 72h at 30C. Cells were scraped from the plate, resuspended in phosphate buffer and OD600 was measured. Congo Red was added and incubated for 10 min at room temperature. Cells were pelleted, supernatant was sampled and OD500 was measured. OD600 was compared to OD500 to determine Congo Red binding. Experiments were done in triplicate. ΔQ^{DLP12} mutant showed less congo red binding, which was recovered in the complement

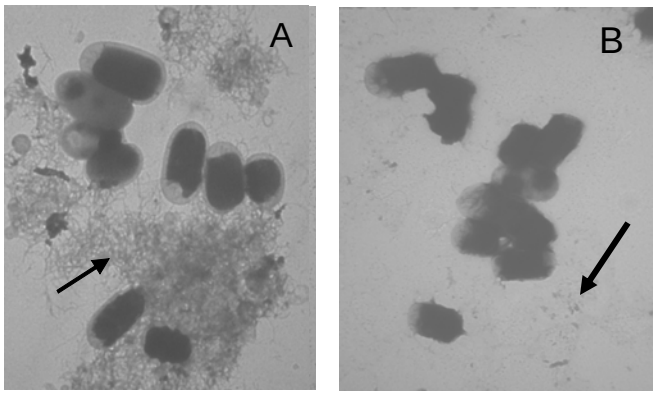

Figure 4.6: Electron Microscopy: 300 mesh Formvar grids were floated on cell cultures, washed and then negatively stained with 3% AMB. Grids were studied under a Philips Electron Microscope 201. Arrows point at curli fibers. (A) PHL628 wild type. (B) ΔQ^{DLP12} mutant.

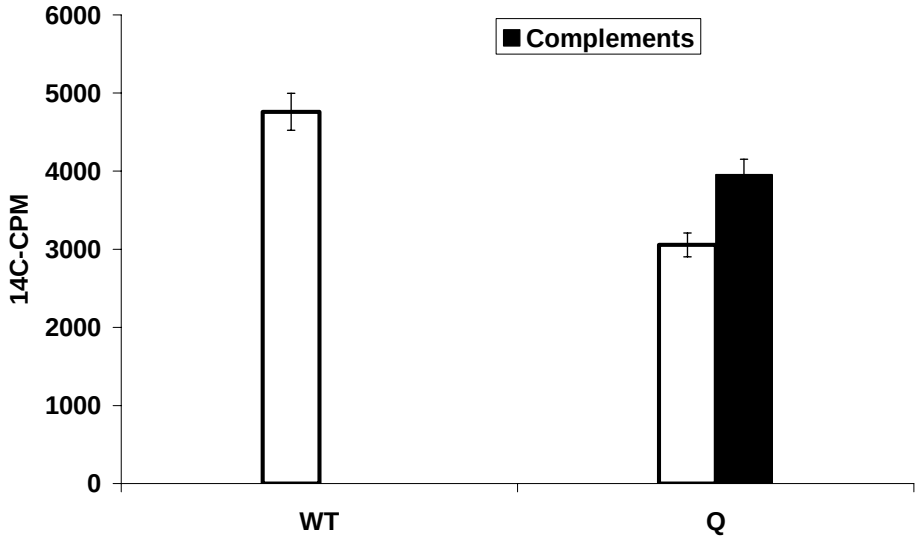

Figure 4.7: ^{14}C labeled NAG incorporation: Cells were grown in the presence of 14C label NAG. After 12 hours, cells were boiled, filtered, and assessed for counts per minutes in a Beckman Coulter® LS 6500 scintillation counter. Experiments were done in triplicates. ΔQ^{DLP12} was significantly affected for incorporation when compared to wild type control (α=0.05). Complement partially recovered the incorporation phenotype.

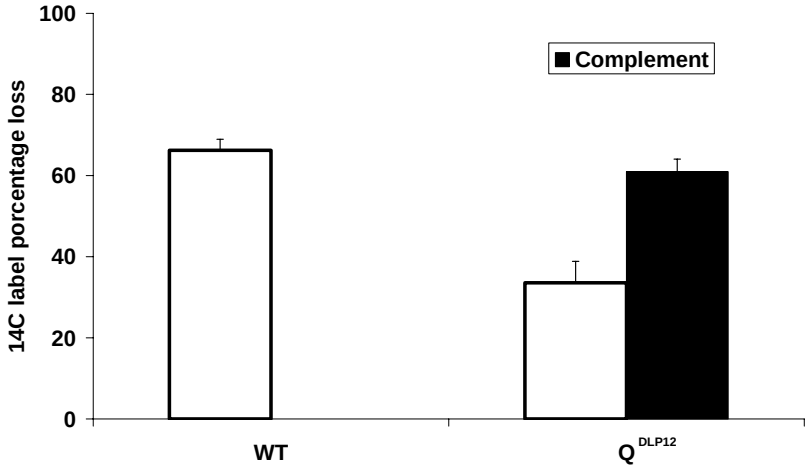

Figure 4.8: Peptidoglycan turnover: Cells were grown in the presence of 14C label NAG. After 12 hours, cells were washed and diluted in fresh media with unlabeled NAG and allowed to grow. 15 hours after re-incubation, cells were boiled, filtered, and assessed for counts per minute in a Beckman Coulter® LS 6500 scintillation counter. Experiments were done in triplicates. ΔQ^{DLP12} mutants were affected for turnover when compared to wild type control. ΔQ^{DLP12} shows some loss of label but this loss was still significantly less than in wild type control (α=0.05). Complement partially recovered the turnover phenotype.

As seen in previous chapters for the DLP12 lysis genes, *E. coli* over-expressing Q^{DLP12} showed the expected and dramatic increase in OD600 when cells entered early stationary phase, as a result from cell lysis (Figure 4.9A). As in the second chapter, lysis was confirmed by a side reader and the release of β-galactosidase activity into the filtered supernatant (Figure 4.9B and Figure 4.10). Figure 4.11 shows that this dramatic Q-mediated lysis did not occur in mutants lacking either S or R, although as expected, some lysis was observed in the Rz mutant since endopeptidases such as Rz have been shown to be important for lysis only under high cation concentrations. Despite *Q* over-expression, cell lysis did not occur until entrance into stationary phase (Figure 4.9A), just as it did in cells over-expressing R^{DLP12} (Chapter 3). This suggests the possibility of another element contributing to the coordination of lysis on-set despite Q^{DLP12} over-expression.

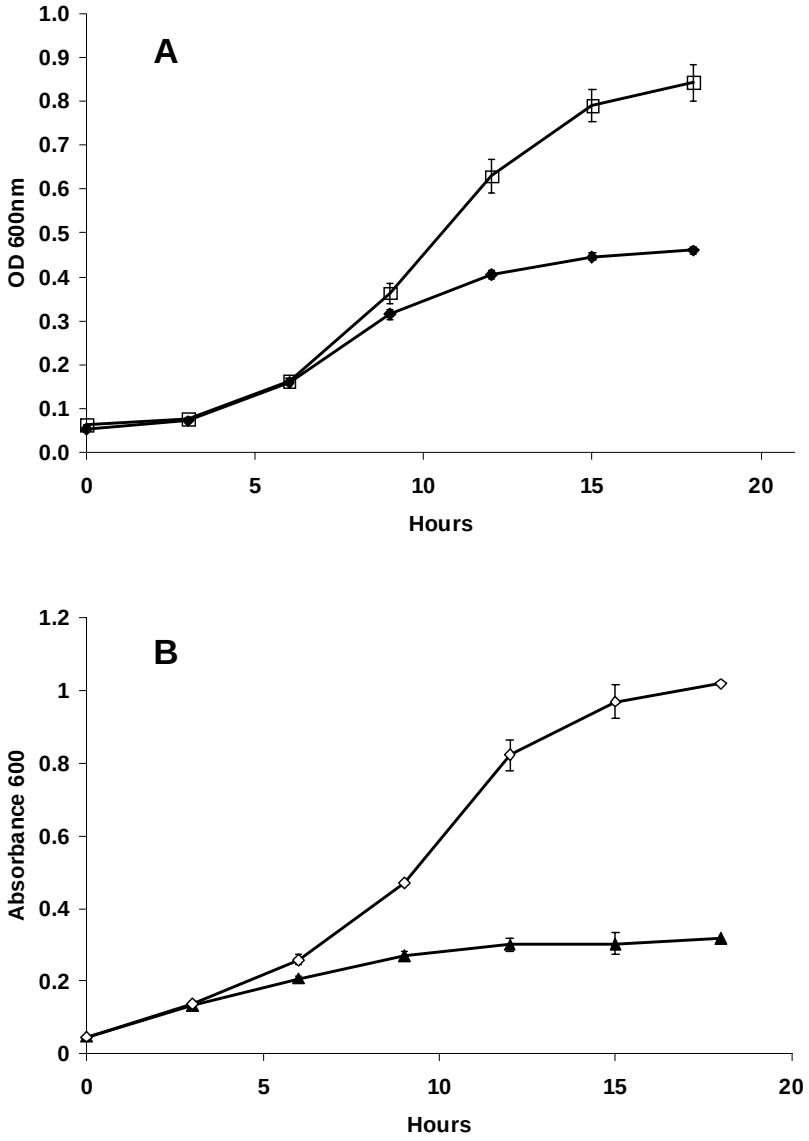

Figure 4.9: Over-espression of Q^{DLP12}: Overnight cultures were diluted in fresh LB and allowed to grow at 37°C. OD600 was monitored in a μQuant spectrophotometer (A) and a a Bechman side reader (B). Experiments were done in triplicates. Interestingly, PHL628 pBBR1MCS:Q^{DLP12} (□) showed an abnormal OD600 increase while wild type control PHL628 (◆) entered stationary phase. This increase in OD600 was interpreted as Q^{DLP12} induced cell lysis based on subsequent results.

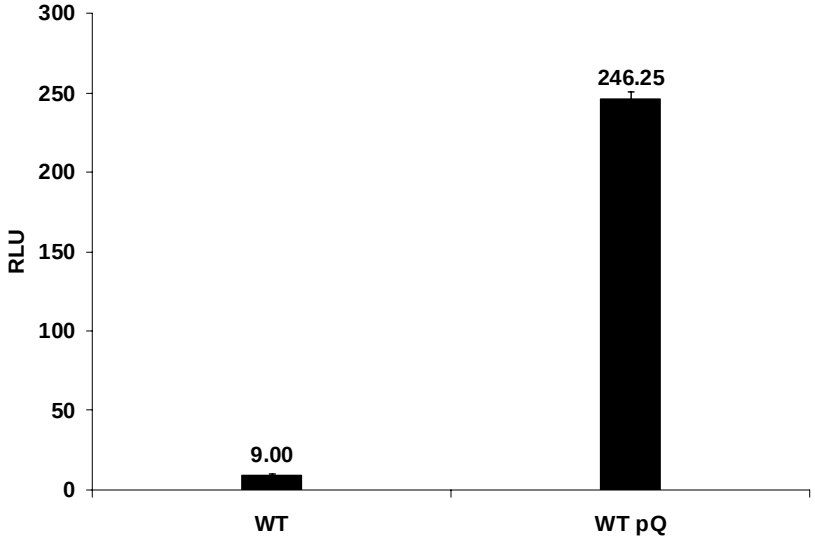

Figure 4.10: β-galactosidase activity: Overnight cultures were diluted in fresh LB and allowed to grow at 37°C. At 36 hours, samples of cell free supernatant of each culture were assayed for β-galactosidase. Galacton plus produces light when cleaved by β-galactosidase. Relative light units (RLU) were measured with a Packard Luminometer. Over-expressing strain PHL628 pBBR1MCS:Q^{DLP12} (WTpQ), showed a significant increase in relative light units (RLUs) when compared to the wild type control strain (WTp) (α=0.05).

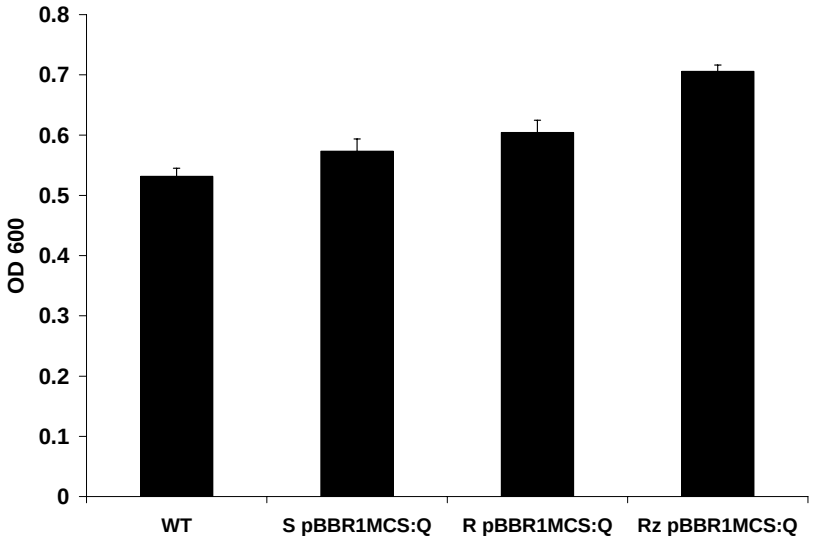

Figure 4.11: Over-espression of Q^{DLP12} in mutant backgrounds: Overnight cultures were diluted in fresh LB and allowed to grow at 37°C. OD600 was monitored in a μQuant spectrophotometer at 16 hours. Experiments were done in triplicates. As expected, S^{DLP12} mutant and R^{DLP12} over-expressing Q^{DLP12} showed less increased OD600 than Rz^{DLP12} mutant over-expressing Q^{DLP12} compared to the wild type control. This suggests that Rz^{DLP12} is not essential for cell lysis in this media.

Our work with transcriptional fusions of the promoter *SRRz*^{DLP12} (*essD*p,-GFP) in Chapter 2 confirmed that transcription of the DLP12 lysis operon is upregulated by RpoE, and that over-expression of RpoE results in increased transcription of *SRRz*^{DLP12} (Figure 4.12). We also demonstrated in Chapter 2 that *S*, *R*, and to a lesser extent *Rz* are required for RpoE-induced stationary cell lysis (23). Taken together with our observations regarding the impact of Q on *SRRz*^{DLP12} transcription, these findings suggest the possibility that Q and RpoE are working together to mediate transcription of the DLP12 lysis operon (Figure 4.12).

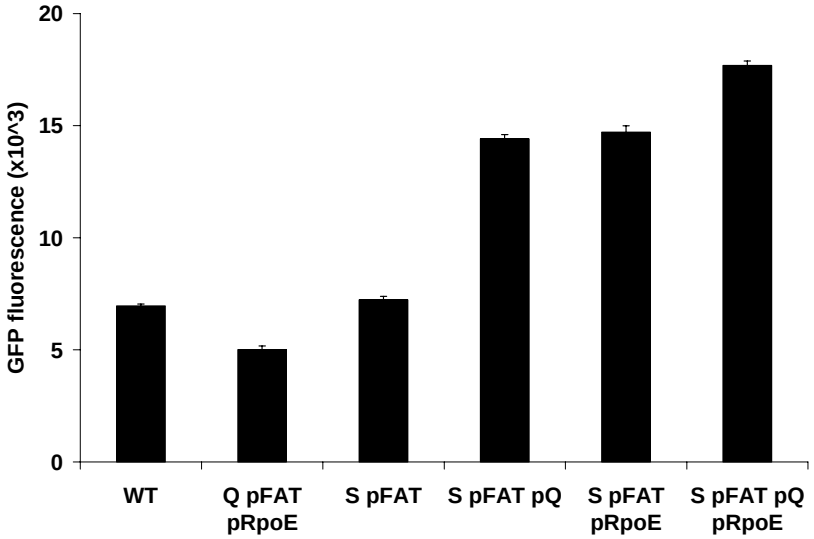

Figure 4.12: RpoE-mediated *essD*p transcription: pFAT1 (pJBA110:*essD*p) was used to assess the RpoE-mediated *essD*p transcription. Overnight cultures were diluted in fresh low salt LB, supplemented with arabinose for *rpoE* induction, and allowed to grow at 30^{o}C. At 12 hours, fluorescence was read using a Bio-Tek Synergy HT spectrophotometer with a 485/20 excitation filter and a 530/25 emission filter and corrected with the OD600 of the corresponding culture. Experiments were done in quadruplicates. Over expression of *Q*^{DLP12} and rpoE increased GFP transcription.

Q-mediated anti-termination requires interaction between the sigma factor of the holoenzyme and a terminator in the initial transcript (10, 30, 31, 36, 44). Although *in-silico* analysis (FinTerm® software from Softberry® at http://www.softberry.com/)

reveals the presence of a putative rho-independent terminator just downstream of the transcriptional start site (Figure 4.13), Q-sigma-termininator interactions have only been described for RpoD and RpoS (27, 31). However, as with most members of the sigma family, RpoE shares significant homology with RpoD, especially in region 4 (27, 29, 34) where lambda Q has been shown to act (10, 30).

```
       430       440       450       460       470       480
ucgauagaaaaaaguucagauaaaaauagagaucuacuucacaaaucaaacgagaaacca
.....................................................(((
       490       500       510       520       530       540
aaacuuacaucuugaaaUAAUCACAUugauuagaugaauauuuaucgcgcagugacauca
((((......)))))).....................................
```

Figure 4.13: Putative terminator sequence near S^{DLP12}: *In silico* analysis, using FindTerm® (from Softberry® at http://www.softberry.com/) suggests the existence of a terminator downstream (circa 490bp) of the transcription starting site and just upstream (circa 100bp) of the start of S^{DLP12} gene. Rho-independent putative terminator sequence is indicated by parenthesis and nucleotides in capital letters.

We therefore hypothesized that Q^{DLP12} and RpoE might be interacting to regulate transcription of $SRRz^{DLP12}$. Since efficient transcription of Q-dependent genes requires contact between the anti-terminator and the sigma factor, the absence of one of these components would be expected to affect the overall transcription of the genes they regulate (10, 18, 31). We therefore assessed the transcription from the *essDp*-GFP reporter in both wild type and in the Q mutant. We found that GFP fluorescence was significantly reduced in the absence of Q even when RpoE was over-expressed (Figure 4.12)

To demonstrate the physiological relevance of the reduced transcription we also assessed the ability of RpoE over-expression to induce lysis in a Q^{DLP12} mutant. As seen in Figure 4.14, the mutant did not lyse when *RpoE* was over expressed. In fact, OD600 values of the Q^{DLP12} mutant over-expressing *rpoE* resembled those of the

control strain not expressing the sigma factor (Figure 4.14**)**. As with the other phenotypes, Q^{DLP12} complementation rescued the RpoE-mediated lyis in the mutant, but in this case actually resulted in greater lysis than observed in the wild type over expressing *rpoE* (α=0.05) (Figure 4.14**),** a result that is likely due to a copy number effect.

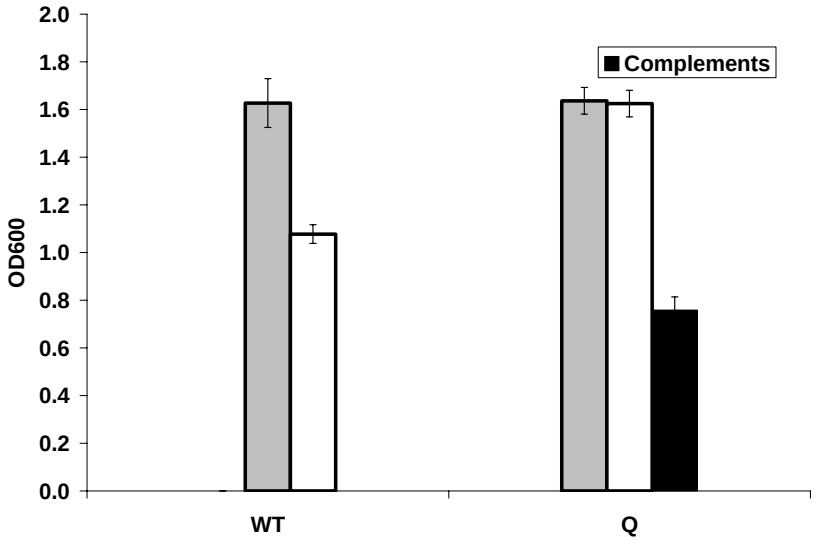

Figure 4.14: RpoE induced lysis: Overnight cultures were diluted in fresh low salt LB, allowed to grow at 30^{o}C, and *rpoE* was induced as previously described (Kahbir). At 36 hours cultures were sampled for OD600. Experiments were done in triplicates. ΔQ^{DLP12} mutant over-expressing rpoE showed less lysis than expected (WT and Q white bars). In both cases, over-expressing *rpoE* (Q white bar) and not over-expressing *rpoE* (Q grey bar), ΔQ^{DLP12} showed comparable growth (α=0.05) to wild type control strain not over-expressing *rpoE* (WT grey bar). When compared with wild type over-expressing *rpoE* (WT white bar), the expected lysis phenotype was recovered in the complement (Q black bar).

Over expression of both Q^{DLP12} and RpoE in the same cells had a modest additive effect on transcription from the *essD*p-GFP reporter vector. Consistent with that, we found that these experiments needed to be performed in an S^{DLP12} mutant since maintenance of plasmids encoding both Q and RpoE at the same time in the wild type lead to high levels of lysis even in the absence of inducers (Data not shown).

These results are consistent with Q^{DLP12} acting as an antiterminator and with the hypothesis that Q^{DLP12} and RpoE act together to regulate transcription of the DLP12 lysis operon.

In *E. coli*, the defective prophage remnant DLP12 encodes a putative antiterminator gene *ybcQ* or Q^{DLP12} which shares high homology with Q^{21}. Here we present results that point to Q^{DLP12} as a possible antiterminator for the $SRRz^{DLP12}$ operon and present genetic evidence for an apparent interation with RpoE. This is the first example of a lamdoid antiterminator participating in host cell physiology outside of the lytic cycle and provides the first preliminary evidence for an interaction between an antiterminator and the alternative sigma factor RpoE.

Although it enhances *SRRz* transcription, Q^{DLP12} antitermination seems not to be essential for $SRRz^{DLP12}$ transcription. We show that this regulatory activity of Q^{DLP12} affects the expression of $SRRz^{DLP12}$ genes, whose products ultimately affect peptidoglycan metabolism, altering the cell wall. It is likely this last effect that indirectly leads to a reduction in curli expression and biofilm formation, although more work needs to be done to determne who this signal is transduced to the *csg* operons. More experiments also need to be done to determine the details about the recognition and interaction between Q^{DLP12}, and the *essD*p promoter on the $SRRz^{DLP12}$ operon. Also, the physical contact between Q^{DLP12} and RpoE in the putative paused complex needs to be addressed; we anticipate that this interaction will be similar to other Q antiterminator and sigma factor interactions, where it has been shown that the antiterminator interacts with region 4 of the sigma factor (10, 18, 31, 34).

4.4 METHODS

Bacterial strains and growth conditions

E. coli strain PHL628 is a MG1655 derivative with an ompR234 mutation (42). *E. coli* PHL628 and mutants were routinely grown on Luria Bertani (LB) media at 37^{o}C overnight with shaking (150 rpm) and supplemented with 50 ul/ml Kanamycin (Kan) unless otherwise stated. For early stationary phase lysis assay and promoter studies low salt LB (5 g/l NaCl) media was used and incubated at 37^{o}C. In ^{14}C labeled N-acetylglucosamine (NAG) incorporation assays *E. coli* PHL628 and mutants were grown on minimal media (MSM) (28) supplemented with casamino acid (0.2%) and 50μl/ml Kan unless otherwise stated. When required, plates and media were supplemented with Ampicillin (Amp 150μl/ml) and/or Chloramphenicol (25μg/ml). For induction, media was supplemented with 1% lactose and 1% arabinose according to the experimental needs.

Mutants and plasmids construction

Deletion mutants were constructed by allele replacement using Lambda RED strategy as described elsewhere (9). Briefly, Chloramphenicol (Cm) interrupted version of the genes were created using PCR-mediated ligation (7). In general, each construct consisted of a 5'and a 3' 500-bp homology region to the gene of interest link by a 1kb Cm cassette flanked by flip recombinase target (FTP) sites. This linear DNA was transformed into wild type *E. coli* PHL628 cell expressing the Lambda RED recombinase. The 5' end and 3'end homology regions allowed RED system to replace the wild type allele with our interrupted version. Clone screening was carried out on plates supplemented with the adequate antibiotic. After confirmed recombination the

Cm marker was removed using FLP-recombinase from plasmid pCP20 (9). All plasmid vectors are temperature sensitive and were cured from the cells by growing cells at 43ºC. The markerless knockout mutants were confirmed by PCR and sequencing.

Complements were constructed using full wild type version of the genes cloned into pBBR1MCS (lactose inducible) (24).

For *rpoE* over-expression, pBAD24:*rpoE* plasmid was obtained from Dr. Yamada (23). In this plasmid, rpoE expression is under control of an arabinose inducible promoter. An empty version of pBAD24 was used as control vector (19). To circumvent same antibiotic selection markers in the promoter experiments (see below), *rpoE* was also cloned into the expression vector pBBAD18T (39), also under the control of the pBAD promoter.

For promoter expression, the promoter-less *gfp* vector pJBA110 was used (2). Plasmid pFAT1 was constructed by cloning the DLP12 lysis gene promoter, *essDp*, upstream of the *gfp* gene in pJBA110 using KpnI and XbaI restriction sites (2).

Wild type control strains and mutant control strains were constructed by transformation of empty vectors as required per experiment.

Table 4.2: Primers, plasmids and bacterial strains.

Name	Sequence	Reference
Primers		
Q^{DLP12} Forward1	5'-ACAGGGCCCAATGCTGGATATCGGCCTGGCTAT-3'	This study
Q^{DLP12} Reverse1	5'-ACAGGATCCACCTTGAAACAGCATCCACGCAAG-3'	This study
Q^{DLP12} internal Reverse	5'-CCCAACGCTCAAGAACCATCTGAA-3'	This study
Q^{DLP12} internal Forward	5'-TAAGGAGGATATTCATATGGCTGAGGGCATAATTGAAGGGA-3'	This study
P1Q	5'-TTCAGATGGTTCTTGAGCGTTGGGGTGTAGGCTGGAGCTGCTTCG-3'	This study
P2Q	5'-TCCCTTCAATTATGCCCTCAGCCATATGAATATCCTCCTTA-3'	This study
Plasmids		
pBAD24		Guzman, 1995
pBAD24:*rpoE*		Kahbir, 2005
pBBAD18T		Sukchawalit, 1999
pBBAD18T:*rpoE*		This study
pBBRMCS		Kovach, 1995
pBBRMCS:Q^{DLP12}		This study
pJBA110		Andersen, 1998
pJBA110:*essD* p		This study
pJBA110:*csgB* p		Junker, 2004
pCP20		Datsenko, 2000
pKD3		Datsenko, 2000
pKD46		Datsenko, 2000
Strains		
E. coli PHL628		Vidal, 1998
E. coli PHL628 ΔQ^{DLP12}		This study
E. coli PHL628 ΔQ^{DLP12} pBBR1MCS:Q^{DLP12}		This study
E. coli PHL628 pBAD24:*rpoE*		This study
E. coli PHL628 ΔQ^{DLP12} pBAD24:rpoE		This study
E. coli PHL628 ΔQ^{DLP12} pBBR1MCS:Q^{DLP12} pBAD24:rpoE		This study
E. coli PHL628 ΔS^{DLP12} pJBA110:*essD* p		This study
E. coli PHL628 ΔS^{DLP12} pBBAD18T:rpoE pJBA110:*essD* p		This study
E. coli PHL628 ΔS^{DLP12} pBBR1MCS:Q^{DLP12} pJBA110:*essD* p		This study
E. coli PHL628 ΔS^{DLP12} pBBR1MCS:Q^{DLP12} pBBAD18T:rpoE pJBA110:*essD* p		This study
E. coli PHL628 ΔQ^{DLP12} pJBA110:csgBp		This study
E. coli PHL628 ΔQ^{DLP12} pBBR1MCS:Q^{DLP12} pJBA110:csgBp		This study
E. coli PHL628 pBBR1MCS:Q^{DLP12}		This study

Growth curve and β-galactosidase activity

Overnight LB cultures were diluted 1:100 on fresh media. The new cultures were grown at 30^{o}C for 24 hours in 96-well plates. OD600 was measured every 30 minutes in a μQant spectrophotometer (Bio-Tek Company Info). To assess the actual growth of the different strains, media samples were taken during growth curve to assess β-galactosidase activity in the supernatant as in previous chapters.

Membrane susceptibility to SDS

Wild type *E. coli* PHL628, deletion mutants and complements, were challenged with an inhibitory concentration of SDS to study their membrane susceptibility (21). Overnight LB cultures of the mutants were diluted 1:100 (OD600=0.05) and exposed to 5% SDS. Then, cultures were incubated shaking for 3h at 37C and OD600 was measured. Absorbance was compared to unchallenged cultures incubated under the same conditions.

^{14}C-NAG Protocol for turnover

Peptidoglycan turnover was measured using a modification of a previously described method (11). Briefly, overnight LB cultures were diluted 1:100 on Media A. ^{14}C labeled NAG (0.2μCi) and unlabeled (0.05%) were added to the growth media. To allow peptidoglycan labeling, cell cultures were grown at 30^{o}C for 12 hours. Then, labeled cells were washed 3 times with fresh Media A; and diluted 1:4 in media supplemented with unlabeled (0.05%). Cells were incubated for an additional 15 hours. Then, samples were boiled in 4% SDS and filtered through 0.2 μm Durapore® filter (Millipore®), Filters were washed once with LiCl buffer and three times with milliQ water. Then, filters were placed in scintillation cocktail, ScentiSafe® Econo 1 (Fisher Scientific®) and read in Beckman Coulter® LS 6500 scintillation counter.

^{14}C labeled NAG incorporation

^{14}C labeled NAG incorporation protocol was modified from a previously described method (11). Briefly, overnight LB cultures were diluted 1:100 on Media A. ^{14}C labeled NAG and unlabeled NAG were added to the growth media. To allow ^{14}C

labeled NAG incorporation, cell cultures were grown at 30^{o}C for 12 hours. Then, samples were boiled in 4% SDS and filtered through 0.2 μm Durapore® filter (Millipore®) filter. Filters were washed once with LiCl buffer and three times with milliQ water. Then, filters were placed in scintillation cocktail, ScentiSafe® Econo 1 (Fisher Scientific®) and read in Beckman Coulter® LS 6500 scintillation counter. Experiments were done in triplicates.

RpoE overexpression

Plasmid pBAD24:*rpoE* was transformed into wild type and mutants. Previously described method with few modifications was used to study the effect of over-expression of *rpoE* in DLP12 mutants. Briefly, overnight cultures were diluted 1:100 in low salt LB (5g/l NaCl) and aliquoted in 96-well plates (COASTAR). Cultures were incubated at 37C and their absorbance (OD600) monitored for 72h. Arabinose (1%) was added at 12h to induce the overexpression of *rpoE* from the pBAD promoter. The experiments were done in quadruplicates. Antibiotics were added when needed.

Promoter fusion studies

Reporter plasmid pJBA110 carrying the *csgB* (curli) promoter was transformed in each of the PHL628 strains (22). Cells were allowed to grow in MSM CA. At various time points fluorescence was measured using a Bio-Tek Synergy HT spectrophotometer with a 485/20 excitation filter and a 530/25 emission filter and corrected with the OD600 of the corresponding culture. Measurements were done in quadruplicate.

Reporter plasmid pFAT1, a pJBA110 (2) derivative constructed in our lab, was transformed into cells and selected for on LB Amp plates. Cells were allowed to grow in low salt LB at 37^{o}C. Arabinose was added at 6h to induce expression from vector

pBBAD18T, which contained a copy of *rpoE*. At various time points fluorescence was measured using a Bio-Tek Synergy HT spectrophotometer with a 485/20 excitation filter and a 530/25 emission filter and corrected with the OD600 of the corresponding culture. Measurements were done in quadruplicate.

Biofilm architecture

Biofilms were grown on MatTek Cultureware glass bottom dishes (Ashland, MA) for imaging. Overnight cultures were inoculated (1% v/v) into 3ml of minimal media (MSM) supplemented with 0.2% casamino acid (CA). Dishes were incubated at 30ºC for 72h with shaking on an orbital shaker at 50rpm. Media was exchange every 24h with new fresh media. After 72h cells biofilms were stained with acridine orange and studied under a Leica confocal microscope. To assess biofilm formation and architectural features a total of 8 Z-image stacks (0.25μm steps) were obtain for each mutant and then analyzed with COMSTAT (20).

Attachment

Attachment to PVC surface was studied as described previously (12). Overnight cultures were diluted 1:5 to a total volume of 120 ul on a 96-well PVC plates and incubated at 30C shaking (50 rpm on an orbital shaker) for 16 hours. After 16 h turbidity (OD600) were measured in each well. Then, 100 ul of a solution of 1% Crystal Violet (CV) was added to each well. After 15 minutes of incubation at room temperature wells were thoroughly washed with water. Plates were allowed to dry for 15 minutes at room temperature. After the plate was dry 100 ul of 95% EtOH was added to each well and incubated for 10 minutes at room temperature. Then, CV absorbances (OD 500) were measured in each well. The original well turbidity

(OD600) was compared to the CV absorbance (OD500). Experiments were done in triplicate.

Autoaggregation

Cell autoaggregation was measured as described previously (13). Briefly, 4ml of overnight LB cultures of each knockout mutants and each of their complements were incubated static for 12h at 37C. OD 600 was measured from samples taken from the top 5mm of each culture with minimal disturbance. Then, cultures were vortexed and sampled again. OD600 values (unvortexed and vortexed) for each mutant were compared as a measurement of cell autoaggregation. Experiments were done in quadruplicate. To determine if fimbriae played a role in autoaggregation 1% a-Methyl-D-Mannopyranoside was added to the media in a separate experiment that was carried out as described above.

Electron microscopy

Cell were grown on LB at 37C shaking overnight. 300 mesh Formvar copper grids (EMS, Hatfield, PA) were floated on 25ul of the overnight cultures for 1 minute. Then the grids were transferred to a solution containing 3% ammonium molybdate (3% AMB) pH=7.0 or a solution containing 1% potassium phosphotungstic acid (1% KPTA) for 2 minutes. Grids were then rinsed in MilliQ water for 1 minute and allow to dry before observation. Cells were studied on a Philips Electron Microscope at different magnification ranging from 7000x to 45000x. Snap shots were taken using a MicroFire digital camera and software by Optronics.

Quantitative Congo Red binding assay

Cells were plated on low salt LB (5g/l NaCl) and incubated at 30C for 72h (15). After three days cells were scraped from the plates and resuspended in 500ul phosphate buffer pH 7.0. Serial dilutions were prepared in phosphate buffer and turbidity of each dilution was measured (OD600). Then, cells were centrifuged at 14000 g for 3 minutes and resuspended in 1ml of a 0.002% solution of Congo Red. Tubes were incubated at room temperature for 10 min to allow Congo Red binding. After the incubation tubes were centrifuged at 14000 g for 10 min, and the Congo Red remaining in the supernatant was measured (OD500) and compared to a cell-free Congo Red tube that was treated as a control tube. Dilution turbidity (OD600) and Congo Red absorbance (OD500) were plotted in graphs. Experiments were done in triplicate.

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