

**CHARACTERIZING THE TRANSCRIPTIONAL REGULATION OF THE  
DLP12 LYSIS CASSETTE AND ITS EFFECTS ON CURLI EXPRESSION AND  
BIOFILM FORMATION IN *Escherichia coli*.**

**A Dissertation Presented to the Faculty of the Graduate School  
of Cornell University  
In Partial Fulfillment of the Requirements for the Degree of  
Doctor of Philosophy**

by

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**Cornell University 2013**

Bacteria often exist in elaborate communities known as biofilms. These biofilms are inherently resistant to environmental insults such as osmotic stress, desiccation, antibiotic treatment, and immune system attack. As such, bacteria in biofilms are regularly the cause of nosocomial infections and represent a significant threat to human health. The resilient nature of biofilm structures has prompted significant research in the regulatory pathways that govern biofilm formation and maintenance. By acquiring comprehensive knowledge of biofilm forming signaling pathways, we could elucidate novel drug targets to either disperse existing biofilms or prevent their formation altogether.

Here, we describe how YbcQ and RpoE affect the regulation of the genes encoding a defective lambdoid prophage's (DLP12) lysis machinery and demonstrate how loss of YbcQ impacts curli production in *E. coli*. By demonstrating that mutants defective in peptidoglycan recycling can rescue the curli deficient phenotype of *ybcQ* mutants we provide preliminary evidence for a mechanistic link between peptidoglycan recycling and curli expression and biofilm formation. Our work ends with the discovery that the peptidoglycan degradation product GlcNAc-6P appears to serve as a previously unappreciated global effector by derepressing NagC regulation of H-NS expression. We

therefore propose that NagC is as actually an important global super-regulator in *E. coli*, sensing changes in peptidoglycan status by responding to changes in GlcNAc-6P and subsequently modulating a significant subset of cellular regulatory pathways via H-NS.

This work furthers our understanding of fundamental processes regulating biofilm formation and lays the foundation for understanding how a previously unappreciated signal of cell wall status is sensed by the bacteria, and how that signal is involved in regulating the production of proteins that are important for adherence to surfaces and the ability to form biofilms. This pathway could prove a potential target for combating biofilm communities associated with chronic infection or biofouling in engineered systems.

## BIOGRAPHICAL SKETCH

Karl-Gustav Rueggeberg was born on June 18<sup>th</sup> 1984 in Houston, Texas. He grew up with a constant fascination with the natural world and harbored a burning desire to understand how life worked. He graduated from Greenwich High School in 2002 and, with inspiration from his Advanced Placement Biology and Chemistry teachers, went on to study Biochemistry at Bates College in Lewiston, Maine. He earned his Bachelors of Science degree in 2006.

In 2006, Karl-Gustav moved to Ithaca, New York and enrolled at Cornell University to pursue his Ph.D. in Microbiology. He joined the laboratory of Dr. Anthony G. Hay, where he began his research on a Defective Prophage Element (DLP12). He focused on the transcriptional regulation and expression of specific lytic genes within DLP12 whose protein products influence biofilm formation by *Escherichia coli*. During this period, Karl-Gustav trained and mentored several undergraduate research assistants that worked diligently on these projects, helping him make this dissertation a reality. While at Cornell, Karl-Gustav became a teaching assistant for the General Microbiology Course where he helped teach the microbiology laboratory sections and lead over a dozen small group discussions. He taught for six semesters, and in his final semester he was promoted to Head TA of the course.

His future plans include pursuing a career in the pharmaceutical sector, with research goals aimed at novel antibiotic drug development.

*Quiero dedicar esta tesis a mi familia, cuyo apoyo me dio la fuerza para lograr este gran éxito. Gracias por todo, los quiero mucho.*

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## CHAPTER 1

### INTRODUCTION: IMPACTS OF FUNCTIONAL AND CRYPTIC PROPHAGE ON BIOFILM FORMATION

#### **1.1 BIOFILMS**

In nature, bacteria frequently assemble into complex communities of adherent cells known as biofilms. This biofilm lifestyle imparts numerous advantages to the constituent bacteria, increasing their overall fitness when confronted with adverse conditions. Biofilms have existed for billions of years, with early evidence of putative biofilm microcolonies appearing in the South African Kornberg formation dating back to 3.4 gya (Westall et al., 2001). In addition, filamentous biofilms were discovered in deep-sea hydrothermal rocks of the Pilbara Craton, Australia, estimated to have originated 3.2 billion years ago (Rasmussen 2000). Modern day biofilm structures that bear striking similarities to these microfossils have been found in hydrothermal environments such as deep-sea vents and hot springs (Taylor, Wirsen et al. 1999; Reysenbach and Cady 2001). Clearly, this form of bacterial growth has proven an effective strategy since bacteria first originated on the primitive earth.

Planktonic, free floating bacteria transition to a biofilm lifestyle in response to environmental stimuli including surface contact, shear stress, nutrient limitation, and elevated levels of quorum sensing molecules (Hall-Stoodley, Costerton et al. 2004). Biofilm formation can be subdivided into five stages: initial attachment, irreversible attachment, microcolony formation, biofilm maturation, and dispersion (Flemming and Wingender 2010). During initial attachment, planktonic cells utilize surface appendages

including fimbriae and flagella to interact with a substrate in a reversible manner. If conditions are suitable, the cells will adhere more tightly to the surface and begin to synthesize additional surface structures such as pili, curli and/or extracellular polymeric substances (EPS) to enhance attachment. Once irreversibly bound, the biofilm cells begin to multiply and grow into discrete structures known as microcolonies. These microcolonies continue to divide and differentiate into elaborate multicellular structures that resemble pillars and mushrooms. Water filled channels form throughout these structures, facilitating nutrient exchange, waste removal, and cell-cell signaling. Mature biofilms are exceedingly resilient structures and are capable of withstanding significant stress from the outside environment. However, on occasion, biofilms undergo dispersion; a process by which cells are released in a planktonic state through digestion of the EPS or as aggregates due to shear stress. The free floating cells or cell clumps can then settle on more suitable surfaces to colonize and restart the biofilm cycle (Sauer, Camper et al. 2002; Stoodley, Sauer et al. 2002).

Biofilm formation is dependent on EPS, whose composition varies by species, but is typically a complex conglomeration of exopolysaccharides, proteins, lipids, and in some cases, genetic material, which immobilizes the cells and serves as a scaffold for the three dimensional biofilm structure (Karatan and Watnick 2009). This biofilm matrix can serve as a reservoir of genes for lateral gene transfer as well as a nutrient source to supplement metabolism during starvation (Flemming and Wingender 2010). In addition, the matrix protects the bacteria from predation, desiccation, ultraviolet radiation, oxidizing agents, charged biocides, certain antibiotics and metal ions, and host immune defenses (Hall-Stoodley, Costerton et al. 2004). Lastly, the EPS layer serves to keep the

cells in close proximity, which enables cell-cell communication and development of synergistic subpopulations (Flemming and Wingender 2010). These characteristics of the biofilm lifestyle endow bacteria with a competitive advantage to overcome substantial competition for limited resources and hostile environmental conditions.

## **1.2 BACTERIOPHAGES AND BIOFILMS**

During biofilm development, various genes involved in attachment, metabolism, and cell wall maintenance are upregulated. Interestingly, recent work has shown that prophage genes are highly upregulated in both gram positive and gram negative bacterial biofilms (Whiteley, Bangera et al. 2001; Stanley and Lazazzera 2004). In *Pseudomonas aeruginosa* biofilms (found in cystic fibrosis patients), a prophage resembling the filamentous prophage Pf1 is involved in cell death, biofilm dissemination, and dispersal of planktonic cells (Webb, Thompson et al. 2003). Further work has demonstrated that prophage in *P. aeruginosa* are responsible for increased attachment and accelerated biofilm development (Webb, Lau et al. 2004). In addition, another filamentous phage, Pf4, is essential for several stages of the *P. aeruginosa* biofilm life cycle including microcolony formation, appearance of small colony variants (SCVs), and cell death (Rice, Tan et al. 2009). In *Shewanella oneidensis* strain MR-1, three different prophages: LambdaSo, MuSo1, and MuSo2 have been shown to significantly affect biofilm formation (Godeke, Paul et al. 2010). *S. oneidensis* strains with individual phage deletions have slightly diminished biofilm formation; however phage-less mutants exhibit a highly impaired biofilm production throughout all stages of development. The authors

propose that prophage lytic action is primarily responsible for the robust biofilm formation capacity of *S. oneidensis*, through the release of extracellular DNA (eDNA) and other crucial biofilm-promoting factors (Godeke, Paul et al. 2010).

*Escherichia coli* MG1655 is also responsive to the action of a bacteriophage; Lacqua and colleagues demonstrated that when *E. coli* cells are exposed to environmental phages, they develop phage-resistant subpopulations that exhibit increased biofilm formation (Lacqua, Wanner et al. 2006). It is plausible that biofilm formation in *P. aeruginosa*, *Shewanella oneidensis*, and *E. coli* is occurring in part as a direct physiological response to the selective pressure arising from phage infection (Sutherland, Hughes et al. 2004), although the mechanistic underpinnings of such a response are not well understood..

Mutation and recombination can result in prophage that are incapable of excising from the host chromosome. When this occurs, these prophage are regarded as defective or cryptic entities (Redfield and Campbell 1987; Campbell 1994). A recent publication, however, demonstrated that these cryptic phage elements can still influence host physiology in *E. coli* K12 (Wang, Kim et al. 2010). Deletion of the nine cryptic prophages decreases cell growth rate, resistance to quinolone and  $\beta$ -lactam antibiotics, as well as resistance to acid, heat, osmotic stress and oxidative stress. Most notably, *E. coli* that lack the defective prophage elements are significantly compromised for biofilm formation, underscoring the vital role these phage genes play in bacterial differentiation and growth (Wang, Kim et al. 2010).

Thought of until recently as genetic baggage or junk (Campbell, 1994; Redfield and Campbell, 1987), these defective prophages are inactive in a purely reproductive

sense; it is evident that certain phage genes are still being expressed within the host and are contributing to host fitness in adverse environments (Wang, Kim et al. 2010). Indeed, expression of the phage lysis genes still occurs in one of the cryptic phages, DLP12 and the gene products are fully functional (Srividhya and Krishnaswamy 2007; Toba, Thompson et al. 2011). Much research still needs to be done to understand the exact regulatory mechanisms by these defective phage genes affect their hosts. These works taken together, however, show conclusive evidence for the involvement of phage genes in bacterial biofilm formation.

### **1.3 LAMBDA BACTERIOPHAGE LYSIS GENE REGULATION**

represent a unique group of temperate phages that infect a wide range of host bacteria (Campbell, Schneider et al. 1992). These phages are lysogenic; they can integrate their chromosome into that of the host through site specific recombination, enabling the phage to undergo duplication as the bacterial host divides. Transcriptional regulation by phage-encoded factors maintains the phage in a dormant, integrated state, preventing the expression of genes encoding phage excision and host cell lysis (Campbell, Schneider et al. 1992). The integrated lambda phage, known as a prophage, remains in the bacterial chromosome until either host DNA is damaged or the bacterial membrane becomes compromised. At this point, the late phage genes involved in viral DNA replication/packaging and subsequent host cell lysis are expressed and the subsequent phage particles can proceed to infect a more stable host (Svenningsen, Costantino et al. 2005). This phase is termed the lytic cycle. The switch between the lytic



and lysogenic life cycles is linked to expression of lambda activator/repressors, *cro* and *cI*. The lysogenic cycle is controlled by the gene product of *cI* which binds specific operators, preventing transcription of all lambda genes excluding those required for chromosomal integration and lysogeny. In contrast, the lytic cycle depends on the abundance of Cro. This factor represses the expression of *cI* and activates the other genes for excision and entry into the lytic cycle (Svenningsen, Costantino et al. 2005)

DLP12, at one point an active lambdoid phage, is present in other enteric bacteria including *Salmonella* which diverged from *E. coli* over 100 million years ago (Lawrence and Ochman 1998). The fact that DLP12 has been conserved among distinct genera of enteric bacteria over such an extensive time period suggests that the phage genes must benefit the host in some way. Recent experimental evidence from the Hay group has elucidated various physiological functions that may be governed by the DLP12 lysis cassette which include: cell wall integrity, biofilm formation, and curli production (Toba, Thompson et al. 2011).

In prophages, gene expression is a process that is dependent on the host transcription apparatus. Within bacteria, transcription is performed by a single core RNA polymerase (RNAP) comprised of  $\alpha$ ,  $\beta$ ,  $\beta'$ , and  $\omega$  subunits. RNAP core is recruited from the cytoplasm by a  $\sigma$  factor which binds the polymerase and targets it to a specific promoter region.  $\sigma^{70}$ , the primary *E. coli*  $\sigma$  factor, binds to the -35 and -10 (position relative to transcription start site) promoter elements via DNA-protein contacts between  $\sigma$  region 4 and 2, respectively. Thus transcription of various genes is directly dependent on the availability of sigma factors that recognize specific promoter sequences (Murakami and Darst 2003). Once RNAP is bound to a  $\sigma$  factor, the complex (known as the

holoenzyme) can melt the DNA double helix at an A/T rich region, forming the open complex. At this point, RNA synthesis begins. Nucleotides likely enter through the secondary channel in  $\beta'$ , where they are directed to the active site. Base specific pairing between the template strand and the incoming nucleotides ensures fidelity. Subsequent interaction with two  $Mg^{2+}$  ions coordinated by the aspartate triad of  $\beta'$ , destabilizes the phosphodiester bond between the NTP's  $\alpha$  and  $\beta$  phosphates and allows for nucleophilic attack by the 3' hydroxyl of the growing RNA chain. The holoenzyme escapes from the promoter by scrunching the DNA as it pulls the downstream strands through the RNAP core. The potential energy stored in the stressed complex can be alleviated through premature RNA release (abortive initiation) or by breaking  $\sigma$ -DNA contacts, promoting RNAP to escape the paused state and begin elongation (Kapanidis, Margeat et al. 2006; Revyakin, Liu et al. 2006). Following promoter escape, RNAP releases the  $\sigma$  factor, thus forming the elongation complex. The elongation complex is very stable until it reaches a terminator, which prompts RNAP to release the RNA transcript and dissociate from the DNA template. Bacteria possess three known methods of transcription termination: intrinsic, rho-dependent, and Mfd-mediated (Santangelo and Roberts, 2004). Intrinsic terminators form G/C rich secondary hairpin structures immediately upstream of a U-rich sequence in the RNA transcript. In this case, termination is caused by the transcript itself, where the hairpin likely disrupts the weak contacts between the U-rich sequence and the DNA template (Yarnell and Roberts 1999). Rho-dependent termination occurs when rho (an ATP dependent helicase) binds *rut* sites on the RNA transcript and translocates toward the 3' end, until it interacts with RNAP and dissociates the complex. Mfd-mediated termination is dependent on Mfd, an ATPase that recognizes stalled elongation

complexes, binds upstream, and pushes the complex downstream. This forward translocation likely causes transcription bubble collapse and complex dissociation (Park, Marr et al. 2002).

One of the alternative sigma factors,  $\sigma^E$  (*rpoE*), which is expressed during the stationary phase of growth, is upregulated during physiological stress caused by high temperature or membrane perturbation (Kabir, Yamashita et al. 2005). It directs its own transcription as well as that of additional sigma factors, such as  $\sigma^{32}$ , a heat shock factor. *In silico* predictions suggest that the DLP12 lysis cassette is transcribed under the control of RpoE, (Rhodius, Suh et al. 2006). However, there is no direct evidence to support this claim. It seems unusual that these prophage genes would be regulated by an alternative sigma factor, since the known lambdoid phage genes are controlled by *rpoD* ( $\sigma^{70}$ ) (Casjens 2003). Yet the substantial homology between  $\sigma^E$  and the primary sigma factor, indicated by conserved DNA binding regions 2 and 4, suggests the potential for a similar mode of action (Missiakas and Raina 1998). In phage lambda and 82,  $\sigma^{70}$  works in concert with core RNAP and additional transcription factors such as NusA to express the late phage genes (Shankar, Hatoum et al. 2007).

One lambda-encoded transcription factor,  $Q^\lambda$ , is critical to the expression of these genes and has been shown to inhibit both rho-dependent and rho-independent (intrinsic) transcription termination (Yarnell and Roberts 1999). Both *in vitro* and *in vivo* evidence has demonstrated the antiterminator activity of  $Q^\lambda$  as it enables the RNAP holoenzyme to read through an intrinsic terminator located downstream of the transcription start site (Yarnell and Roberts 1992; Deighan and Hochschild 2007). In the absence of  $Q^\lambda$ , terminator readthrough is abrogated and the lysis genes aren't expressed. Despite

similarities among the different phage lysis cassettes, there is limited sequence homology among the phage Q factors (Guo, Kainz et al. 1991). This fact makes classification of additional Q factors a tedious process. Nevertheless, sequence comparison between DLP12 and the other lambdoid phages has revealed a putative transcriptional antiterminator ( $Q^{DLP12}$ ), encoded by the gene *ycbQ* (Lindsey, Mullin et al. 1989). In functional lambdoid phages this gene is found just upstream of the late phage genes. However, in DLP12 it is located a few thousand nucleotides upstream of the lysis cassette (Lindsey, Mullin et al. 1989) because of an insertion element (ins-H2) that is located just downstream of *ycbQ* (Ecocyc.org). That said, the nature of Q-mediated antitermination is independent of the physical proximity of the Q gene and its target promoter; in p21, p $\lambda$ , and p82,  $Q^\lambda$  acts in *trans* on the *SRRz* promoter (Yang and Roberts 1989; Guo, Kainz et al. 1991).

Upon expression, the Qs studied to date dimerize and bind the DNA in the untranscribed region of the promoter and region 4 of  $\sigma^{70}$  (Nickels, Roberts et al. 2002). In addition, contacts form between Q and RNAP core, possibly at the flap domain of the  $\beta$  subunit (Nickels, Roberts et al. 2006). Once Q docks with RNAP, it modifies the overall holoenzyme structure and becomes a stable component of the transcription elongation complex (Deighan and Hochschild 2007).  $Q^{DLP12}$  may act in a similar manner to promote transcription of the DLP12 lysis cassette. Efficient docking of Q is dependent on the presence of two specific DNA sequences at the promoter. One sequence, termed QBE for Q binding element, is typically located between the -35 and -10 promoter regions and is essential for bringing Q in close proximity to RNAP. The other sequence, called the pause sequence, is a -10 like element found immediately downstream of the transcription

start site that pauses the RNAP holoenzyme through an interaction between the nontemplate strand and region 2 of  $\sigma^{70}$  (Marr, Datwyler et al. 2001). This paused complex seems to resemble the scrunched complex that occurs during initiation. While in this paused state, RNAP can be contacted by Q (Marr, Datwyler et al. 2001; Nickels, Roberts et al. 2002). Q interacts with  $\sigma^{70}$  region 4, stabilizing its binding to the -35 like sequence and subsequently causes the release of RNAP from the paused state (Nickels, Roberts et al. 2006). In the absence of Q, RNAP will remain paused at this site for an extended time period until it either backtracks or frees itself from the site (Grayhack, Yang et al. 1985).

#### **1.4 IMPACT OF PHAGE GENES ON CURLI EXPRESSION**

Deletion of any of the genes encoding the DLP12 lysis machinery (the holin *ybcR* or  $S^{\text{DLP12}}$ , the lysozyme *ybcS* or  $R^{\text{DLP12}}$ , or the spanin *rzoD* or  $Rz^{\text{DLP12}}$ , collectively known as  $SRRz^{\text{DLP12}}$ ) impaired curli production and prevented *E. coli* from making normal biofilms (Toba et al. 2011). Given  $Q^{\text{DLP12}}$ 's predicted role in regulating  $SRRz^{\text{DLP12}}$  we anticipated that  $Q^{\text{DLP12}}$  mutants would also be defective in biofilm formation. *E. coli* biofilms are dependent on the production of various different extracellular structures such as curli, fimbriae, and flagella, that mediate surface attachment and tether adjoining cells together in a matrix of extracellular polymeric substances (Flemming and Wingender 2010). Curli are proteinaceous amyloid fibers, appearing as a meshwork of repeating curlin subunits (CsgA) which are anchored to the cell surface and polymerized by the nucleator protein, CsgB (Barnhart and Chapman 2006). Previous work revealed the

importance of curli fibers in the biofilm lifestyle of certain *E. coli* strains (Vidal, Longin et al. 1998; Barnhart and Chapman 2006). In addition, several enteric bacteria including *Salmonella* and *Shigella spp* are also capable of producing curli fibers, commonly called Tafi (Thin Aggregative Filaments) and their genomes contain similarly structured operons (Romling, Bian et al. 1998; Gerstel, Park et al. 2003).

Curli production is determined by the divergently transcribed *csgDEFG* and *csgBAC* operons which are linked by a 520 base pair (bp) intergenic region. Transcription of the curli genes is performed primarily by the stationary phase sigma factor, RpoS, however it is not solely responsible for curli regulation. Expression from this promoter region is tightly regulated by over a dozen transcription factors including Crp, IHF, H-NS, MlrA, OmpR, RstA, CpxR, and CsgD (Gerstel, Park et al. 2003; Ogasawara, Yamada et al. 2010). Currently, there are also 5 known sRNA regulators that bind to the curli promoter region and modulate curli expression by interacting with the 5' untranslated region (UTR) of the *csgD* transcript and either preventing ribosome binding and/or enhancing transcript degradation (Holmqvist, Reimegard et al. ; Jorgensen, Nielsen et al. ; Mika, Busse et al. ; Boehm and Vogel 2012; Thomason, Fontaine et al. 2012).

One of the transcription factors, CsgD, is directly involved in modulating curli production and is encoded within the operon itself. CsgD upregulates expression of the curli structural genes, *csgBAC*. Furthermore, CsgD promotes expression of the *csgDEFG* operon as well, acting as a positive regulator for its own expression and that of the accessory proteins CsgE, F, and G (Barnhart and Chapman 2006). CsgE and CsgF are periplasmic proteins that interact with CsgG, an outer membrane lipoprotein to transport

both the curlin subunits and the nucleator proteins out of the cell (Barnhart and Chapman 2006). Transcriptional control of the *csgDEFG* operon, however, is highly complex and not simply controlled by CsgD.

Several of the transcription factors regulating curli production are members of two component signal transduction systems; these include OmpR, CpxR, and RstA. Typically, signal recognition by a sensor histidine kinase induces ATP-hydrolysis and transfer of a phosphoryl group to some associated response regulator. The phosphorylated response regulator can then interact with specific genes to modulate their expression and potentially influence downstream signaling pathways. These signaling pathways allow for adaptive responses to a variety of stimuli, such as oxygen levels, osmolarity, and quorum sensing (Hoch 2000; Koretke, Lupas et al. 2000; Ferrieres and Clarke 2003; Hengge 2008; MacRitchie, Buelow et al. 2008).

OmpR, the response regulator of the OmpR/EnvZ two-component system, positively regulates *csgDEFG* transcription and responds to changes in osmolarity (Igo, Ninfa et al. 1989). CpxR, the response regulator of the CpxR/CpxA two-component system, responds to general outer membrane stress and protein misfolding and negatively regulates *csgDEFG* (Dorel, Lejeune et al. 2006). RstA, the response regulator of the RstA/RstB two-component system, negatively regulates *csgDEFG* transcription and responds to pH and magnesium ion levels (Minagawa, Ogasawara et al. 2003; Ogasawara, Hasegawa et al. 2007).

Crp, IHF, and MlrA act as positive effectors for curli production. Crp, the cAMP receptor protein, positively regulates *csgDEFG* transcription and responds to nutrient availability (Grainger, Hurd et al. 2005). IHF, a ubiquitous heterodimeric protein, acts as

a global regulator through DNA bending and positively regulates *csgDEFG* transcription. This factor increases in concentration as cells enter into stationary phase (Swinger and Rice 2004; Ogasawara, Yamada et al. 2010). MlrA, a member of the MerR family, positively regulates *csgDEFG* transcription, and its expression is dependent upon RpoS (Brown, Dozois et al. 2001). H-NS was recently described as a negative regulator of curli expression, mediating its repressive effects by directly binding the promoter region and indirectly, through repression of RpoS (Stanley and Lazazzera 2004; Pruss, Besernann et al. 2006; Ogasawara, Yamada et al. 2010). H-NS, is a nucleoid-associated protein that is capable of condensing and supercoiling DNA, which in turn influences the expression of nearby genes (usually through transcriptional silencing) (Dame, Wyman et al. 2000; Zimmerman 2006). This DNA-binding factor regulates over 5% of all *E. coli* genes, underscoring the protein's involvement in host physiology (Hommais, Krin et al. 2001). H-NS has been shown to participate in a variety of cellular functions including: flagella production, transcriptional control of type I fimbria structural genes, acid resistance, osmotic control, and protease production (Donato and Kawula 1999; Hommais, Krin et al. 2001; Rajkumari and Gowrishankar 2001; Landini and Zehnder 2002; Forns, Juarez et al. 2005; Shin, Song et al. 2005).

Due to the plethora of factors involved in the regulation of curli production, the curli operon can be considered one of the most complex in the *E. coli* genome (Romling, Bian et al. 1998; Gerstel, Park et al. 2003; Gerstel and Romling 2003). This diverse range of mechanisms permits subtle physiological changes to greatly alter curli production; and in turn alter biofilm formation in these enteric bacteria. The goal of this dissertation was



to understand the how  $Q^{DLP12}$  regulates  $SRR_z^{DLP12}$  and how loss of these proteins lead to curli downregulation.

## **LITERATURE CITED**

- Barnhart, M. M. and M. R. Chapman (2006). "Curli biogenesis and function." Annu Rev Microbiol **60**: 131-47.
- Boehm, A. and J. Vogel (2012). "The csgD mRNA as a hub for signal integration via multiple small RNAs." Mol Microbiol **84**(1): 1-5.
- Brown, P. K., C. M. Dozois, et al. (2001). "MlrA, a novel regulator of curli (AgF) and extracellular matrix synthesis by Escherichia coli and Salmonella enterica serovar Typhimurium." Mol Microbiol **41**(2): 349-63.
- Campbell, A. (1994). "Comparative Molecular-Biology of Lambdoid Phages." Annual Review of Microbiology **48**: 193-222.
- Campbell, A., S. J. Schneider, et al. (1992). "Lambdoid Phages as Elements of Bacterial Genomes (Integrase Phage21 Escherichia-Coli K-12 Icd Gene)." Genetica **86**(1-3): 259-267.
- Casjens, S. (2003). "Prophages and bacterial genomics: what have we learned so far? ." Mol Microbiol **49**: 277-300.
- Dame, R. T., C. Wyman, et al. (2000). "H-NS mediated compaction of DNA visualised by atomic force microscopy." Nucleic Acids Res **28**(18): 3504-10.
- Deighan, P. and A. Hochschild (2007). "The bacteriophage lambda Q anti-terminator protein regulates late gene expression as a stable component of the transcription elongation complex." Molecular Microbiology **63**(3): 911-920.
- Donato, G. M. and T. H. Kawula (1999). "Phenotypic analysis of random hns mutations differentiate DNA-binding activity from properties of fimA promoter inversion modulation and bacterial motility." J Bacteriol **181**(3): 941-8.
- Dorel, C., P. Lejeune, et al. (2006). "The Cpx system of Escherichia coli, a strategic signaling pathway for confronting adverse conditions and for settling biofilm communities?" Res Microbiol **157**(4): 306-14.
- Ferrieres, L. and D. J. Clarke (2003). "The RcsC sensor kinase is required for normal biofilm formation in Escherichia coli K-12 and controls the expression of a regulon in response to growth on a solid surface." Mol Microbiol **50**(5): 1665-82.
- Flemming, H. C. and J. Wingender (2010). "The biofilm matrix." Nat Rev Microbiol **8**(9): 623-33.
- Forns, N., A. Juarez, et al. (2005). "Osmoregulation of the HtrA (DegP) protease of Escherichia coli: An Hha-H-NS complex represses HtrA expression at low osmolarity." Fems Microbiology Letters **251**(1): 75-80.
- Gerstel, U., C. Park, et al. (2003). "Complex regulation of csgD promoter activity by global regulatory proteins." Molecular Microbiology **49**(3): 639-654.
- Gerstel, U. and U. Romling (2003). "The csgD promoter, a control unit for biofilm formation in Salmonella typhimurium." Res Microbiol **154**(10): 659-67.
- Godeke, J., K. Paul, et al. (2010). "Phage-induced lysis enhances biofilm formation in Shewanella oneidensis MR-1." ISME J **5**(4): 613-26.
- Grainger, D. C., D. Hurd, et al. (2005). "Studies of the distribution of Escherichia coli cAMP-receptor protein and RNA polymerase along the E. coli chromosome." Proc Natl Acad Sci U S A **102**(49): 17693-8.

- Grayhack, E. J., X. J. Yang, et al. (1985). "Phage Lambda Gene-Q Antiterminator Recognizes Rna-Polymerase near the Promoter and Accelerates It through a Pause Site." Cell **42**(1): 259-269.
- Guo, H. C., M. Kainz, et al. (1991). "Characterization of the Late-Gene Regulatory Region of Phage-21." Journal of Bacteriology **173**(4): 1554-1560.
- Hall-Stoodley, L., J. W. Costerton, et al. (2004). "Bacterial biofilms: from the natural environment to infectious diseases." Nat Rev Microbiol **2**(2): 95-108.
- Hengge, R. (2008). "The two-component network and the general stress sigma factor RpoS (sigma S) in Escherichia coli." Adv Exp Med Biol **631**: 40-53.
- Hoch, J. A. (2000). "Two-component and phosphorelay signal transduction." Curr Opin Microbiol **3**(2): 165-70.
- Holmqvist, E., J. Reimegard, et al. "Two antisense RNAs target the transcriptional regulator CsgD to inhibit curli synthesis." EMBO J **29**(11): 1840-50.
- Hommais, F., E. Krin, et al. (2001). "Large-scale monitoring of pleiotropic regulation of gene expression by the prokaryotic nucleoid-associated protein, H-NS." Mol Microbiol **40**(1): 20-36.
- Igo, M. M., A. J. Ninfa, et al. (1989). "Phosphorylation and dephosphorylation of a bacterial transcriptional activator by a transmembrane receptor." Genes Dev **3**(11): 1725-34.
- Jorgensen, M. G., J. S. Nielsen, et al. "Small regulatory RNAs control the multi-cellular adhesive lifestyle of Escherichia coli." Mol Microbiol **84**(1): 36-50.
- Kabir, M. S., D. Yamashita, et al. (2005). "Cell lysis directed by sigmaE in early stationary phase and effect of induction of the rpoE gene on global gene expression in Escherichia coli." Microbiology **151**(Pt 8): 2721-35.
- Kapanidis, A. N., E. Margeat, et al. (2006). "Initial transcription by RNA polymerase proceeds through a DNA-scrunching mechanism." Science **314**(5802): 1144-1147.
- Karatan, E. and P. Watnick (2009). "Signals, regulatory networks, and materials that build and break bacterial biofilms." Microbiol Mol Biol Rev **73**(2): 310-47.
- Koretke, K. K., A. N. Lupas, et al. (2000). "Evolution of two-component signal transduction." Mol Biol Evol **17**(12): 1956-70.
- Lacqua, A., O. Wanner, et al. (2006). "Emergence of biofilm-forming subpopulations upon exposure of Escherichia coli to environmental bacteriophages." Appl Environ Microbiol **72**(1): 956-9.
- Landini, P. and A. J. B. Zehnder (2002). "The global regulatory hns gene negatively affects adhesion to solid surfaces by anaerobically grown Escherichia coli by modulating expression of flagellar genes and lipopolysaccharide production." Journal of Bacteriology **184**(6): 1522-1529.
- Lawrence, J. G. and H. Ochman (1998). "Molecular archaeology of the Escherichia coli genome." Proc Natl Acad Sci U S A **95**(16): 9413-7.
- Lindsey, D. F., D. A. Mullin, et al. (1989). "Characterization of the cryptic lambdoid prophage DLP12 of Escherichia coli and overlap of the DLP12 integrase gene with the tRNA gene argU." J Bacteriol **171**(11): 6197-205.
- MacRitchie, D. M., D. R. Buelow, et al. (2008). "Two-component signaling and gram negative envelope stress response systems." Adv Exp Med Biol **631**: 80-110.

- Marr, M. T., S. A. Datwyler, et al. (2001). "Restructuring of an RNA polymerase holoenzyme elongation complex by lambdaoid phage Q proteins." Proc Natl Acad Sci U S A **98**(16): 8972-8.
- Mika, F., S. Busse, et al. "Targeting of csgD by the small regulatory RNA RprA links stationary phase, biofilm formation and cell envelope stress in Escherichia coli." Mol Microbiol **84**(1): 51-65.
- Minagawa, S., H. Ogasawara, et al. (2003). "Identification and molecular characterization of the Mg<sup>2+</sup> stimulon of Escherichia coli." Journal of Bacteriology **185**(13): 3696-3702.
- Missiakas, D. and S. Raina (1998). "The extracytoplasmic function sigma factors: role and regulation." Molecular Microbiology **28**(6): 1059-1066.
- Murakami, K. S. and S. A. Darst (2003). "Bacterial RNA polymerases: the whole story." Curr Opin Struct Biol **13**(1): 31-9.
- Nickels, B. E., C. W. Roberts, et al. (2006). "RNA-mediated destabilization of the sigma(70) region 4/beta flap interaction facilitates engagement of RNA polymerase by the Q antiterminator." Molecular Cell **24**(3): 457-468.
- Nickels, B. E., C. W. Roberts, et al. (2002). "The sigma(70) subunit of RNA polymerase is contacted by the lambda Q antiterminator during early elongation." Molecular Cell **10**(3): 611-622.
- Ogasawara, H., A. Hasegawa, et al. (2007). "Genomic SELEX search for target promoters under the control of the PhoQP-RstBA signal relay cascade." Journal of Bacteriology **189**(13): 4791-4799.
- Ogasawara, H., K. Yamada, et al. (2010). "Regulation of the Escherichia coli csgD promoter: interplay between five transcription factors." Microbiology **156**(Pt 8): 2470-83.
- Park, J. S., M. T. Marr, et al. (2002). "E. coli Transcription repair coupling factor (Mfd protein) rescues arrested complexes by promoting forward translocation." Cell **109**(6): 757-67.
- Pruss, B. M., C. Besernann, et al. (2006). "A complex transcription network controls the early stages of biofilm development by Escherichia coli." Journal of Bacteriology **188**(11): 3731-3739.
- Rajkumari, K. and J. Gowrishankar (2001). "In vivo expression from the RpoS-dependent P1 promoter of the osmotically regulated proU operon in Escherichia coli and Salmonella enterica serovar typhimurium: Activation by rho and hns mutations and by cold stress." Journal of Bacteriology **183**(22): 6543-6550.
- Rasmussen, B. (2000). "Filamentous microfossils in a 3,235-million-year-old volcanogenic massive sulphide deposit." Nature **405**(6787): 676-9.
- Redfield, R. J. and A. M. Campbell (1987). "Structure of Cryptic Lambda-Prophages." Journal of Molecular Biology **198**(3): 393-404.
- Revyakin, A., C. Liu, et al. (2006). "Abortive initiation and productive initiation by RNA polymerase involve DNA scrunching." Science **314**(5802): 1139-43.
- Reysenbach, A. L. and S. L. Cady (2001). "Microbiology of ancient and modern hydrothermal systems." Trends Microbiol **9**(2): 79-86.
- Rhodijs, V. A., W. C. Suh, et al. (2006). "Conserved and variable functions of the sigmaE stress response in related genomes." PLoS Biol **4**(1): e2.

- Rice, S. A., C. H. Tan, et al. (2009). "The biofilm life cycle and virulence of *Pseudomonas aeruginosa* are dependent on a filamentous prophage." ISME J **3**(3): 271-82.
- Romling, U., Z. Bian, et al. (1998). "Curli fibers are highly conserved between *Salmonella typhimurium* and *Escherichia coli* with respect to operon structure and regulation." Journal of Bacteriology **180**(3): 722-731.
- Romling, U., Z. Bian, et al. (1998). "Curli fibers are highly conserved between *Salmonella typhimurium* and *Escherichia coli* with respect to operon structure and regulation." J Bacteriol **180**(3): 722-31.
- Sauer, K., A. K. Camper, et al. (2002). "*Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm." J Bacteriol **184**(4): 1140-54.
- Shankar, S., A. Hatoum, et al. (2007). "A transcription antiterminator constructs a NusA-Dependent shield to the emerging transcript." Molecular Cell **27**(6): 914-927.
- Shin, M., M. Song, et al. (2005). "DNA looping-mediated repression by histone-like protein H-NS: specific requirement of Esigma70 as a cofactor for looping." Genes Dev **19**(19): 2388-98.
- Srividhya, K. V. and S. Krishnaswamy (2007). "Subclassification and targeted characterization of prophage-encoded two-component cell lysis cassette." J Biosci **32**(5): 979-90.
- Stanley, N. R. and B. A. Lazazzera (2004). "Environmental signals and regulatory pathways that influence biofilm formation." Molecular Microbiology **52**(4): 917-924.
- Stoodley, P., K. Sauer, et al. (2002). "Biofilms as complex differentiated communities." Annu Rev Microbiol **56**: 187-209.
- Sutherland, I. W., K. A. Hughes, et al. (2004). "The interaction of phage and biofilms." FEMS Microbiol Lett **232**(1): 1-6.
- Svenningsen, S. L., N. Costantino, et al. (2005). "On the role of Cro in lambda prophage induction." Proc Natl Acad Sci U S A **102**(12): 4465-9.
- Swinger, K. K. and P. A. Rice (2004). "IHF and HU: flexible architects of bent DNA." Curr Opin Struct Biol **14**(1): 28-35.
- Taylor, C. D., C. O. Wirsen, et al. (1999). "Rapid microbial production of filamentous sulfur mats at hydrothermal vents." Appl Environ Microbiol **65**(5): 2253-5.
- Thomason, M. K., F. Fontaine, et al. (2012). "A small RNA that regulates motility and biofilm formation in response to changes in nutrient availability in *Escherichia coli*." Mol Microbiol **84**(1): 17-35.
- Toba, F. A., M. G. Thompson, et al. (2011). "Role of DLP12 lysis genes in *Escherichia coli* biofilm formation." Microbiology **157**(Pt 6): 1640-50.
- Vidal, O., R. Longin, et al. (1998). "Isolation of an *Escherichia coli* K-12 mutant strain able to form biofilms on inert surfaces: involvement of a new ompR allele that increases curli expression." J Bacteriol **180**(9): 2442-9.
- Wang, X., Y. Kim, et al. (2010). "Cryptic prophages help bacteria cope with adverse environments." Nat Commun **1**(9): 147.
- Webb, J. S., M. Lau, et al. (2004). "Bacteriophage and phenotypic variation in *Pseudomonas aeruginosa* biofilm development." J Bacteriol **186**(23): 8066-73.
- Webb, J. S., L. S. Thompson, et al. (2003). "Cell death in *Pseudomonas aeruginosa* biofilm development." J Bacteriol **185**(15): 4585-92.

- Whiteley, M., M. G. Banger, et al. (2001). "Gene expression in *Pseudomonas aeruginosa* biofilms." Nature **413**(6858): 860-4.
- Yang, X. J. and J. W. Roberts (1989). "Gene Q antiterminator proteins of *Escherichia coli* phages 82 and lambda suppress pausing by RNA polymerase at a rho-dependent terminator and at other sites." Proc Natl Acad Sci U S A **86**(14): 5301-5.
- Yarnell, W. S. and J. W. Roberts (1992). "The phage lambda gene Q transcription antiterminator binds DNA in the late gene promoter as it modifies RNA polymerase." Cell **69**(7): 1181-9.
- Yarnell, W. S. and J. W. Roberts (1999). "Mechanism of intrinsic transcription termination and antitermination." Science **284**(5414): 611-5.
- Zimmerman, S. B. (2006). "Cooperative transitions of isolated *Escherichia coli* nucleoids: Implications for the nucleoid as a cellular phase." Journal of Structural Biology **153**(2): 160-175.

## CHAPTER 2

### A Q-LIKE TRANSCRIPTION FACTOR REGULATES BIOFILM DEVELOPMENT IN *E. coli* BY CONTROLLING EXPRESSION OF THE DLP12 LYSIS CASSETTE.

#### 2.1 ABSTRACT

The DLP12 lysis cassette (*essD*, *ybcT*, *rzpD/rzoD*) is required in certain *Escherichia coli* strains for normal curli expression and biofilm development. Tightly controlled regulation of the lysis cassette is of particular importance, since its overexpression causes host cell lysis. *In silico* analysis revealed a putative intrinsic transcriptional terminator 100bp upstream of *essD* and within 2000bp of *ybcQ* ( $Q^{DLP12}$ ), a putative lambda ( $\lambda$ ) Q-like antiterminator. We hypothesized that  $Q^{DLP12}$  may be required for effective expression of the lysis cassette. In this work we report on the role of  $Q^{DLP12}$  as a positive regulator of DLP12 lysis cassette expression. Mutants lacking  $Q^{DLP12}$  exhibited a biofilm defective phenotype analogous to that of the lysis cassette knockouts. This defect occurred through the downregulation of curli transcription, which is also consistent with that seen in the lysis cassette mutants and was restored by complementation by ectopic expression of  $Q^{DLP12}$ . In addition,  $Q^{DLP12}$  overexpression caused cell lysis as demonstrated by leakage of *beta*-galactosidase activity from cells. This was accompanied by upregulation of the DLP12 lysis cassette as demonstrated by increased *essD* transcription which was documented with *gfp*-reporter assays, RT-PCR, and ChIP. We provide evidence that this Q-mediated effect resulted from direct interaction of  $Q^{DLP12}$  with the lysis cassette promoter (*essDp*) as demonstrated by EMSA. We propose that  $Q^{DLP12}$  encodes a functional transcriptional regulator, which promotes

expression of the DLP12 lysis cassette. This work provides evidence of a regulator from a defective prophage impacting host cell physiology

## 2.2 INTRODUCTION

Biofilm forming bacteria can colonize biotic and abiotic surfaces. Initial attachment is mediated by extracellular polysaccharides and monomeric proteins, or by more structured components such as pili, flagella, and curli (Vidal, Longin et al. 1998; Sheikh, Hicks et al. 2001). Once adhered, the bacteria adopt a multicellular lifestyle through the formation of microcolonies which later grow into a mature biofilm. The bacteria within these biofilms are more resistant to environmental insults such as nutrient starvation, desiccation, and antibiotics (Sauer, Camper et al. 2002; Stoodley, Sauer et al. 2002; Stewart and Franklin 2008; Karatan and Watnick 2009).

Curli, in particular, have been implicated in the initial adhesion phase which leads to biofilm formation in several strains of *E. coli* (Fink, Black et al. 2012; Lee, Kim et al. 2012). In most *E. coli* the biofilm master regulator, *csgD* is critical for modulating curli production and serves as a hub for integrating various extracellular signals, thereby dictating whether or not the bacteria initiate biofilm formation (Ogasawara, Yamamoto et al. ; Pesavento, Becker et al. 2008; Ogasawara, Yamada et al. 2010). Recently, several groups have demonstrated that in addition to its regulation by over a dozen transcription factors (Boehm and Vogel 2012), CsgD expression is also regulated post transcriptionally. This latter effect is mediated by at least five known sRNAs, which bind to the 5' UTR of the *csgD* transcript preventing ribosome binding and/or enhancing transcript degradation (Holmqvist, Reimegard et al. ; Jorgensen, Nielsen et al. ; Mika,



Busse et al. ; Boehm and Vogel 2012; Thomason, Fontaine et al. 2012). This diverse range of mechanisms permits subtle physiological changes to greatly alter curli production.

In addition to the genes encoding surface structures, early transcriptional studies demonstrated that bacteriophage genes were highly upregulated within gram-positive and gram-negative bacteria in biofilms (Whiteley, Bangera et al. 2001; Stanley, Britton et al. 2003). The importance of prophages was confirmed by the deletion of Pf4 and Pf1 prophages in *Pseudomonas aeruginosa*, resulting in defects in the maturation and dispersal of biofilms, respectively. *E. coli* K12 biofilms have been shown to be dramatically affected by the deletion of specific prophage, with the loss of cryptic prophage CP4-57 dramatically increasing biofilm formation, whereas the loss of others decreased it (Wang, Kim et al. 2010).

In *E. coli*, lambdoid prophage genes such as *bor* and *lom* had previously been linked to resistance to animal sera and increased surface adhesion (Canchaya 2003; Casjens 2003). Although previously considered genetic baggage recent evidence suggests that these cryptic phage elements encode functional proteins that have numerous benefits to their hosts (Casjens 2003; Wang, Kim et al. 2010). This extends beyond their ability to contribute to biofilm formation. For example, Wang and colleagues have recently shown that many of the cryptic prophages in BW25113 contribute to traits such as oxidative, osmotic, and acid stress resistance, as well as antibiotic resistance. They also found that many of these prophages actually increased growth rate (Wang, Kim et al. 2010).

One of these prophage, DLP12, a **d**efective **l**ambdoid **p**rophage present in the 12 minute of *E. coli's* genome, has likely been around for more than 100 million years since

it is also found in *Salmonella* (Lawrence and Ochman 1998; Casjens 2003). DLP12 was recently shown to contribute to stress resistance and biofilm formation in *E. coli* (Wang, Kim et al. 2010). While the exact mechanisms behind the multifaceted impact of DLP12 on host physiology remains unclear, Toba et al. (2011) recently demonstrated that deletion of the lysis genes of this lambdoid-like prophage (the holin S encoded by *essD*, the lysozyme R, encoded by *ybcS*, and the spannin Rz/Rz1, encoded by *rzpD/rzoD*) reduced biofilm formation by impacting curli production in a curli overproducing K12 derivative (Toba, Thompson et al. 2011). This evidence suggests that specific defective prophage genes are impacting host cell physiology and have been co-opted by the host because of their contributions to host survival.

The lysis cassette of lambda ( $\lambda$ ) phage itself encodes a holin, an endolysin, and a spannin, which work together to rupture the cell envelope and allow viral particles to escape their cellular confines (Young, Wang et al. 2000; Berry 2008). Phage  $\lambda$  regulates expression of these lysis genes through the production of Q, an antiterminator that associates with the RNA polymerase (RNAP) holoenzyme and permits readthrough past an intrinsic terminator located near the lysis cassette promoter,  $P_R'$  (Guo and Roberts 2004). In the absence of Q, the majority of transcripts produced from  $P_R'$  fail to extend past the terminator upstream of the lysis genes, thus preventing unnecessary expression of these potentially lethal genes (Oppenheim, Kobiler et al. 2005; Zhou, Shi et al. 2006; Salazar 2007). Q, however, may bind to DNA found adjacent to sigma factor binding regions called the Q binding element (QBE), enabling Q to interact with paused RNAP and become stably incorporated into the elongation complex (Nickels, Roberts et al.

2002; Deighan and Hochschild 2007). This event releases RNAP from its stalled state and renders the complex resistant to downstream terminators.

Lindsey et al. (1989) mapped the genome of DLP12 and noted the presence of an open reading frame upstream of the lysis cassette that encodes a Q-like protein. Sequence alignment between  $Q^{DLP12}$  (*ybcQ*) and  $Q^{21}$  shows 84% sequence identity, strongly suggesting that  $Q^{DLP12}$  has a function similar to that of the canonical Q antiterminators, although there is an insertion sequence between Q and the DLP12 lysis genes in K12 (Lindsey, Mullin et al. 1989; Casjens 2003).

In this work we present evidence that  $Q^{DLP12}$  encodes a functional protein that regulates expression of the DLP12 lysis cassette by binding directly to its promoter and in turn influences biofilm formation in *E. coli*. Taken together, these data present a demonstration of a Q-like protein regulating expression of functional lysis genes in a defective phage and suggest that a well characterized regulatory mechanism governing phage lysis cassette expression has been co-opted by the bacterial host.

## 2.3 MATERIALS AND METHODS

### Bacterial strains and growth conditions

*E. coli* strain PHL628 is an MG1655 derivative with an ompR234 mutation (Vidal, Longin et al. 1998). *E. coli* PHL628  $\Delta S^{DLP12}$  (628.1), a strain with *S* (*essD*) deleted from the chromosome (Toba, Thompson et al. 2011), was utilized in cell count, fluorometry, and chromatin immunoprecipitation (ChIP) assays unless otherwise stated because Q overexpression is less toxic in this strain (Data not shown). *E. coli* strains were routinely grown on lysogen broth (LB) supplemented with 50  $\mu$ l/ml Kanamycin (Kan) at

37°C overnight with shaking (150 rpm) and subsequently diluted 1:100 into fresh media and cultured at 30°C for the experiments unless otherwise stated. For early stationary phase lysis assays and promoter studies low salt LB (5 g/l NaCl) was used. When required, plates and media were supplemented with Ampicillin (Amp 150µl/ml) and Chloramphenicol (25µg/ml) or Spectinomycin (100µl/ml). For induction, media was supplemented with 1mM isopropyl-β-D-thiogalactopyranoside (IPTG) according to the experimental needs. For growth curves, overnight LB cultures were diluted 1:100 into fresh media. The new cultures were grown at 30°C for 36 hours in 96-well plates. Optical Density at 600nm (O.D.600) was measured every 30 minutes in a µQuant spectrophotometer (Bio-Tek Company Info).

#### Mutants and plasmid construction

Deletion mutants were constructed by allele replacement using a λ-RED strategy described elsewhere (Datsenko and Wanner 2000). Briefly, a Chloramphenicol (Cm) interrupted version of the gene of interest was created using PCR-mediated ligation (Choi and Schweizer 2005). In general, each construct consisted of a 5' and a 3' 500-bp homology region to the gene of interest linked 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 λ-RED recombinase. The 5' end and 3' end homology regions allowed the RED system to replace the wild type allele with our interrupted version. Clone screening was carried out on plates supplemented with chloramphenicol. After confirmed recombination via PCR, the Cm marker was removed using FLP-recombinase from plasmid pCP20 (Datsenko and Wanner 2000). All plasmid vectors are temperature

sensitive and were cured from the cells by growth at 43°C. 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) (Kovach, Elzer et al. 1995). For Q overexpression, two vectors were used: pBBR1MCS (medium copy number) and pOFX (low copy number).  $Q^{DLP12}$  was cloned into pBBRMCS1 and labeled pQ1. A C-His<sub>6</sub> tagged version of  $Q^{DLP12}$  was cloned into pOFX using BamHI and SacI restriction sites and labeled pQ. For promoter reporter assays, the promoter-less *gfp* vector pJBA110 was used (Andersen, Sternberg et al. 1998). Plasmid *pcsgBp-GFP* was described by Toba et al. (2011). Plasmid *pessDp-GFP* was constructed by cloning the DLP12 lysis gene promoter, *essDp*, upstream of the *gfp* gene in pJBA110 using KpnI and XbaI restriction sites (Anderson *et al.*, 1998). Wild type control strains and mutant control strains were constructed by transformation of empty vectors as required per experiment.

### 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 acids (CA). Dishes were incubated at 30°C for 72h with shaking on an orbital shaker at 50 rpm. The spent media was exchanged every 24h with new media. After 72h, 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 obtained at 40X magnification for each mutant and then analyzed with COMSTAT (Heydorn, Nielsen et al. 2000).

### Attachment

Attachment to PVC surface was studied as described previously (Genevaux, Muller et al. 1996). Overnight cultures were diluted 1:5 to a total volume of 120  $\mu$ l in 96-well PVC plates and incubated at 30°C shaking (50 rpm) for 16 hours. After 16 h, turbidity (O.D.600) was measured in each well. Then, 100  $\mu$ l 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  $\mu$ l of 95% ethanol (EtOH) was added to each well and incubated for 10 minutes at room temperature. Then the absorbance of the EtOH solubilized CV (O.D.595) was measured in each well. The original well turbidity was compared to the CV absorbance (O.D.595/600). Experiments were done in quadruplicate.

### Autoaggregation

Cell autoaggregation was measured as described previously (Barrios, Zuo et al. 2006). Briefly, 4ml of overnight (30°) LB culture were incubated statically for 12h at 30°C. O.D.600 was measured from samples taken from the top 5mm of each culture with minimal disturbance. Then, cultures were vortexed and sampled again. O.D.600 values (unvortexed /vortexed) for each mutant were compared as a measurement of cell autoaggregation. Experiments were done in quadruplicate.

### Electron microscopy

Cells were grown on low salt LB at 30°C with shaking overnight. 300 mesh Formvar copper grids (EMS, Hatfield, PA) were floated on 25µl of the overnight cultures for 1 minute. Then the grids were transferred to a solution containing 3% ammonium molybdate (3% AMB) pH=7. Grids were then rinsed in MilliQ water for 1 minute and allowed to dry before observation. Cells were examined on a Philips Electron Microscope at different magnifications ranging from 7000x to 45000x. Snap shots were taken using a MicroFire digital camera and software from Optronics at 10000x magnification.

### Antiterminator Predictions

*essDp* sequence encompassing 400bp upstream of the translation start site was analyzed for the presence of putative Rho-independent transcriptional terminators using FindTerm software from Softberry© (Hagen, Tramp et al. 2010). The resulting terminator site was mapped by inserting the terminator sequence (58bp) into the mfold RNA folding program.

### Extracellular $\beta$ -Galactosidase activity assay

To assess the lysis of the different strains culture samples were obtained at 36hrs and  $\beta$ -galactosidase activity was determined in the cell-free supernatant using *ortho*-nitrophenyl- $\beta$ -galactoside (ONPG). The production of *ortho*-nitrophenol (ONP) from ONPG in the supernatant was used as an indicator of the relative abundance of extracellular  $\beta$ -galactosidase. ONP was measured in a Bio-Tek Synergy HT spectrophotometer (O.D.415). Activity was normalized to O.D.600 of each respective culture.

### Promoter fusion studies

Reporter plasmids *pcsgBp-GFP* (Toba, Thompson et al. 2011) and *pessDp-GFP* containing the curli and *essD* promoters respectively was transformed into each of the PHL628 strains and selected on low salt LB Amp plates. Cells were allowed to grow with shaking (150 rpm) in low salt LB at 30°C. At indicated 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 normalized to the O.D.600 of the corresponding culture. Measurements were done in quadruplicate.

### Quantitative real time reverse transcription PCR assay

Cells were cultured overnight with shaking (150 rpm) at 37°C in LB with 0.2% glucose and diluted 1:100 in low salt LB with glucose. Diluted cells were grown at 30°C; for the  $\Delta$ Q pQ1 strain, glucose was omitted from the culture to allow for Q expression from the lac promoter. At 12hrs, cultures were pelleted and lysed. RNA was extracted using standard phenol chloroform extraction protocol. Briefly, cell pellets were resuspended (50mM NaOAc, 10mM EDTA pH5.5) then lysed by addition of 1% SDS and an equal volume water-saturated phenol (pH 4.3). Lysate was centrifuged for 15 minutes @ 4°C, 3500 rpm and poured into a new tube. An equal volume of chloroform was added, then mixed by inverting, centrifuged as before, then the aqueous phase was transferred to a new tube. RNA was isolated from supernatant using isopropanol precipitation. RNA was treated with DNaseI to remove residual DNA contamination. The pure RNA was subsequently used in the RT-PCR assay. 1ug of purified RNA was



incubated with a reverse primer specific to the readthrough *essDp* transcript (transcription past putative intrinsic terminator discussed below) in 11µl total volume at 70°C for 5 minutes. Mixtures were chilled on ice for 5 minutes and then added to a 25µl reverse transcription reaction comprised of 1X AMV RT Buffer (Promega), 1mM dNTP mixture, 40u of rRNasin Inhibitor (Promega), and 10u AMV reverse transcriptase (Promega). Reactions were incubated for 1hr at 48°C. Reactions were terminated by incubating for 2 minutes at 80°C. 1µl cDNA from RT reactions was used as template for PCR reactions containing a forward *essDp* transcript primer (Forward Tran Primer, Table 2.1) which anneals to the beginning of the transcript and the same reverse primer used to generate the cDNA (Table 2). PCR products were run on 1% agarose gels, stained with ethidium bromide, and visualized with a UV transilluminator.

**Table 2.1.** Primer sequences used in RT and ChIP assays.

For Tran primer	(5' GATAAATATTCATCTAATCAATGTG)
SRRz Rev 685 primer	(5' CACCTCGCAGACAAAGCGGGTG)
<i>essDp</i> Rev no term. primer	(5' CGGGTTTAAGCTGTGTGACGAAGT)
<i>essDp</i> For downstream of term. primer	(5' CGCTTTGTCTGCGAGGTGGGG)
SRRz Rev primer	(5' CTATCTGCACTGCTCATTAAATATAC)
5' BqIF For primer	(5' GGAGTTAGCCAGAAAAATAGTC)
5' BqIF Rev primer	(5' GTACCTCTGCTTGCGCTTTG)

### Purification of Q<sup>DLP12</sup>

Q-His was cloned into pRSET-A and transformed into BL21DE3 pLysS. Cells were grown at 37°C with shaking until an O.D.600 of 0.6. Then expression was induced

with 1mM IPTG followed by a 4hr incubation. Cells were centrifuged, lysed, and processed as described in the PrepEase™ His-Tagged Purification Denaturing Protocol. Purified Q<sup>DLP12</sup> fractions were analyzed by SDS-PAGE, pooled, and dialyzed against decreasing urea concentrations (4M-1M) at 4°C. The final dialysis at 4°C was performed into storage buffer (10mM KH<sub>2</sub>PO<sub>4</sub> pH 6.5, 1M KCl, 5mM TCEP, 1mM EDTA, 50% Glycerol). Protein was stored at -20°C

#### Electrophoretic gel mobility shift assay of Q<sup>DLP12</sup> DNA binding

A 151bp, double-stranded DNA probe encompassing nucleotides -86 to +65 of *essDp* was made by PCR with a Cy5-labeled primer and then gel purified. Q<sup>DLP12</sup> protein in storage buffer (10mM KH<sub>2</sub>PO<sub>4</sub> pH 6.5, 1M KCl, 5mM TCEP, 1mM EDTA, 50% Glycerol) was diluted 1:4 into 1X binding buffer (20 mM Tris-HCl (pH 8.0), 25 mM KCl, 12% glycerol, 0.1 mM EDTA,) prior to mixing with 7.5ng purified probe and a 100-fold excess of sonicated herring sperm DNA (competitor DNA) in a 25µl binding reaction; the final buffer was 20 mM Tris-HCl (pH 8.0), 2.5mM KH<sub>2</sub>PO<sub>4</sub> 165mM KCl, 22% glycerol, 1 mM DTT, 1.25mM TCEP, 0.25mM EDTA, and 40ug of BSA/ml. Binding reactions were incubated for 20 min at room temperature and stored on ice before loading onto a prechilled and prerun 3-8% polyacrylamide gel in 0.5X TAE running buffer (20 mM Tris-acetate (pH 8.5), 1 mM sodium EDTA). Samples were electrophoresed at 4°C in a Bio-Rad Protean III apparatus (Bio-Rad, Hercules, Calif.) in an ice-water bath. Gels were scanned with a Typhoon Imager and fluorescent bands were quantified using ImageJ. The  $K_d$  is reported as the concentration of Q<sup>DLP12</sup> protein giving 50% shift.

### Chromatin immunoprecipitation assay

Strain 628.1 pQ was grown with shaking at 30°C in low salt LB for 12hrs either with or without 1mM IPTG. Cultures were collected and crosslinked. Cross-linking of protein/protein and protein/DNA complexes was induced by the addition of formaldehyde to a final concentration of 1% for 20 minutes at 20°C. The cross-linking reaction was then quenched by the addition of glycine (0.5M final concentration). Cells were pelleted and washed twice in cold TBS, resuspended in 0.5 ml lysis buffer [10 mM Tris (pH 8), 50 mM NaCl, 10 mM EDTA, 2 mg/ml lysozyme, 1mM PMSF] and incubated for 30 min at 37°C. The lysate was then equilibrated in 0.5 ml 2X immunoprecipitation buffer [1X IP buffer: 50 mM HEPES (pH 7), 150 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, 1 mM PMSF] for 10 minutes at 4°C. The DNA was sheared by sonication to an average size of ~500 bp. Following centrifugation, the lysate was pre-cleared by incubation with 50 µl of protein-A CL4B sepharose slurry (Invitrogen) for 1 h at 4°C. Samples of the pre-cleared lysates were retained as unenriched input samples. Protein/DNA complexes were immunoprecipitated using monoclonal antibody reactive against the  $\beta$  subunit of RNAP (Neoclone), or a polyclonal antibody reactive against the His tags (Sigma) and 25 µl of protein-A CL4B sepharose. A mock immunoprecipitation control contained only the protein-A sepharose. Complexes were washed five times with cold IP buffer, once with cold IP buffer containing 0.4 M NaCl, and once with cold Tris-EDTA buffer [50 mM Tris (pH 7.5), 10 mM EDTA]. Immunoprecipitated protein/DNA complexes and the input samples were then incubated in elution buffer (TE buffer containing 1% SDS) for 10 minutes at 65°C. RNaseA was

added (100µg/ml final conc.) and incubated for 90 minutes at 42°C. The cross-links were reversed by boiling samples for 10 minutes. The immunoprecipitated DNAs, as well as the products of the mock immunoprecipitation controls and the input samples, were purified using Zippy spin columns. Multiplex PCR was employed to quantify the relative enrichment at *essDp* both proximal to (immediately following) the transcription start site and within the DLP12 lysis genes. These amplicons were normalized to that of the 5' region of *bglF*. For primers see Table 2. PCR reactions were performed with 30 amplification cycles. PCR products were run on 1% agarose gel, stained with ethidium bromide, and bands quantified using ImageJ software.

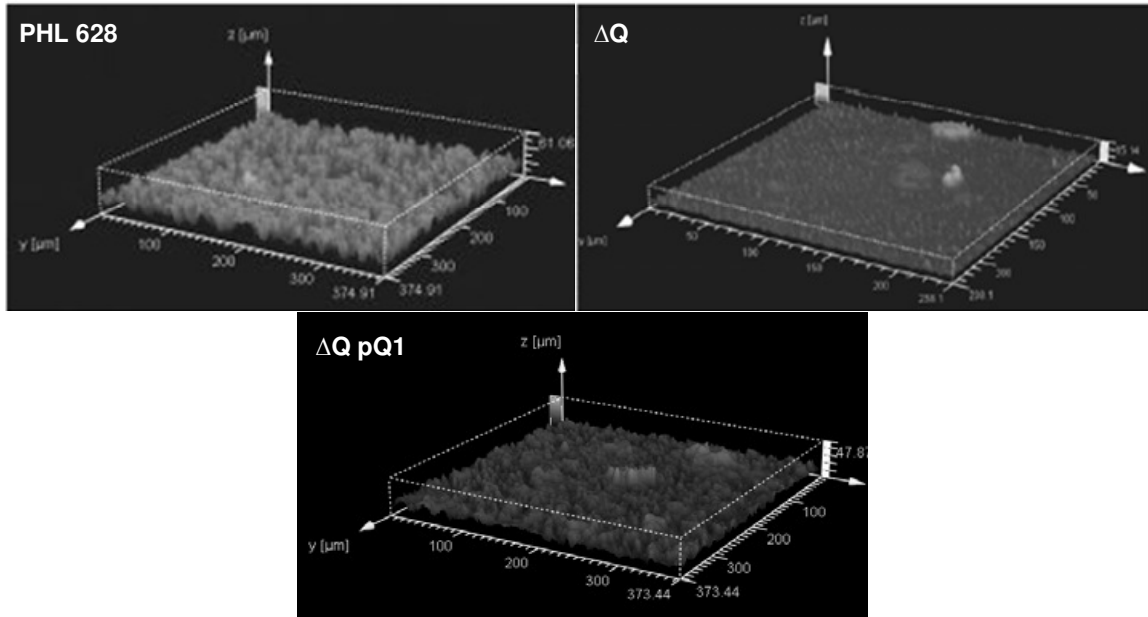
## 2.4 RESULTS

### Effects of $\Delta Q^{DLP12}$ on growth and biofilm formation

Growth curves performed with WT cells,  $\Delta Q^{DLP12}$  mutants, and  $\Delta Q^{DLP12}$  mutants complemented with pQ1 (a medium copy plasmid that encodes  $Q^{DLP12}$ ) showed no significant difference in the time required to reach equivalent cell densities upon entry into stationary phase (Data not shown). However, the  $\Delta Q^{DLP12}$  mutant exhibited significantly attenuated biofilm development ( $P < 0.05$ ) (Figure 2.1 and Table 2.2). The total biofilm biomass and thickness of  $\Delta Q^{DLP12}$  biofilms decreased 4 fold when compared to WT biofilms (Table 2.2).  $\Delta Q^{DLP12}$  cells also exhibited a 33% reduction in attachment versus WT cells (Table 2.2) and were 3-fold less capable of aggregating. Exogenous complementation of the  $\Delta Q^{DLP12}$  mutant with pQ1, restored biofilm formation, attachment, and autoaggregation to WT levels (Figure 2.1 and Table 2.2).

## Effects of $\Delta Q^{DLP12}$ on curli production

Transmission electron micrographs of the mutant revealed that it had far less curli than the wild type (Figure 2a). GFP fluorescence from the  $\Delta Q^{DLP12}$  strain containing the



**Figure 2.1.**  $\Delta Q^{DLP12}$  mutant exhibits defects in biofilm formation. Biofilms were grown in MatTek microwell dishes in minimal media with casamino acids for 72h at 30°C 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.  $\Delta Q^{DLP12}$  was complemented by pQ1, restoring biofilm formation.

**Table 2.2.**  $\Delta Q^{DLP12}$  mutants exhibited defects in biofilm formation. Biofilms were grown in MatTek microwell dishes in minimal media with casamino acids for 72h at 30°C with moderate shaking (50rpm). Biofilm features were determined as described in the methods section. The  $\Delta Q^{DLP12}$  mutant was affected for biomass, thickness, attachment and autoaggregation ( $\alpha=0.05$ ). These phenotypes were rescued by complementation (PHL 628  $\Delta Q$  pQ1). Experiments were done in quadruplicates. Standard error is indicated in parentheses.

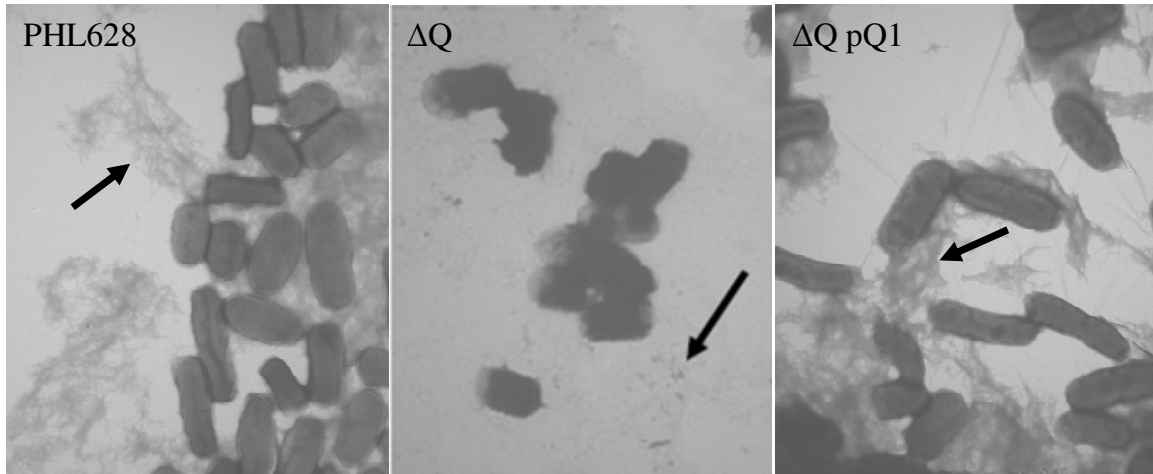
	<b>Biomass</b> ( $\mu\text{m}^3/\mu\text{m}^2$ )	<b>Average</b> thickness ( $\mu\text{m}$ )	<b>Attachment</b> (O.D.595/600)	<b>Autoaggregation</b> O.D.600 (unvor/vortexed)
<b>PHL 628</b>	6.289 (1.117)	6.544 (0.996)	3.393 (0.479)	0.234 (0.014)
<b>PHL 628 <math>\Delta Q</math></b>	1.669 (0.722)	1.654 (0.706)	2.298 (0.241)	0.772 (0.044)
<b>PHL 628 <math>\Delta Q</math> pQ1</b>	4.721 (0.838)	4.912 (0.747)	3.384 (0.515)	0.274 (0.054)

*pcsgBp-GFP* was reduced 7 fold compared to wild type, indicating that decreased *csgB* transcription was responsible for the reduced curli phenotype (Figure 2.2b). Expression of  $Q^{DLP12}$  from pQ in the  $\Delta Q^{DLP12}$  strain restored WT levels of curli expression (Figure 2.2a, b).

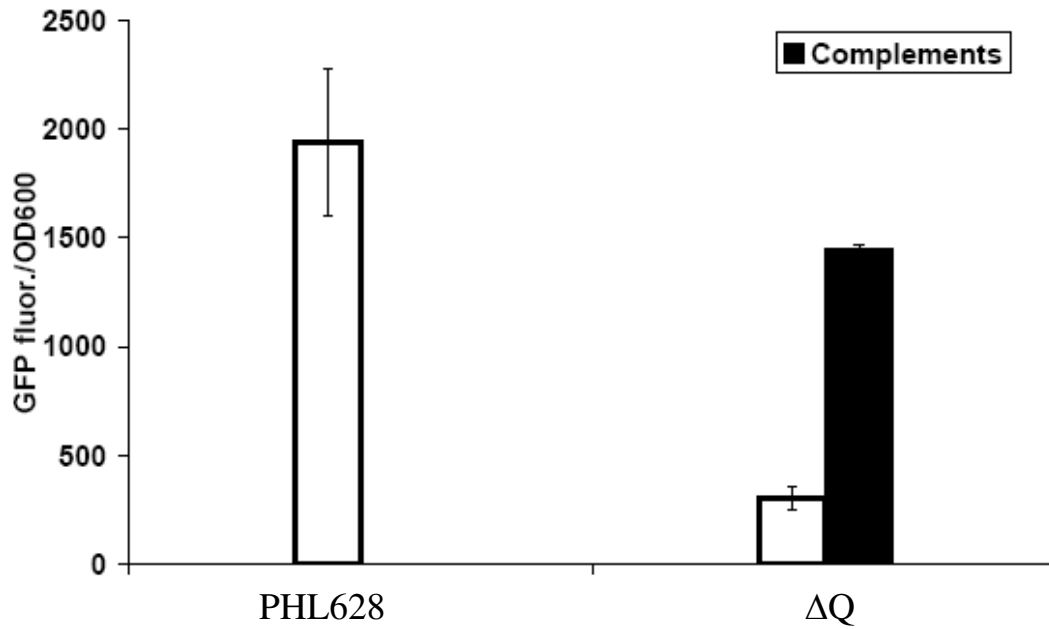
#### Intrinsic transcriptional terminator prediction

Since lambdoid Q proteins function as antiterminators, we postulated that  $Q^{DLP12}$  would be acting in a similar manner. We queried the promoter region using Softberry© and found a potential terminator ~100bp upstream of the translation start site. We next mapped the structure of the putative terminator by inserting the *essDp* sequence from -138bp to -80bp relative to the translation start site using mfold (Figure 2.6). The structure consisted of a hairpin and had a  $\Delta G$  value of -12.5 kcal/mol. This suggests that *essDp* contains an intrinsic transcriptional terminator.

a.



b.



**Figure 2.2.** (a)  $\Delta Q^{DLP12}$  mutant exhibits reduced curli phenotype. Electron micrographs were prepared by floating cells on 300 mesh Formvar grids which were washed and then negatively stained with 3% AMB. Grids were visualized in a Philips Electron Microscope 201. Arrows point at curli fibers. (b)  $\Delta Q^{DLP12}$  exhibit decreased *csgBp* expression. Cells bearing the reporter vector pJBA110 carrying the *csgB* promoter (*csgBp*) 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 normalized to O.D.600.  $\Delta Q^{DLP12}$  mutant showed reduced curli operon expression, which was restored by complementation with pQ1. Experiments were done in quadruplicate.

## Q<sup>DLP12</sup> Regulates Transcription of DLP12 Lysis Cassette

The prediction of an intrinsic terminator suggested to us that Q<sup>DLP12</sup> regulated the DLP12 lysis genes in a manner analogous to that of other lambdoid Q proteins. To test this hypothesis, Q<sup>DLP12</sup> was overexpressed in WT cells and cell lysis was inferred by the release of  $\beta$ -galactosidase activity into the supernatant. Q<sup>DLP12</sup> overexpression promoted a 27-fold increase in  $\beta$ -galactosidase activity in the supernatant, implying that it was causing cell lysis (Data not shown).

Consistent with that observation, we found that IPTG-induced overexpression of Q<sup>DLP12</sup> caused a 22-fold increase in GFP fluorescence from the *essDp-GFP* promoter reporter (Figure 2.3a). These results were of similar magnitude to those obtained using semi quantitative RT-PCR which provided evidence for a 7-fold increase in the relative abundance of the read-through *essDp* transcript when Q<sup>DLP12</sup> was overexpressed (Figure 2.3b). Deletion of Q<sup>DLP12</sup> resulted in a modest 60% decrease in the basal production of read-through transcript from *essDp* when compared to the WT (Figure 2.3b). These results were all highly significant (P<0.05) and support the hypothesis that Q<sup>DLP12</sup> functions as a positive regulator of the lysis cassette.

## Q<sup>DLP12</sup> overexpression results in RNA polymerase enrichment at *essDp* in vivo

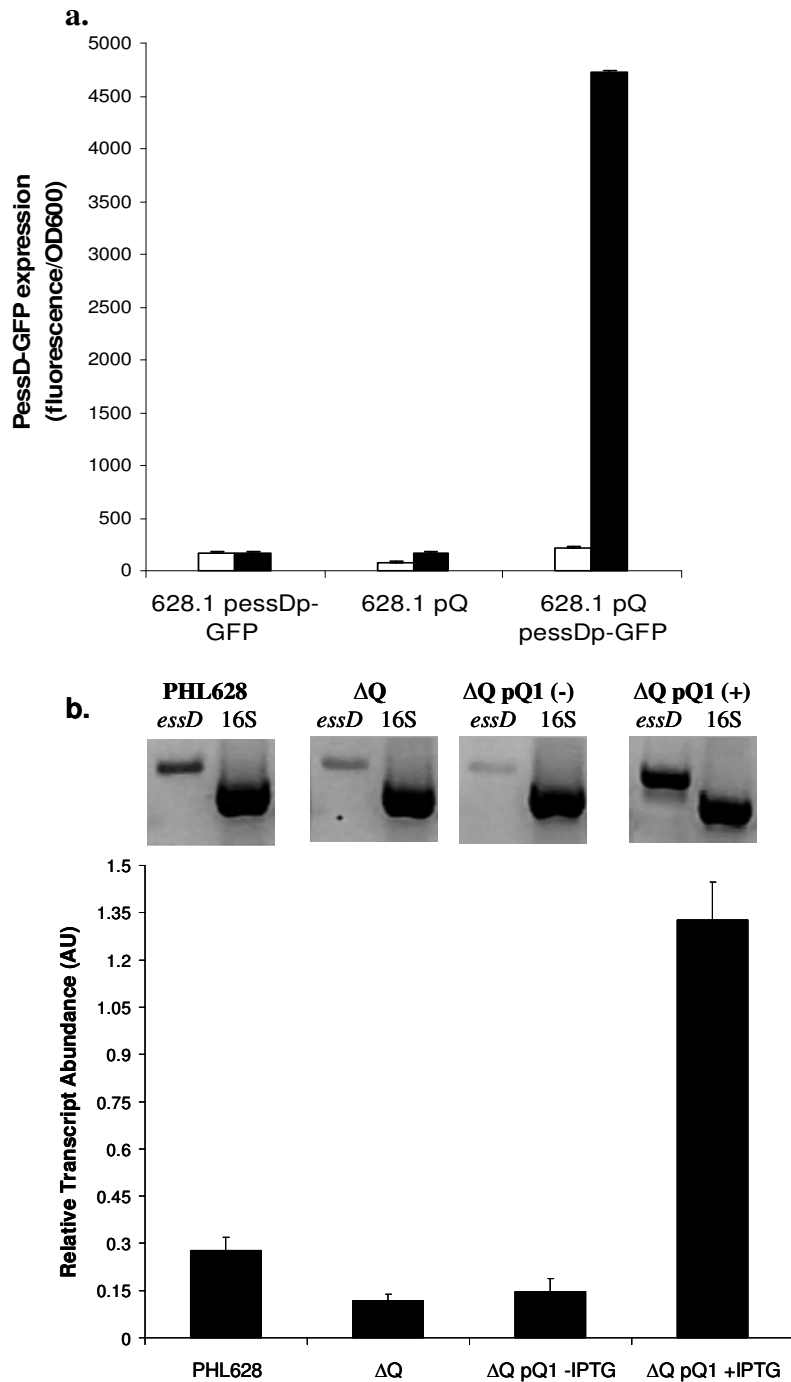
We sought to determine if Q<sup>DLP12</sup> increased the amount of RNAP at the lysis cassette promoter *in vivo*. We performed chromatin immunoprecipitation (ChIP) on cells expressing Q-His<sup>DLP12</sup> using anti-His and anti-RNAP beta subunit (anti $\beta$ ) antibodies. Upon Q-His<sup>DLP12</sup> overexpression, analysis of the anti $\beta$  pulldown revealed that there was a significant increase in the relative abundance of RNAP (29 fold) at the region of *essDp*



immediately downstream of the transcription start site (promoter proximal site) when compared to the control (Figure 2.4). There was also a marked increase in RNAP enrichment downstream of the intrinsic terminator as a result of  $Q^{DLP12}$  overexpression (5 fold), which corroborates the RT-PCR results displaying increased abundance of readthrough transcript (Fig 2.3, 2.4). Repeated attempts to pull down  $Q$ -His<sup>DLP12</sup> with either anti-His antibodies or with nickel columns were unsuccessful.

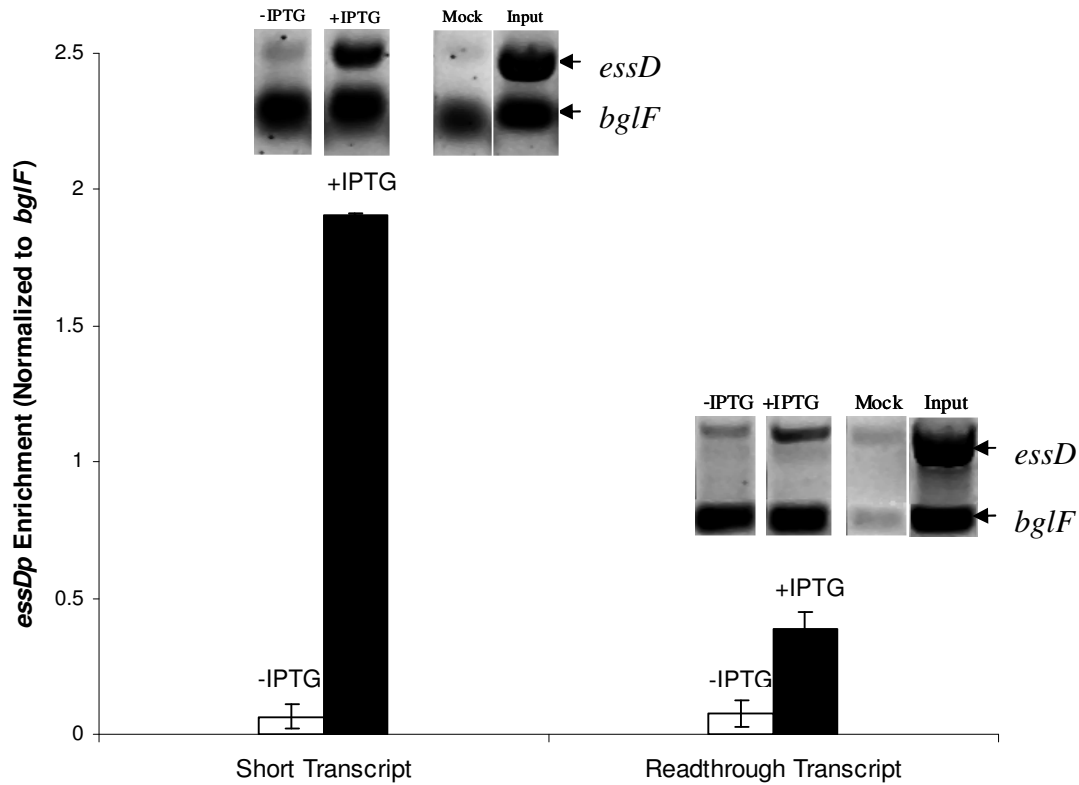
#### $Q^{DLP12}$ Binds to *essDp* DNA in vitro

In lambda,  $Q^\lambda$  regulates late gene expression through direct binding to the QBE DNA site so we attempted to determine if  $Q^{DLP12}$  directly bound the promoter of the DLP12 lysis cassette. We purified  $Q^{DLP12}$  and employed a gel mobility shift assay to assess its ability to bind *essDp*. Figure 2.5 shows that there was an observable band shift, indicating that  $Q^{DLP12}$  binds the lysis cassette promoter. The presence of 100 fold excess nonspecific competitor DNA failed to block  $Q$  from causing a shift, whereas a 10 fold excess of unlabeled probe prevented most of the labeled probe from shifting up. These results confirm that  $Q$ 's interaction was specific to *essDp* ( $K_d$  5.3uM) (Figure 2.5).

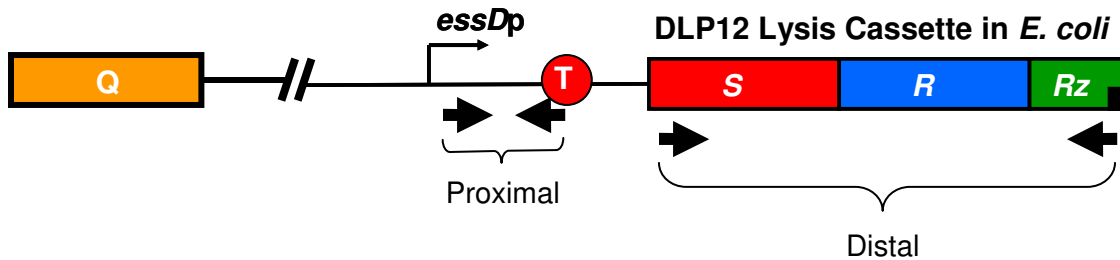


**Figure 2.3.**  $Q^{DLP12}$  upregulates transcription of *essDp*. (a) Fluorescence from *pessDp-GFP* was quantified to assess the effects of Q expression on *essDp* transcription 12 hours post IPTG-addition. Overnight cultures were diluted in fresh LB and allowed to grow at 30°C in the presence (black bars) or absence (white bars) of 1mM IPTG. Fluorescence was read using a Bio-Tek Synergy HT spectrophotometer with a 485/20 excitation filter and a 530/25 emission filter and normalized to O.D.600. (b) WT and the  $\Delta Q^{DLP12}$  mutant were grown in low salt LB. At 12hrs RNA was harvested from cells, quantified and subjected to RT-PCR. The amount of readthrough transcript (*essD*) was normalized to that of 16S rRNA transcript. The  $\Delta Q^{DLP12}$  mutant exhibited significantly reduced levels of readthrough transcript when compared to WT. IPTG mediated overexpression of  $Q^{DLP12}$  (indicated by (+)) increased relative abundance of *essD* mRNA. Experiments were quantified in quadruplicate (one representative result shown in the above images).

**a.**



**b.**

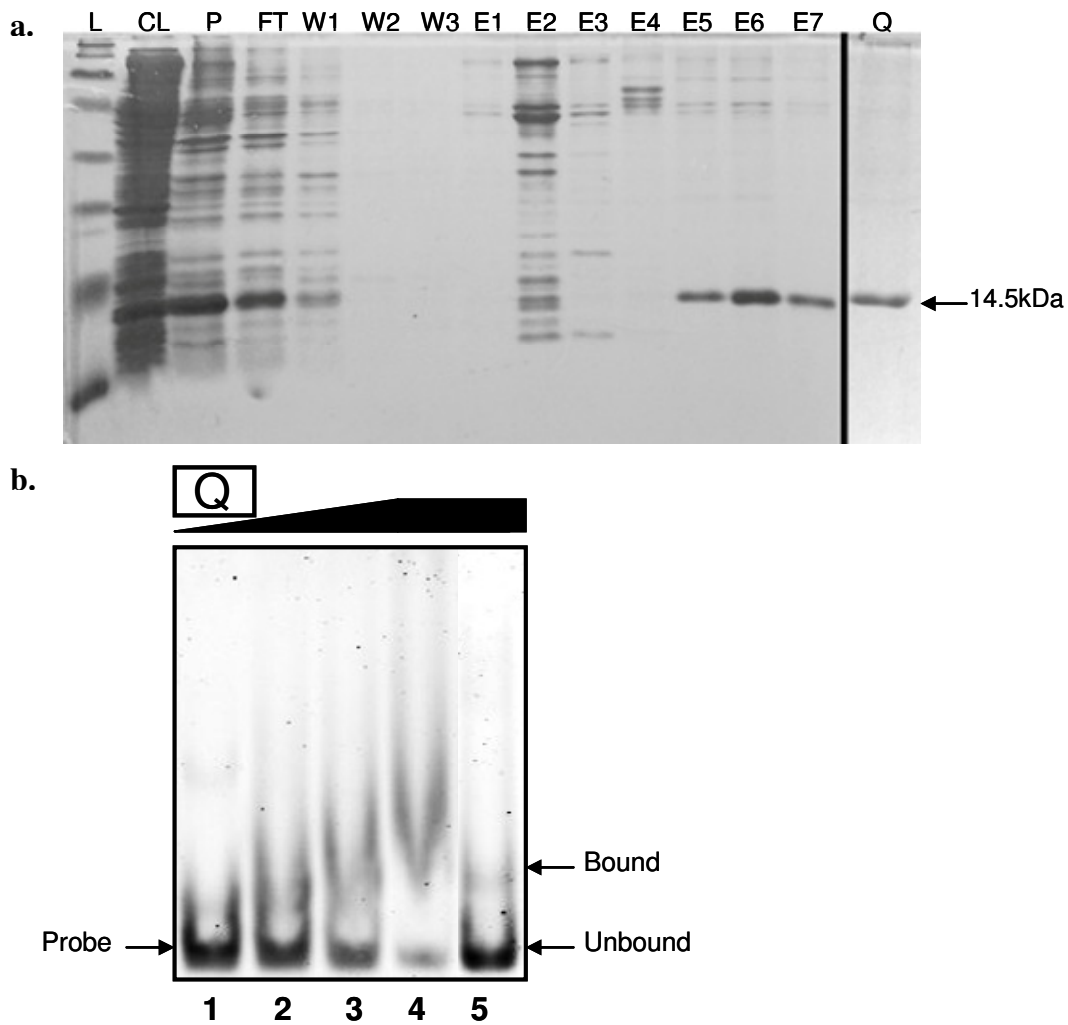


**Figure 2.4. (a)** PCR quantification after ChIP using anti-rpoB antibodies demonstrated that Q overexpression increased RNAP enrichment at the *essDp* both upstream (proximal) and downstream of the putative intrinsic terminator (distal). 628.1 pQ cells were cultured at 30C in low salt LB shaking for 12hrs either without (-IPTG) or with Q overexpression (+IPTG). Cells were crosslinked, lysed, and ChIP was performed. PCR analysis was employed to determine relative enrichment of *essDp* DNA by comparison with those of *bglF*. Mock: No antibody control, Input: Total DNA control. Gel bands were quantified using ImageJ software. Experiments were performed in triplicate (images shown from one representative replicate). **(b)** Gene map showing regions amplified by short transcript and readthrough primers within *essDp* operon (not to scale).

## **2.5 DISCUSSION**

This study shows that  $Q^{DLP12}$  positively regulates the expression of the DLP12 lysis genes, and in turn influences biofilm formation in *E. coli*. Our work implicates  $Q^{DLP12}$  in direct expression of the DLP12 lysis genes, where it likely acts as an antiterminator like other Q homologs (Guo and Roberts 2004). The fact that  $\Delta Q^{DLP12}$  mutants behave almost identically to the lysis cassette knockouts (Toba, Thompson et al. 2011) with respect to biofilm defects (Figure 2.1, 2.2 and Table 2.2), suggests that the two cistrons are involved in the same physiological pathway. Curli production was rescued in the lysis cassette mutants by deleting *nagK* (Toba, Thompson et al. 2011), demonstrating that curli regulation was sensitive to changes in peptidoglycan recycling. Thus, we expected that loss of  $Q^{DLP12}$  would affect curli production given our evidence that  $Q^{DLP12}$  regulates the lysis cassette. Future work, however, needs to be done to elucidate the exact manner in which  $Q^{DLP12}$  affects curli expression.

Prophage genes have been shown to impact bacterial physiology in many different ways including altering growth rate (Wang, Kim et al. 2010), escaping host immune responses (*bor* and *lom*) (Barondess and Beckwith 1995; Vica Pacheco S 1997), altering endogenous mutation rates (Chikova and Schaaper 2006; Pal, Macia et al. 2007), increasing resistance to antibiotics and biocides (Wang, Kim et al. 2010), and altering biofilm formation (Rice *et al.*, 2009; Wang *et al.*, 2010; Webb *et al.*, 2004). In some cases the impact on biofilm formation was a direct result of the production of filamentous prophage such as Pf1 and Pf4 in *Pseudomonas aeruginosa* (Webb, Lau et al. 2004; Rice, Tan et al. 2009). In other cases the effect was more subtle and not well understood from a mechanistic standpoint (Wang, Kim et al. 2010). This is especially true for defective



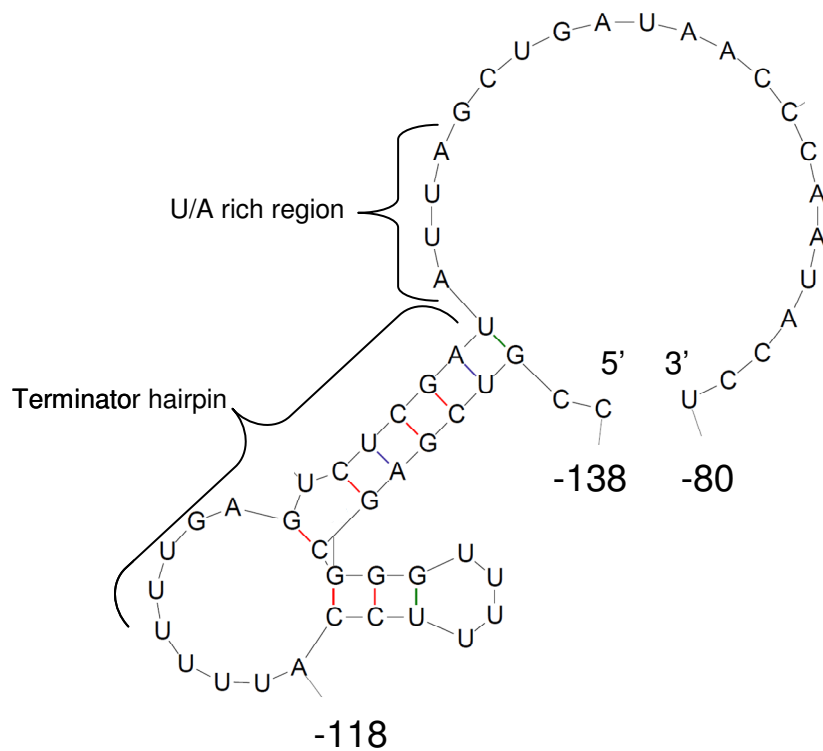
**Figure 2.5.** Purified  $Q^{DLP12}$  can bind the *essDp* region proximal to transcription start site *in vitro*. **(a)**  $Q^{DLP12}$  was purified under denaturing conditions and gradually refolded through dialysis as described in the methods section. L: Ladder, CL: Cell lysate, P: Pellet, FT: Flow through, W1-W3: Wash 1-3, E1-E7: Elution 1-7 stepwise with increasing [Imidazole], Q: Refolded pure Q fraction. **(b)** Gelshift analysis of  $Q^{DLP12}$  binding to *essDp* DNA (6.4 nM).  $Q^{DLP12}$  was present in lanes 1-5 at 0, 2.3, 4.6, 9.2 and 9.2 uM respectively. Lane 5 contained 10 fold excess of unlabeled probe.  $K_d$  for  $Q^{DLP12}$  binding: 5.3 uM.

prophages which have long been considered genetic baggage (Casjens 2003), since they have lost the ability to excise and replicate.

Recent evidence has shown that some defective prophages can still influence the physiology of their bacterial hosts (Wang, Kim et al. 2010; Toba, Thompson et al. 2011).

There are no published reports, however, demonstrating a role for defective prophage-encoded antiterminators contributing to the regulation of host cell processes such as curli production. Transcriptional antitermination occurs within bacterial operons such as *hly*, *rfa*, and *kps* which are involved in the production of hemolysin, lipopolysaccharide, and exopolysaccharide respectively (Bailey, Hughes et al. 1997; Santangelo and Roberts 2002). Expression of these genes is dependent upon the host encoded antiterminator, *rfaH* which interacts with the RNAP holoenzyme in a manner analogous to that of  $Q^\lambda$  (Bailey, Hughes et al. 1997; Santangelo and Roberts 2002). While we provide *in vivo* and *in vitro* evidence demonstrating that  $Q^{DLP12}$  directly regulates lysis cassette expression, the exact mechanism requires further elucidation. In addition, there is a need for more information about the signals that regulate expression of  $Q^{DLP12}$  itself. Huerta et al. predicted a putative sigma 32-specific promoter 182bp upstream of the translation start site of  $Q^{DLP12}$  (Huerta and Collado-Vides 2003), although neither heat (50°C) nor ethanol exposure were sufficient to up regulate  $Q^{DLP12}$  transcription in our hands (Data not shown).

Taken together, this work demonstrates that over expression of  $Q^{DLP12}$  upregulates expression of the DLP12 lysis cassette due to the direct interaction of  $Q^{DLP12}$  with *essDp*. To our knowledge, this is the first report of a functional Q from a defective prophage that is relevant to normal host physiology and suggests that this well-characterized phage mechanism of transcription regulation has been co-opted by *E. coli* in a novel way.



5' **CCGTCGAGCG** GGTTTTCCA TTTTGTGAGT **CTCGATATTA** GCTGATAACC  
**CAATACCT** 3'

**Figure 2.6.** Analysis using the program FindTerm indicated the presence of a putative Rho-independent transcriptional terminator ~100bp upstream of the DLP12 lysis cassette translation start. The terminator structure was mapped using the mfold folding program on *essDp* sequence starting at -138bp relative to translation start site of *essD* and ending at -80bp. Red base pair lines indicate normal G-C pairing, blue base pair lines: normal A-U pairing, and green base pair lines: atypical G-U pairing. The coding DNA sequence is depicted under the RNA structure. Nucleotides that base pair to form the hairpin are shown in grey.

## **LITERATURE CITED**

- Andersen, J. B., Sternberg, C., Poulsen, L. K., Bjorn, S. P., Givskov, M. & Molin, S. (1998).** New unstable variants of green fluorescent protein for studies of transient gene expression in bacteria. *Appl Environ Microbiol* **64**, 2240-2246.
- Bailey, M. J., Hughes, C. & Koronakis, V. (1997).** RfaH and the ops element, components of a novel system controlling bacterial transcription elongation. *Mol Microbiol* **26**, 845-851.
- Barondess, J. J. & Beckwith, J. (1995).** bor gene of phage lambda, involved in serum resistance, encodes a widely conserved outer membrane lipoprotein. *J Bacteriol* **177**, 1247-1253.
- Barrios, A. F., Zuo, R., Ren, D. & Wood, T. K. (2006).** Hha, YbaJ, and OmpA regulate Escherichia coli K12 biofilm formation and conjugation plasmids abolish motility. *Biotechnol Bioeng* **93**, 188-200.
- Berry, J., Summer, E.J., Struck, D.K., and Young, R. (2008).** The final step in the phage infection cycle: the Rz and Rz1 lysis proteins link the inner and outer membranes. *Mol Microbiol* **70**, 341-351.
- Boehm, A. & Vogel, J. (2012).** The csgD mRNA as a hub for signal integration via multiple small RNAs. *Mol Microbiol* **84**, 1-5.
- Canchaya, C., C, Proux, G. Fournous, A. Bruttin, and H. Brussow (2003).** Prophage genomics. *Microbiology and Molecular Biology Reviews* **67**, 238-276.
- Casjens, S. (2003).** Prophages and bacterial genomics: what have we learned so far? . *Mol Microbiol* **49**, 277-300.
- Chikova, A. K. & Schaaper, R. M. (2006).** Mutator and antimutator effects of the bacteriophage P1 hot gene product. *J Bacteriol* **188**, 5831-5838.
- Choi, K. H. & Schweizer, H. P. (2005).** An improved method for rapid generation of unmarked Pseudomonas aeruginosa deletion mutants. *BMC Microbiol* **5**, 30.
- Datsenko, K. A. & Wanner, B. L. (2000).** One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. *Proc Natl Acad Sci U S A* **97**, 6640-6645.
- Deighan, P. & Hochschild, A. (2007).** The bacteriophage lambda Q anti-terminator protein regulates late gene expression as a stable component of the transcription elongation complex. *Molecular Microbiology* **63**, 911-920.



- Fink, R. C., Black, E. P., Hou, Z., Sugawara, M., Sadowsky, M. J. & Diez-Gonzalez, F. (2012).** Transcriptional responses of *Escherichia coli* K-12 and O157:H7 associated with lettuce leaves. *Appl Environ Microbiol* **78**, 1752-1764.
- Genevaux, P., Muller, S. & Bauda, P. (1996).** A rapid screening procedure to identify mini-Tn10 insertion mutants of *Escherichia coli* K-12 with altered adhesion properties. *FEMS Microbiol Lett* **142**, 27-30.
- Guo, J. S. & Roberts, J. W. (2004).** DNA binding regions of Q proteins of phages lambda and phi 80. *Journal of Bacteriology* **186**, 3599-3608.
- Hagen, K. E., Tramp, C. A., Altermann, E., Welker, D. L. & Tompkins, T. A. (2010).** Sequence analysis of plasmid pIR52-1 from *Lactobacillus helveticus* R0052 and investigation of its origin of replication. *Plasmid* **63**, 108-117.
- Heydorn, A., Nielsen, A. T., Hentzer, M., Sternberg, C., Givskov, M., Ersboll, B. K. & Molin, S. (2000).** Quantification of biofilm structures by the novel computer program COMSTAT. *Microbiology* **146** ( Pt 10), 2395-2407.
- Holmqvist, E., Reimegard, J., Sterk, M., Grantcharova, N., Romling, U. & Wagner, E. G.** Two antisense RNAs target the transcriptional regulator CsgD to inhibit curli synthesis. *EMBO J* **29**, 1840-1850.
- Huerta, A. M. & Collado-Vides, J. (2003).** Sigma70 promoters in *Escherichia coli*: specific transcription in dense regions of overlapping promoter-like signals. *J Mol Biol* **333**, 261-278.
- Jorgensen, M. G., Nielsen, J. S., Boysen, A., Franch, T., Moller-Jensen, J. & Valentin-Hansen, P.** Small regulatory RNAs control the multi-cellular adhesive lifestyle of *Escherichia coli*. *Mol Microbiol* **84**, 36-50.
- Karatan, E. & Watnick, P. (2009).** Signals, regulatory networks, and materials that build and break bacterial biofilms. *Microbiol Mol Biol Rev* **73**, 310-347.
- Kovach, M. E., Elzer, P. H., Hill, D. S., Robertson, G. T., Farris, M. A., Roop, R. M., 2nd & Peterson, K. M. (1995).** Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* **166**, 175-176.
- Lawrence, J. G. & Ochman, H. (1998).** Molecular archaeology of the *Escherichia coli* genome. *Proc Natl Acad Sci U S A* **95**, 9413-9417.
- Lee, J. H., Kim, Y. G., Cho, M. H., Wood, T. K. & Lee, J. (2012).** Transcriptomic analysis for genetic mechanisms of the factors related to biofilm formation in *Escherichia coli* O157:H7. *Curr Microbiol* **62**, 1321-1330.

**Lindsey, D. F., Mullin, D. A. & Walker, J. R. (1989).** Characterization of the cryptic lambdoid prophage DLP12 of *Escherichia coli* and overlap of the DLP12 integrase gene with the tRNA gene *argU*. *J Bacteriol* **171**, 6197-6205.

**Mika, F., Busse, S., Possling, A., Berkholz, J., Tschowri, N., Sommerfeldt, N., Pruteanu, M. & Hengge, R.** Targeting of *csgD* by the small regulatory RNA RprA links stationary phase, biofilm formation and cell envelope stress in *Escherichia coli*. *Mol Microbiol* **84**, 51-65.

**Nickels, B. E., Roberts, C. W., Sun, H. T., Roberts, J. W. & Hochschild, A. (2002).** The sigma(70) subunit of RNA polymerase is contacted by the lambda Q antiterminator during early elongation. *Molecular Cell* **10**, 611-622.

**Ogasawara, H., Yamamoto, K. & Ishihama, A.** Role of the biofilm master regulator CsgD in cross-regulation between biofilm formation and flagellar synthesis. *J Bacteriol* **193**, 2587-2597.

**Ogasawara, H., Yamada, K., Kori, A., Yamamoto, K. & Ishihama, A. (2010).** Regulation of the *Escherichia coli* *csgD* promoter: interplay between five transcription factors. *Microbiology* **156**, 2470-2483.

**Oppenheim, A. B., Kobilier, O., Stavans, J., Court, D. L. & Adhya, S. (2005).** Switches in bacteriophage lambda development. *Annu Rev Genet* **39**, 409-429.

**Pal, C., Macia, M. D., Oliver, A., Schachar, I. & Buckling, A. (2007).** Coevolution with viruses drives the evolution of bacterial mutation rates. *Nature* **450**, 1079-1081.

**Pesavento, C., Becker, G., Sommerfeldt, N., Possling, A., Tschowri, N., Mehli, A. & Hengge, R. (2008).** Inverse regulatory coordination of motility and curli-mediated adhesion in *Escherichia coli*. *Genes Dev* **22**, 2434-2446.

**Rice, S. A., Tan, C. H., Mikkelsen, P. J. & other authors (2009).** The biofilm life cycle and virulence of *Pseudomonas aeruginosa* are dependent on a filamentous prophage. *ISME J* **3**, 271-282.

**Salazar, O., Ansejo JA (2007).** Enzymatic lysis of microbial cells. *Biotechnol Lett* **29**, 985-994.

**Santangelo, T. J. & Roberts, J. W. (2002).** RfaH, a bacterial transcription antiterminator. *Mol Cell* **9**, 698-700.

**Sauer, K., Camper, A. K., Ehrlich, G. D., Costerton, J. W. & Davies, D. G. (2002).** *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. *Journal of Bacteriology* **184**, 1140-1154.

- Sheikh, J., Hicks, S., Dall'Agnol, M., Phillips, A. D. & Nataro, J. P. (2001).** Roles for Fis and YafK in biofilm formation by enteroaggregative *Escherichia coli*. *Mol Microbiol* **41**, 983-997.
- Stanley, N. R., Britton, R. A., Grossman, A. D. & Lazizzera, B. A. (2003).** Identification of catabolite repression as a physiological regulator of biofilm formation by *Bacillus subtilis* by use of DNA microarrays. *Journal of Bacteriology* **185**, 1951-1957.
- Stewart, P. S. & Franklin, M. J. (2008).** Physiological heterogeneity in biofilms. *Nat Rev Microbiol* **6**, 199-210.
- Stoodley, P., Sauer, K., Davies, D. G. & Costerton, J. W. (2002).** Biofilms as complex differentiated communities. *Annual Review of Microbiology* **56**, 187-209.
- Thomason, M. K., Fontaine, F., De Lay, N. & Storz, G. (2012).** A small RNA that regulates motility and biofilm formation in response to changes in nutrient availability in *Escherichia coli*. *Mol Microbiol* **84**, 17-35.
- Toba, F. A., Thompson, M. G., Campbell, B. R., Junker, L. M., Rueggeberg, K. G. & Hay, A. G. (2011).** Role of DLP12 lysis genes in *Escherichia coli* biofilm formation. *Microbiology* **157**, 1640-1650.
- Vica Pacheco S, G. G. O., Paniagua Contreras GL (1997).** The lom gene of bacteriophage lambda is involved in *Escherichia coli* K12 adhesion to human buccal epithelial cells. *FEMS Microbiol Lett*, 129–132.
- Vidal, O., Longin, R., Prigent-Combaret, C., Dorel, C., Hooreman, M. & Lejeune, P. (1998).** Isolation of an *Escherichia coli* K-12 mutant strain able to form biofilms on inert surfaces: involvement of a new ompR allele that increases curli expression. *J Bacteriol* **180**, 2442-2449.
- Wang, X., Kim, Y., Ma, Q., Hong, S. H., Pokusaeva, K., Sturino, J. M. & Wood, T. K. (2010).** Cryptic prophages help bacteria cope with adverse environments. *Nat Commun* **1**, 147.
- Webb, J. S., Lau, M. & Kjelleberg, S. (2004).** Bacteriophage and phenotypic variation in *Pseudomonas aeruginosa* biofilm development. *J Bacteriol* **186**, 8066-8073.
- Whiteley, M., Bangera, M. G., Bumgarner, R. E., Parsek, M. R., Teitzel, G. M., Lory, S. & Greenberg, E. P. (2001).** Gene expression in *Pseudomonas aeruginosa* biofilms. *Nature* **413**, 860-864.
- Young, I., Wang, I. & Roof, W. D. (2000).** Phages will out: strategies of host cell lysis. *Trends Microbiol* **8**, 120-128.

**Zhou, Y., Shi, T., Mozola, M. A., Olson, E. R., Henthorn, K., Brown, S., Gussin, G. N. & Friedman, D. I. (2006).** Evidence that the promoter can influence assembly of antitermination complexes at downstream RNA sites. *Journal of Bacteriology* **188**, 2222-2232.

## CHAPTER 3

### CHARACTERIZING THE TRANSCRIPTIONAL REGULATION OF THE DLP12 LYSIS CASSETTE

#### 3.1 ABSTRACT

Expression of the lysis cassette (*essD*, *ycbT*, *rzpD/rzoD*) from the Defective Lambdoid Prophage at the 12<sup>th</sup> minute of *Escherichia coli*'s genome (DLP12) is required in some strains for proper curli expression and biofilm formation. Regulating production of the lytic enzymes encoded by these genes is critical for maintaining cell wall integrity. In Lambdoid phages, late gene regulation is mediated by the vegetative sigma factor RpoD and the lambda antiterminator Q<sup>λ</sup>. We previously demonstrated that DLP12 contains a Q-like protein (Q<sup>DLP12</sup>) which positively regulates transcription of the lysis cassette, but the sigma factor responsible for this transcription initiation remains to be elucidated. *In silico* analysis of *essDp* revealed the presence of a putative -35 and -10 site recognized by the extracytoplasmic stress response sigma factor, RpoE. In this work we report that RpoE promotes transcription from *essDp* *in vivo* and *in vitro* using purified RNAP. We demonstrate that the -35 region is important for RpoE binding *in vitro* and that this region is also important for Q<sup>DLP12</sup>-mediated transcription of *essDp* *in-vivo*. This suggests that Q<sup>DLP12</sup> and RpoE physically interact *in vivo* and is consistent with the results from a bacterial 2-hybrid assay. We propose that RpoE regulates transcription of the DLP12 lysis genes through interaction with Q<sup>DLP12</sup> and that proper expression is dependent on an intact -35 sigma region in *essDp*. This work provides evidence that the unique Q-dependent regulatory mechanism of lambdoid phages has been co-opted by *E.*

*coli* harboring defective DLP12 and has been integrated into the tightly controlled RpoE regulon.

### 3.2 INTRODUCTION

In nature, bacteria are frequently preyed upon by bacteriophages. However, this process can also allow bacteria to acquire beneficial genes that can facilitate their survival (O'Brien, LaVeck et al. 1982; Strockbine, Jackson et al. 1988; Brussow, Canchaya et al. 2004). Bacteriophage can thus have a profound influence on the physiology of the host, impacting both the individual and multicellular lifestyle (Edlin, Tait et al. 1984; Forde, Thompson et al. 2008; Rice, Tan et al. 2009). Bacteriophages have been implicated in the production of hypervirulent bacteria, bacterial acquisition of antibiotic resistant genes, and biofilm formation (Barondess and Beckwith 1995; Boyd, Davis et al. 2001; Wang, Kim et al. 2010). Pathogenic bacteria such as *Vibrio cholera*, *Escherichia coli* O157-H7, and *Shigella dysenteriae* contain prophage elements coding for virulence factors which play a role in colonization of the target organism (Boyd, Davis et al. 2001; Canchaya, Fournous et al. 2004). Phage transduction, a process in which bacterial genes are shuttled from one organism to another when phage erroneously incorporate host DNA into virions, plays an active role in spreading antibiotic resistance (Schmieger and Schicklmaier 1999; Tenover 2006). Lastly, biofilm development is greatly impacted by the presence of either active prophage as is the case for *Pseudomonas aeruginosa*, or cryptic prophage elements as has been reported for *Escherichia coli* (Webb, Lau et al. 2004; Rice, Tan et al. 2009; Wang, Kim et al. 2010; Toba, Thompson et al. 2011).

Defective cryptic prophages of *E. coli* are particularly interesting due to their inherent inability to excise and enter the lytic phase of growth. Over millions of years, the host has retained these phage remnants despite the apparent metabolic cost of maintaining the extra DNA (Casjens 2003). Recently, it has been demonstrated that several defective phage genes play a role in *E. coli* stress responses and biofilm formation (Wang, Kim et al. 2010; Toba, Thompson et al. 2011). Of these cryptic phages, DLP12 is of particular interest due to its relatively intact 20 kilobase genome (Lindsey, Mullin et al. 1989). DLP12, (Defective Lambdoid Prophage present at the 12<sup>th</sup> minute) contains a functional lysis cassette homologous to that of lambda ( $\lambda$ ) phage, which has been shown to be important for cell attachment and biofilm formation (Toba, Thompson et al. 2011). For  $\lambda$ , these enzymes disrupt the cell envelope and allow viral particles to escape the cell (Young, Wang et al. 2000; Berry 2008). We recently showed that the expression of the DLP12 lysis cassette genes (the holin S encoded by *essD*, the lysozyme R, encoded by *ybcS*, and the spannin Rz/Rz1, encoded by *rzpD/rzoD*) are dependent on a Q-like protein, Q<sup>DLP12</sup> (*ybcQ*). Sequence alignment between Q<sup>DLP12</sup> and Q<sup>21</sup> shows an 84% sequence identity, which suggests that Q<sup>DLP12</sup> has a similar function to that of the canonical antiterminators. Phage  $\lambda$  regulates the expression of its lysis cassette through the production of Q which is located near the lysis cassette promoter, P<sub>R'</sub> (Guo and Roberts 2004). Q is necessary to extend transcripts produced from P<sub>R'</sub> past the intrinsic terminator situated upstream of the lysis genes. Thus, expression of these potentially lethal genes is directly tied to the abundance of Q within the cell (Oppenheim, Kobiler et al. 2005; Zhou, Shi et al. 2006; Salazar 2007). In lambdoid phages 21, 82, and  $\lambda$ , the engagement of Q with RNAP is dependent on the presence of *cis*-acting DNA elements within P<sub>R'</sub>, known

collectively as the Q utilization site (qut). The qut site contains a Q binding element (QBE), as well as a pause site that stalls RNAP (Guo, Kainz et al. 1991; Roberts, Yarnell et al. 1998; Guo and Roberts 2004). Once Q binds to the QBE, it can interact with paused RNAP and become stably incorporated into the elongation complex (Nickels, Roberts et al. 2002; Deighan and Hochschild 2007). This event releases RNAP from its stalled state and renders the complex resistant to downstream terminators. It is believed that phages have adopted this method of gene regulation as opposed to the conventional repression/derepression system used to dictate early phage gene expression due to its rapid response time (Casjens 2003).

In *E. coli*, expression of lambdoid phage genes has been reported to be under the control of the ubiquitous vegetative sigma factor RpoD or the stationary phase factor RpoS and phage promoters contain readily identifiable RpoD or RpoS consensus sequences (Nakamura, Kurihara et al. 1979; Nickels, Roberts et al. 2002; Roucourt and Lavigne 2009). Computational analysis of the DLP12 lysis cassette promoter (*essDp*), however, predicted the presence of a -35 and -10 sigma binding region that is specific for RpoE, the extracellular stress sigma factor (Rhodius, Suh et al. 2006).

In this work, we present evidence that the DLP12 lysis genes are controlled by RpoE, through direct binding to the -35 position of *essDp*. In addition, we present evidence that  $Q^{DLP12}$  interacts with both RpoE and RNAP  $\beta$  subunit in a manner analogous to that of  $Q^\lambda$  with RpoD and  $\beta$ . Furthermore, we establish that  $Q^{DLP12}$  remains stably bound to the RNAP core complex throughout transcription of the lysis cassette. Taken together these data demonstrate that *E. coli* has co-opted the Q-based



transcriptional regulator mechanism of DLP12 and domesticated it via integration into the RpoE regulon.

### **3.3 MATERIALS AND METHODS**

#### Bacterial strains and growth conditions

*E. coli* strain PHL628 is an MG1655 derivative with an *ompR234* mutation (Vidal, Longin et al. 1998). *E. coli* PHL628  $\Delta S^{DLP12}$  (628.1), a strain with *S (essD)* deleted from the chromosome (Toba, Thompson et al. 2011), was utilized in cell count, fluorometry, and Chromatin Pulldown assays unless otherwise stated because Q overexpression is less toxic in this strain (Data not shown). *E. coli* strains were routinely grown in lysogeny broth (LB) supplemented with 50  $\mu$ l/ml kanamycin (Kan) at 37°C overnight with shaking (150 rpm) and subsequently diluted 1:100 into fresh media and cultured at 30°C for the experiments unless otherwise stated. For promoter studies low salt LB (5 g/l NaCl) was used. When required, plates and media were supplemented with ampicillin (150 $\mu$ l/ml), tetracycline (10 $\mu$ g/ml) or spectinomycin (100 $\mu$ l/ml). For induction, media was supplemented with 1mM isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) according to the experimental needs. Optical Density at 600nm (O.D.600) was measured using a  $\mu$ Quant spectrophotometer (Bio-Tek).

### Promoter fusion studies

Reporter plasmid *pessDp-GFP* containing the *essD* promoter (Rueggeberg et al., 2013) was transformed into each of the PHL628 strains and selected on low salt LB Amp plates. Cells were allowed to grow with shaking (150 rpm) in low salt LB at 30°C. At indicated 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 normalized to the O.D.600 of the corresponding culture. Measurements were done in quadruplicate.

### Plasmid Construction

For RpoE over-expression, *rpoE* was also cloned into the expression vector *pBBAD18T* to form *pBAD18T: rpoE*. In this plasmid, RpoE expression is under the control of an arabinose inducible promoter (Sukchawalit, Vattanaviboon et al. 1999). For *in vitro* transcription reactions, the promoter proximal region of *essD* was cloned into *pM650* (Amp<sup>r</sup>), upstream of the intrinsic terminator of *rpoC* using the following primers: *essDp For2 Upstream BamHI* and *essDp Rev Short EcoRI*. For fluorometry measurements, *pessDp-GFP* was utilized (Rueggeberg et al., 2013). *pessDp-GFP rpoE* -10 and -35 region mutants were created with site directed mutagenesis of *pessDp-GFP* using primers listed in Table 3.1 (Stratagene). For Q<sup>DLP12</sup> overexpression, *pOFX-Q-His* was utilized (Rueggeberg et al., 2013)

**Table 3.1.**

Primer Name	Sequence (Mutated Bases in <b>Bold</b> )	Reference
-35-2 For	5' <b>G</b> ACTATTATTAATAAATGATGTCACTGCGCG	This work
-35-3 For	5' <b>A</b> TCTATTATTAATAAATGATGTCACTGCGCG	This work
-35-4 For	5' <b>AAG</b> TATTATTAATAAATGATGTCACTGCGCG	This work
-35 Rev	5'CAAAAAAAGGGCTCACGATGAAAAA	This work
-10-1 For	5' <b>GA</b> ACTGCGCGATAAATATTCATCT	This work
-10-2 For	5' <b>G</b> CACTGCGCGATAAATATTCATCT	This work
-10-3 For	5' <b>T</b> A <b>A</b> CTGCGCGATAAATATTCATCT	This work
-10 Rev	5'CATCATTTTTTAATAATAGTTCAAAAAAAGGGC	This work
rpoD -10 Mut For.	5'GCTACAGCAGAAATTGCCACTGTT <b>C</b> ATT <b>G</b> TTTCATCGTGAGCCC	This work
rpoD -10 Mut Rev.	5'GGGCTCACGATGAAA <b>C</b> AAT <b>G</b> AACAGTGGCAATTTCTGCTGTAGC	This work
essDp For2 Upstream BamHI	5'GTTTCTGGATCCCATATTCCCACTGACCGAAAC	This work
essDp Rev Short EcoRI	5'GTACTCGAATTCCGATTCGATAGAAAAAAGTTCAGAT	This work

### RpoE, Core RNAP, and Q<sup>DLP12</sup> overexpression and purification

His<sub>6</sub>-RpoE was expressed and purified as previously described (Rouvierre et al., 1995) with certain modifications. Briefly, pPER76 was electroporated into BL21DE3 (pLysS) to create the expression strain. A 1L culture of this expression strain was grown in LB broth shaking at 30°C. When the OD<sub>600</sub> reached 0.7, isopropyl-thio-β-galactoside (IPTG) (1mM) was added to the culture. Cells were harvested 3hrs post induction,

pelleted, resuspended in lysis-equilibration-wash (LEW) buffer (50mM NaH<sub>2</sub>PO<sub>4</sub>, 300mM NaCl, pH 8.0), and lysed via sonication. The lysate was centrifuged and the soluble fraction was mixed with 1.5g (3mL) Ni-IDA resin (PrepEase) precharged with Ni<sup>2+</sup> and equilibrated with LEW buffer. The resin was then transferred to a new falcon tube and washed with LEW buffer (10 resin volumes). The protein was eluted with elution buffer (50mM NaH<sub>2</sub>PO<sub>4</sub>, 300mM NaCl, and 250mM Imidazol pH 8.0) in three fractions (3 resin volumes each). SDS-PAGE analysis showed sample purity >95%. The protein was dialyzed overnight against storage buffer (20mM Tris-HCl, 20% glycerol, 500mM NaCl, 1mM EDTA, 1mM dithiothreitol). The percent glycerol was increased to 50% via dialysis and the His<sub>6</sub>-rpoE fractions were stored at -20°C. Core RNAP was purified as described in Hager et al., 1990. Q<sup>DLP12</sup> was purified as described in Rueggeberg et al., 2013.

#### Electrophoretic Mobility Shift Assay of RpoE binding

A 151bp double stranded DNA probe encompassing nucleotides -86 to +65 containing the putative RpoE binding region in *essDp* was made by PCR and gel purified. The probe was then radiolabeled using  $\gamma^{32}\text{P}$ -dATP and T4 PNK enzyme (Promega). His<sub>6</sub>-RpoE in storage buffer was added to 2.5ng of purified probe (~5000 cpm) and 100-fold excess of herring sperm DNA (competitor DNA) in a 25uL binding reaction; the final buffer was 20mM Tris HCl (pH 8.0), 25mM KCl, 12% glycerol, 1mM DTT, 0.1mM EDTA, and 40ug/mL BSA. The samples were mixed, incubated at room temperature for 25 min, and briefly stored on ice. A 3-8% gradient polyacrylamide gel in 0.5X TAE

(20mM Tris-acetate (pH 8.5), 1mM EDTA) running buffer was prechilled and prerun on ice for 30 min prior to loading reaction mixtures. Samples were electrophoresed for 2hrs (75V) at ~4°C in a Bio-Rad Mini Protean III apparatus (Bio-Rad, Hercules, Calif.) suspended in an ice bath. The glass cover plate was removed and the gel was wrapped in 1 layer of saran wrap and exposed to a phosphor screen for 1 day. The screen was then scanned with a Molecular Dynamics Typhoon PhosphorImager. Bands were quantified using ImageJ software to determine the Kd of His<sub>6</sub>-RpoE at *essDp*.

### *In vitro* transcription assay

Reactions containing 2 nM linear template (PCR amplification of *pM650-essDp* with *essDp* For2 Upstream BamHI and *essDp* Rev Short EcoRI primers) and 20 nM RNAP (20 nM core reconstituted with 100 nM  $\sigma^{70}$  or  $\sigma^E$ ) were incubated in TB [20 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 1 mM DTT and 50 mM KCl], 0.1 mg ml<sup>-1</sup> BSA and 10% glycerol, plus 200  $\mu$ M ATP, GTP and CTP and 50  $\mu$ M UTP (supplemented with 0.5  $\mu$ Ci/ $\mu$ l [ $\alpha$ -<sup>32</sup>P]-UTP) at 37°C for 10 min to form the open complex. RNAP core was used with 6xHis-RpoE. Transcription was initiated by addition of MgCl<sub>2</sub> to a final concentration of 5 mM and rifampicin to 10  $\mu$ g ml<sup>-1</sup>; this scheme allows a single round of synthesis because initiation occurs before rifampicin can bind to inhibit further rounds. Aliquots were taken at indicated times after initiation and added to 5 volumes cold 1.2X STOP buffer [600 mM Tris-HCl (pH 8.0), 12 mM EDTA, 0.16 mg ml<sup>-1</sup> tRNA] on ice. Samples were extracted with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1), 2.5 volumes 100% EtOH was added, and samples were precipitated overnight

at  $-20^{\circ}\text{C}$ . RNAs were resolved on a 12% polyacrylamide denaturing gel and detected by a Molecular Dynamics Typhoon PhosphorImager.

### Bacterial 2-Hybrid Assay

pAC $\lambda$ cI  $\beta$ -831-1057 was used to construct all plasmids encoding fusion proteins containing the DNA binding protein  $\lambda$ cI. This vector is a derivative of the plasmid pAC- $\lambda$ cI32 which itself is a derivative of the cloning vector pACYC184. These vectors are all low copy number plasmids that contain a chloramphenicol resistance cassette (25  $\mu\text{g}/\text{mL}$ ) (Hu, Kornacker et al. 2000; Dove and Hochschild 2004). pAC $\lambda$ cI  $\beta$ -831-1057 encodes both domains of  $\lambda$ cI with the C terminus domain fused to a truncated version (residues 831 to 1057) of the  $\beta$  subunit of RNA polymerase connected by a short three alanine linker region. The transcription of this protein is under the control of the *lacUV5* promoter, and can be induced by addition of IPTG. The  $\beta$  subunit truncated gene can be substituted with any other desired nucleotide sequence, because two restriction enzyme cut sites flank the sequence encoding this portion of the fusion protein, NotI before and BamHI after. Conveniently, the NotI restriction site also encodes the short three alanine residue linker. These alanines serve as a flexible linker region between the two proteins, reducing the risk of steric interactions inhibiting the function of either protein (Dove and Hochschild 2004). To synthesize the pAC $\lambda$  cI-*ycbQ* bait fusion plasmid the *ycbQ* gene was cloned into pAC $\lambda$ cI  $\beta$ -831-1057, replacing the  $\beta$  subunit gene. This was done by PCR amplifying *ycbQ* and simultaneously introducing a NotI restriction enzyme cut site before the gene and BamHI restriction enzyme cut site directly before the gene's stop

codon using specially designed oligonucleotide primers. This PCR amplicon was sequentially digested with the restriction enzymes NotI & BamHI. It was then ligated into a pAC $\lambda$ cI backbone obtained by sequentially digesting (NotI & BamHI) pAC $\lambda$ cI  $\beta$ -831-1057 to remove the  $\beta$ -831-1057 fragment. The pAC $\lambda$ cI  $\beta$ -831-1057 vector backbone was isolated using an alkaline lysis large scale plasmid prep protocol (Felicciello and Chinali 2003). Ligation products were transformed by electroporation into *E. coli* JM109, and selected on LB plates containing Chl. Colonies were PCR screened and positive colonies were sequenced to confirm their identity and check for any mutations. All confirmatory PCR reactions and sequencing reactions utilized a forward primer specific to the vector backbone and a reverse primer specific to the inserted sequence.

pBR $\alpha$   $\beta$ -831-1057 was used in the synthesis of all pBR derivative prey plasmids created for this system. This plasmid is a derivative of the low copy number cloning vector pBR332, and contains a cassette for ampicillin resistance (Dove and Hochschild 2004). It encodes the N terminal domain of the  $\alpha$  subunit of bacterial RNA polymerase whose C terminus is fused to the same truncated version (residues 831 to 1057) of the  $\beta$  subunit of RNA polymerase found in pAC $\lambda$ cI  $\beta$ -831-1057 and is also under the control of the *lacUV5* promoter. This plasmid also contains NotI and BamHI cloning sites that can be utilized in the same manner as the pAC plasmid to create fusion protein constructs (Dove and Hochschild 2004). All pBR derivative plasmids created for this system were constructed in the same manner as pAC derivative plasmids, except that Amp was utilized instead of Chl for selection purposes.

Bait and prey plasmids (Table 3.2, 3.3) were transformed into *E. coli* BN469, a *lac*<sup>-</sup> strain specifically designed for bacterial two hybrid systems. It harbors an F' plasmid

bearing an artificial test promoter that contains a binding site for  $\lambda$ cI ( $\lambda$  operator) and the *lac* core promoter, *plac*O<sub>R</sub>2-62, linked to *lacZ*. It also encodes a marker for Kan resistance (50ug/mL) (Dove and Hochschild 2004; Nickels 2009). Plasmid combinations were simultaneously transformed into the reporter strain through electroporation. Transformants were simultaneously selected for with Kan (reporter strain specific), Amp (selects for pBR derivative plasmids), and Chl (selects for pAC derivative plasmids) supplemented LB. The presence of all plasmids except for empty vector plasmids (pBR $\alpha$  & pAC $\lambda$ cI) was confirmed through PCR reactions utilizing a vector specific forward primer and an insert specific reverse primer.

#### Q-His pulldown assay

Overnight cultures (200mL) of PHL628.1 *pBAD18T: rpoE, pOFX-Q-His* were diluted into LB Kan with 1mM IPTG and grown for 9hrs, at which point arabinose (0.2% final concentration w/v) was added to the media to induce RpoE overexpression. The cultures were then incubated for an additional 3hrs. Cells were crosslinked with formaldehyde (1% v/v final concentration), shaking @100rpm for 20 min at 20°C. Glycine was added to final concentration of 0.5M and cells were shaken for 5 min to quench crosslinking. Cells were pelleted by centrifugation at 3000 x g @4°C, 10 min and resuspended in 5ml denaturing lysis buffer (50mM NaH<sub>2</sub>PO<sub>4</sub> pH 8.0, 300mM NaCl, 0.5mg/mL lysozyme, 8M Urea, 1% Triton X-100). Purification was carried out as described in PrepEase<sup>TM</sup> His-Tagged Purification Denaturing Protocol. Protein fractions



**Table 3.2.**

Plasmid	Relevant Details	Reference
pAC $\lambda$ cI $\beta$ -831-1057	Positive control bait plasmid & fusion plasmid (bait) construction template plasmid. Encodes $\lambda$ cI fused via three alanine residues to a truncated RNA pol. $\beta$ subunit (residues 831-1057)	Deighan et al. 2008
pAC $\lambda$ cI	Empty vector bait plasmid. Encodes $\lambda$ cI only.	Deighan et al. 2008
pBR $\alpha$ $\beta$ -831-1057	Fusion plasmid (prey) construction template plasmid. Encodes the N terminal domain of the $\alpha$ subunit of RNA pol. (residues 1-248) fused to a truncated RNA pol. $\beta$ subunit (residues 831-1057)	Deighan et al. 2008
pBR $\alpha$ $\sigma^{70}$ D581G	Prey plasmid. Encodes the N terminal domain of the $\alpha$ subunit of RNA pol. (residues 1-248) directly fused to domain 4 of $\sigma^{70}$ (residues 528-613) carrying the D581G substitution (increased affinity to the $\beta$ region of RNA polymerase)	Deighan et al. 2008
pBR $\alpha$	Empty vector bait plasmid. Encodes the N terminal domain of the $\alpha$ subunit of RNA pol. (residues 1-248)	Deighan et al. 2008
pBR $\alpha$ $\sigma^E$	Prey plasmid. Encodes the N terminal domain of the $\alpha$ subunit of RNA pol. (residues 1-248) fused via three alanine residues to the N terminus of full length $\sigma^E$ .	This work
pAC $\lambda$ cI- ybcQ	Bait plasmid. Encodes $\lambda$ cI fused via three alanine residues to full length YbcQ.	This work
pBR $\alpha$ $\sigma^E$ (3.0-4.2)	Prey plasmid. Encodes the N terminal domain of the $\alpha$ subunit of RNA pol. (residues 1-248) fused via three alanine residues to the N terminus regions 3.0 to 4.2 of $\sigma^E$ (residues 94-191).	This work
pBR $\alpha$ $\sigma^E$ (4.2)	Prey plasmid. Encodes the N terminal domain of the $\alpha$ subunit of RNA pol. (residues 1-248) fused via three alanine residues to the N terminus region 4.2 of $\sigma^E$ (residues 155-191).	This work
pBR $\alpha$ $\sigma^E$ (1.2-2.4)	Prey plasmid. Encodes the N terminal domain of the $\alpha$ subunit of RNA pol. (residues 1-248) fused via three alanine residues to the N terminus regions 1.2 to 2.4 of $\sigma^E$ (residues 1-93).	This work

were collected in 1.5ml volumes with 5 steps of increasing imidazole concentrations from 20mM to 250mM. Samples were mixed with loading dye (500mM Tris HCl, pH 6.8, 8% SDS, 40% glycerol, 20%  $\beta$ -mercaptoethanol, 5mg/ml bromophenol blue), boiled/unboiled, and

**Table 3.3.**

Plasmid combination	Abbreviation	Function
pAC $\lambda$ cI- $\beta$ 831-1057 + pBR $\alpha$ $\sigma^{70}$ D581G	$\beta$ (831-1057) + $\sigma^{70}$ D581G	Positive Control, test for a known interaction (strong) between this portion of the $\beta$ subunit of RNA pol. and domain 4 of $\sigma^{70}$ (residues 528-613) carrying the D581G substitution.
pAC $\lambda$ cI-ybcQ + pBR $\alpha$ $\sigma^E$	ybcQ + $\sigma^E$	Tests for an interaction between $\sigma^E$ and YbcQ.
pAC $\lambda$ cI-ybcQ + pBR $\alpha$ $\sigma^E$ (1.2-2.4)	ybcQ + $\sigma^E$ (1.2-2.4)	Tests for an interaction between $\sigma^E$ and the 1.2 to 2.4 regions of $\sigma^E$ .
pAC $\lambda$ cI-ybcQ + pBR $\alpha$ $\sigma^E$ (3.0-4.2)	ybcQ + $\sigma^E$ (3.0-4.2)	Tests for an interaction between $\sigma^E$ and the 3.0 to 4.2 regions of $\sigma^E$ .
pAC $\lambda$ cI-ybcQ + pBR $\alpha$ $\sigma^E$ (4.2)	ybcQ + $\sigma^E$ (4.2)	Tests for an interaction between $\sigma^E$ and the 4.2 region of $\sigma^E$ .
pAC $\lambda$ cI-ybcQ + pBR $\alpha$ $\sigma^{70}$ D581G	ybcQ + $\sigma^{70}$ D581G	Tests for an interaction between domain 4 of $\sigma^{70}$ (residues 528-613) carrying the D581G substitution (increased affinity to the $\beta$ region of RNA polymerase) and YbcQ.
pAC $\lambda$ cI + pBR $\alpha$	$\lambda$ cI only + $\alpha$ only	Negative control (no expected interaction between protein products)
pAC $\lambda$ cI- $\beta$ 831-1057 + pBR $\alpha$	$\beta$ (831-1057) + $\alpha$ only	Negative control (no expected interaction between protein products)
pAC $\lambda$ cI + pBR $\alpha$ $\sigma^{70}$ D581G	$\lambda$ cI only + $\sigma^{70}$ D581G	Negative control (no expected interaction between protein products)
pAC $\lambda$ cI-ybcQ + pBR $\alpha$	ybcQ + $\alpha$ only	Negative control (no expected interaction between protein products)
pAC $\lambda$ cI + pBR $\alpha$ $\sigma^E$	$\lambda$ cI only + $\sigma^E$	Negative control (no expected interaction between protein products)
pAC $\lambda$ cI + pBR $\alpha$ $\sigma^E$ (3.0-4.2)	$\lambda$ cI only + $\sigma^E$ (3.0-4.2)	Negative control (no expected interaction between protein products)
pAC $\lambda$ cI + pBR $\alpha$ $\sigma^E$ (4.2)	$\lambda$ cI only + $\sigma^E$ (4.2)	Negative control (no expected interaction between protein products)
pAC $\lambda$ cI + pBR $\alpha$ $\sigma^E$ (1.2-2.4)	$\lambda$ cI only + $\sigma^E$ (1.2-2.4)	Negative control (no expected interaction between protein products)

analyzed by 6-12% gradient SDS-PAGE and Western blotting. For Western blot analysis, proteins were transferred via a wet transfer procedure on polyvinylidene difluoride membranes (MilliPore), blocked for 1hr at room temp with 5% milk powder (w/v) in PBST (phosphate buffered saline (PBS), 0.1% Tween20), and incubated with monoclonal anti-polyHistidine antibody (Sigma) 1:3,000 overnight at 4°C. Membranes were incubated with goat anti-mouse HRP (Neoclone) 1:20,000 for 1hr at room temp. HRP signal was detected with chemiluminescence solution (ECL, Pierce). Quantitation of immunoblot was performed using ImageJ.

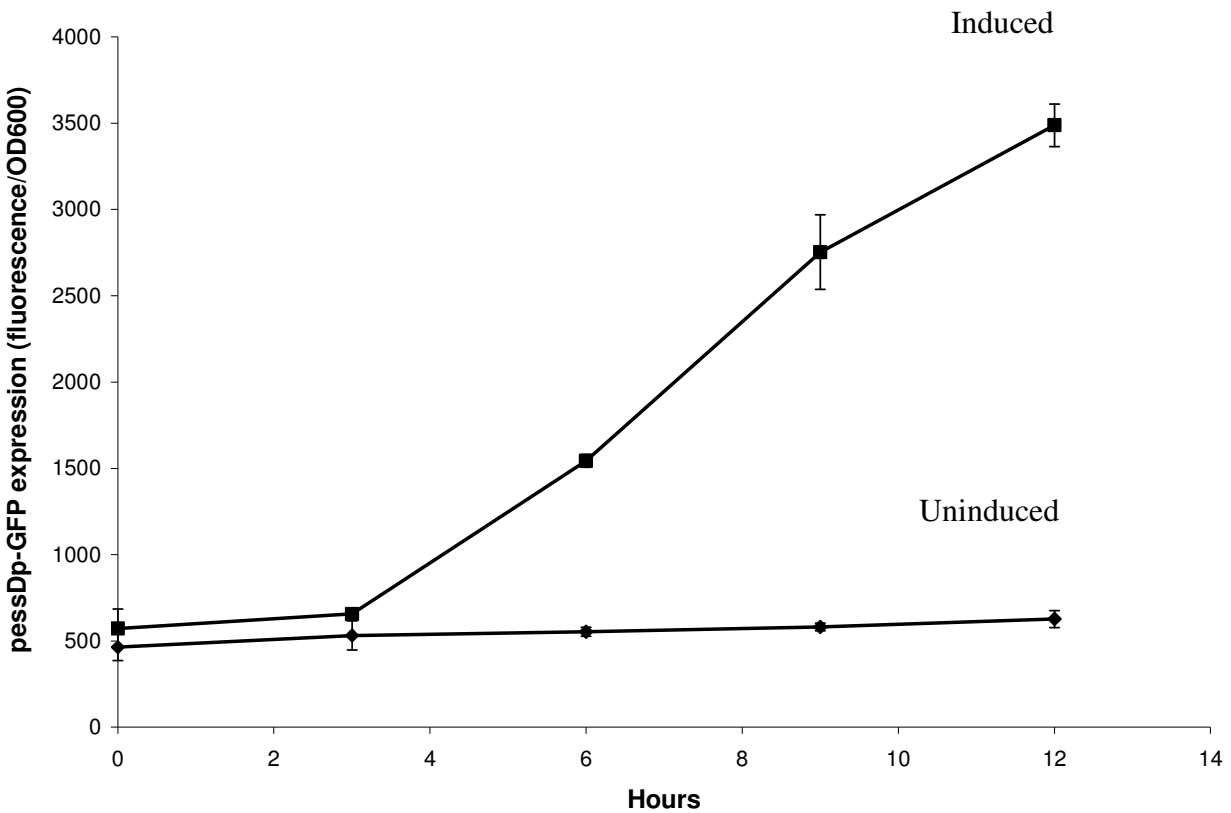
### Chromatin Pulldown Assay

Overnight cultures (200mL) of PHL628.1 *pOFX-Q-His* were prepared as in the Q-His pulldown assay, except immediately following crosslinking and quenching, pelleted cells were washed twice in 200mL cold PBS (pH 7.5) then resuspended in denaturing lysis buffer. The DNA was sheared by sonication to an average size of ~500 bp. Following centrifugation at 12000 xg, 4°C for 10min, the lysate was loaded onto PrepEase™ Ni-IDA resin column and denaturing purification was conducted as per manufacturer's instructions. A subsample of the lysate was retained as input control. The eluted Q-His/DNA complex was boiled for 10min to reverse crosslinks and the free DNA was purified using Zippy Spin columns and eluted in water. RNaseA was added (100µg/ml final conc.) and incubated for 90 minutes at 42°C. Multiplex PCR was employed to quantify the relative enrichment at *essDp* both proximal to (immediately following) the transcription start site and within the DLP12 lysis genes. These amplicons

were normalized to that of 16Ss rRNA gene. For primers see Table 1. PCR reactions were performed with 25 amplification cycles. PCR products were run on 1% agarose gel, stained with ethidium bromide, and bands quantified using ImageJ software.

### 3.4 RESULTS

When RpoE was overexpressed there was a 7 fold increase in *essDp*GFP fluorescence, indicating that RpoE was involved in controlling expression of the DLP12 lysis cassette (Fig. 3.1).

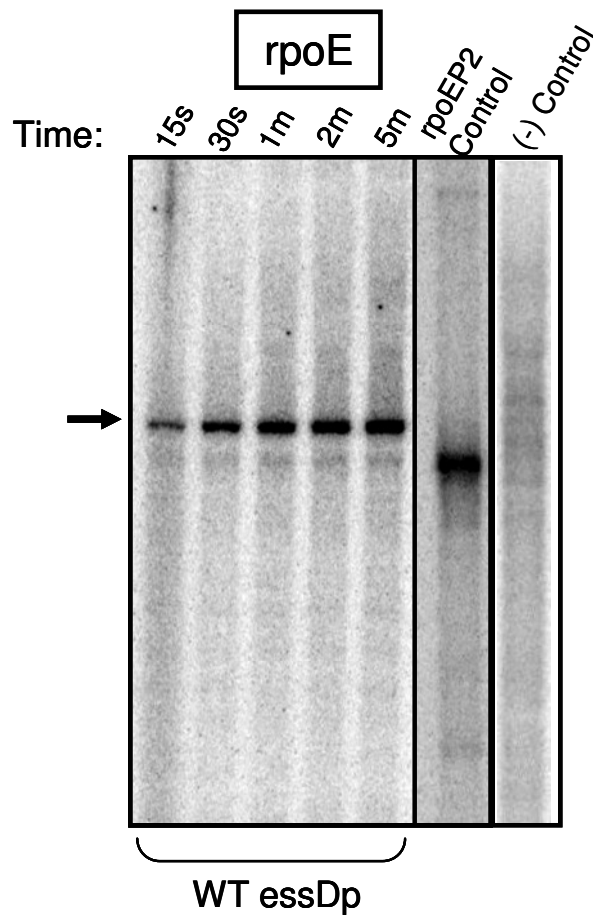


**Figure 3.1.** RpoE overexpression causes an increase in transcription from *essDp*. Fluorescence from *pEssDp-GFP* was quantified to assess the effects of RpoE overexpression on *essDp* transcription over a 12hr time course following arabinose addition (0.2%). Overnight cultures were diluted in fresh LB and allowed to grow at 30°C in the presence (■) or absence (◆) of arabinose. Experiments were performed in triplicate.

To determine whether this was a direct result of RpoE interacting with *essDp* and RNAP we performed a single round of *in vitro* transcription assays and found that *essDp* mRNA was produced in a time dependent manner only when RpoE was present (Fig. 3.2). The results in Figures 3.3a-c demonstrate that the predicted -35 region (Rhodius et al. 2006) was critical for RpoE binding of *essDp*. Mutations in the predicted -10 region, however, failed to alter RpoE binding (Fig. 3.3b). The -35 was also important for Q mediated expression of *essDp in vivo*; mutations that affected RpoE binding *in vitro* resulted 3 to 4 fold lower GFP fluorescence *in vivo* even when Q was overexpressed (Fig. 3.4). This suggested to us that RpoE and Q might be physically interacting with one another. To test this hypothesis we employed a bacterial 2-hybrid assay where Q<sup>DLP12</sup> was fused to  $\lambda$ cI and rpoE or its subunits (1.2-2.4, 3.0-4.2, or 4.2) were fused to  $\alpha$ CTD (Fig. 3.5b). Coexpression of fusions to Q and the entirety of RpoE was toxic. Coexpression of Q and either RpoE domains 1.2-2.4 or 3.0-4.2 showed 2 and 4 fold increases in activity over background, respectively (Fig. 3.5a). These data indicate that Q<sup>DLP12</sup> interacts with RpoE subunit 3.0-4.2 and, to a lesser degree, 1.2-2.4 *in vivo*.

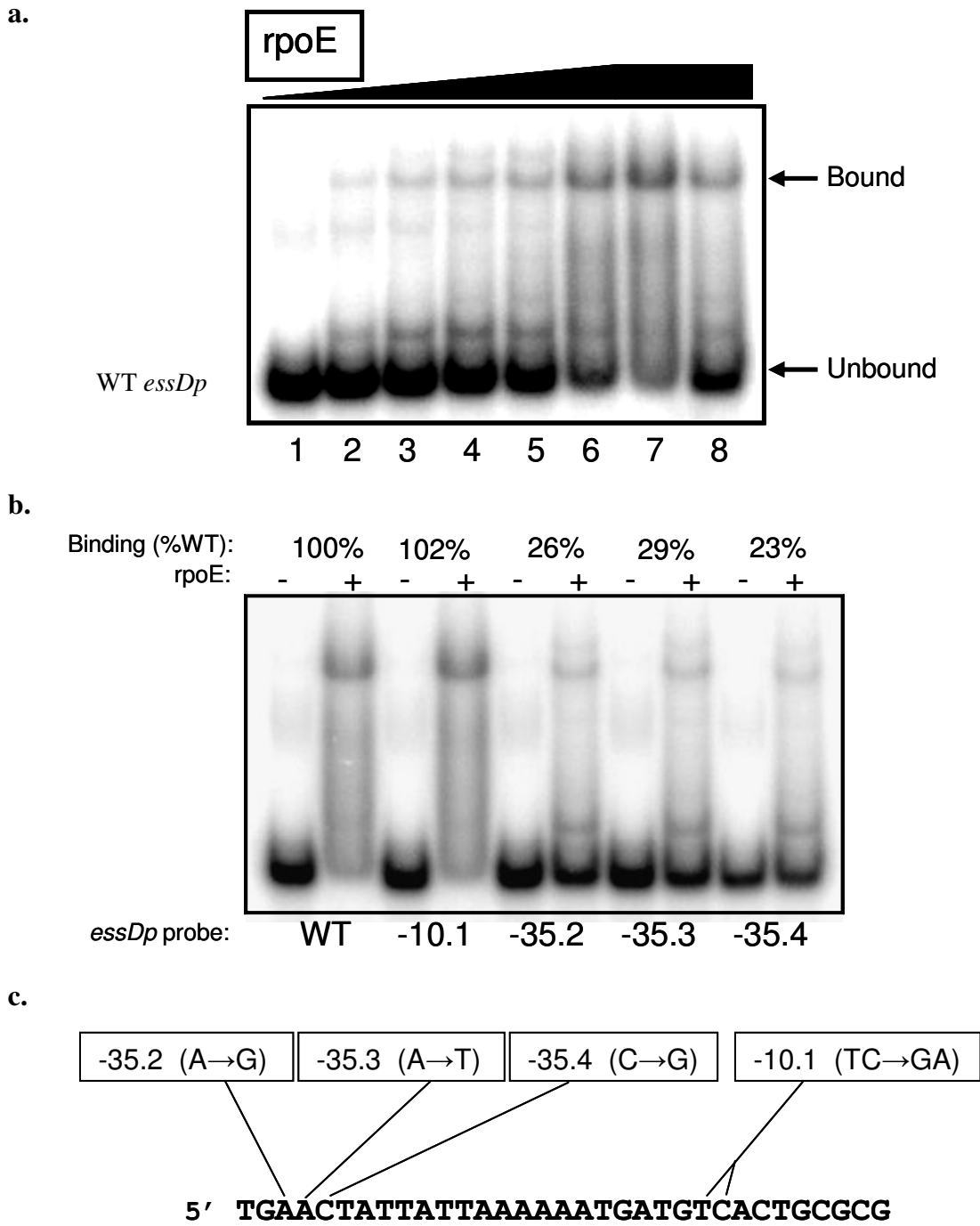
To confirm the *in vivo* interactions observed between Q<sup>DLP12</sup> and RpoE in the two hybrid assay, we utilized an affinity pulldown assay to determine if Q<sup>DLP12</sup> and RpoE copurify. However, nanoLC/MS failed to identify RpoE as a component of the protein pulldown fraction (data not shown).

Based on the Q <sup>$\lambda$</sup>  model we expected that Q<sup>DLP12</sup> would also interact with the  $\beta$  subunit of RNAP. Western blotting of the lysate from the Q-affinity pulldown assay with anti-His and anti- $\beta$  antibodies revealed the presence of both  $\beta$ -specific and Q<sup>DLP12</sup> specific bands. The crosslinked fraction showed a high molecular weight complex band and a

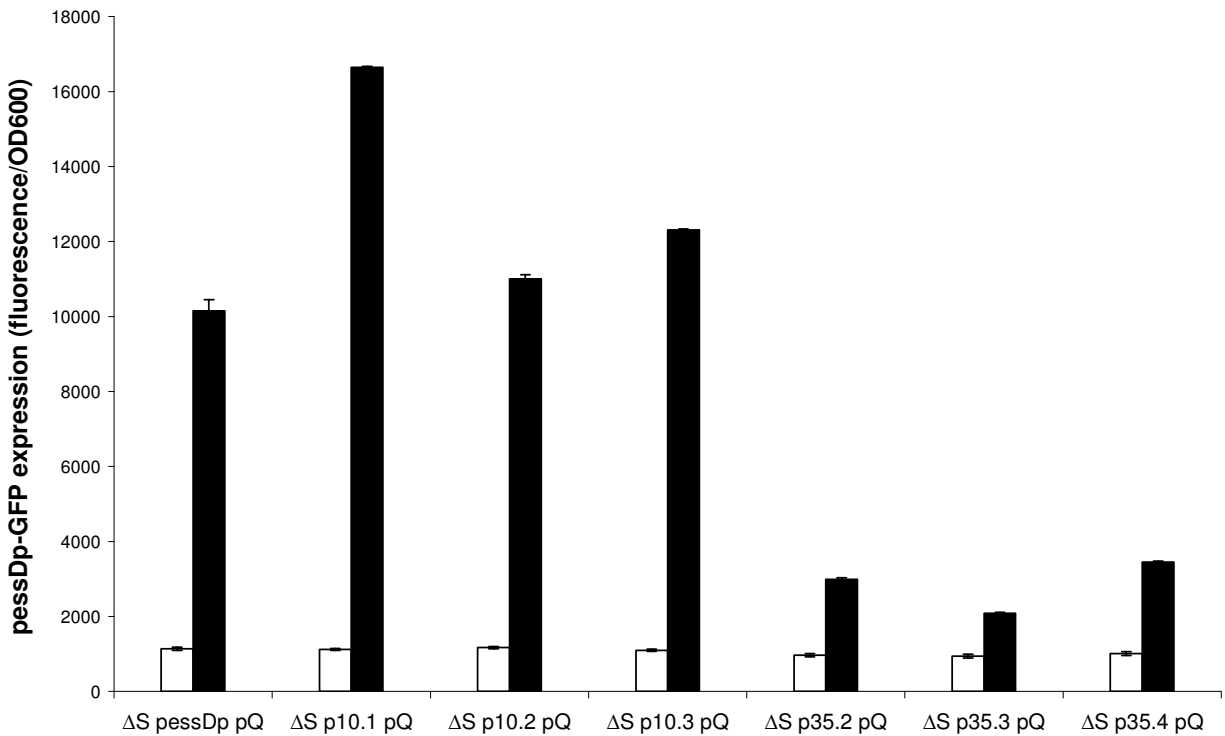


**Figure 3.2.** Purified RpoE allows RNAP to transcribe *essDp* *in vitro*. 100 nM of RpoE was mixed with 20 nM core RNAP to reconstitute holoenzyme and incubated with *essDp* template at 37°C prior to transcription initiation. Reactions were sampled over a five minute time course (Lanes 1-5). *rpoEP2* Control: Positive RpoE control containing RNAP holoenzyme incubated with *rpoEP2* promoter template and allowed to transcribe for 5 min. RpoE-dependent transcript production occurs in a time dependent manner. Arrow indicates mRNA transcript location. No transcript was detected in the absence of RpoE after 5 min of transcription ((-) Control).

Q<sup>DLP12</sup>-specific band when probed with the anti-His antibody. In contrast, the boiled fraction in which the crosslinks were reversed had very little signal from the high molecular weight fraction although it still retained the Q<sup>DLP12</sup>-specific band. This suggests that Q<sup>DLP12</sup> is interacting with other proteins in a high molecular weight complex. In addition, when the same blot was stripped and re-probed with anti-β, two



**Figure 3.3.** Purified RpoE binds *essDp* proximal to the transcription start site *in vitro*. (a) Gel shift analysis of RpoE binding to *essDp* DNA (1.02 nM). RpoE was present in lanes 1-7 at 0, 0.17, 0.35, 0.52, 0.7, 1.4, and 2.1  $\mu$ M respectively. Lane 8 contained 2.1  $\mu$ M plus 8-fold excess of unlabeled probe.  $K_d$  for RpoE binding: 0.92  $\mu$ M. (b) Gel shift analysis of RpoE binding to mutated -10 and -35 regions of *essDp* DNA (1.02 nM). -35 mutants showed significantly reduced RpoE binding when compared to WT and -10.1 mutant. Gel bands were quantified using ImageJ software. Experiments were performed in triplicate (images shown from one representative replicate). (c) *essDp* region depicting putative -35 and -10 regions (underlined) that were mutated at most highly conserved residues based on the consensus sequence for RpoE promoters (Rhodius, Suh et al. 2006). Underlined guanine represents transcription start site.

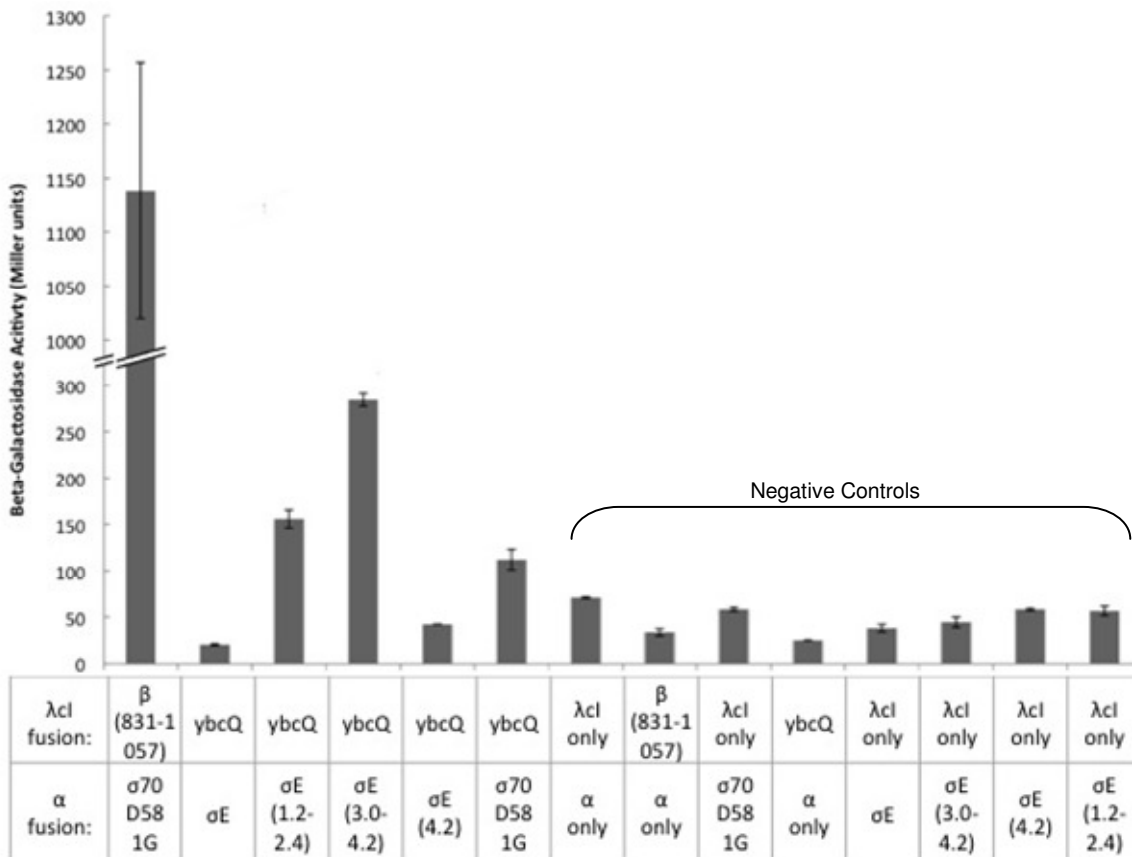


**Figure 3.4.** Q-mediated upregulation of lysis cassette is dependent on the RpoE -35 promoter region of *essDp*. Fluorescence from *pEssDp* and -10/-35 mutant versions (p10.1-10.3, p35.2-35.4) was quantified to assess the effects of mutating conserved -35 and -10 residues on *essDp* transcription 12 hours after IPTG addition. Overnight cultures were diluted in fresh LB and allowed to grow at 30°C in the presence (black bars) or absence (white bars) of 1mM IPTG. Fluorescence was read using a Bio-Tek Synergy HT spectrophotometer and normalized to OD<sub>600</sub>. Experiments were performed in triplicate.

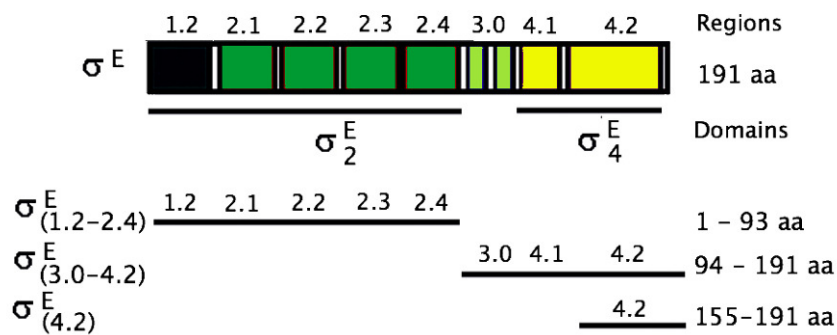
notable bands appear: a  $\beta$ -specific band and a band of high molecular weight at the same location as the anti-His band. As before, the high molecular weight band was only present in the crosslinked sample, while the sample in which the crosslinks were reversed contained only a  $\beta$ -specific band at the predicted molecular weight (Fig. 3.6). Taken together these data indicate that Q<sup>DLPI2</sup> and  $\beta$  are present in the same protein complex. In addition, analysis with nanoLC-MS confirmed the presence of  $\beta'$  subunit in the Q pulldown (data not shown).



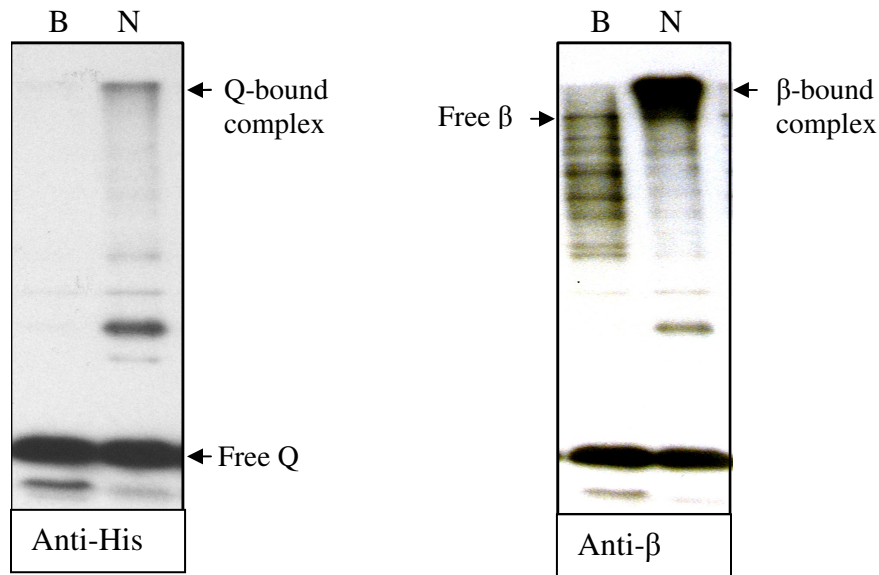
a.



b.



**Figure 3.5.** RpoE directly interacts with Q *in vivo*. (a) Reporter strains containing various combinations of Q<sup>DLPI2</sup> (YbcQ), RpoE (σ<sup>E</sup>), σ<sup>E</sup> subunit 1.2-2.4, σ<sup>E</sup> subunit 3.0-4.2, and σ<sup>E</sup> subunit 4.2 fused to either λcl or α subunit of RNAP (see above chart) were tested for β-galactosidase expression in a bacterial 2-hybrid assay (bac2-hybrid). Cell density was recorded, and then β-galactosidase activity was measured as described in Methods. Relative β-galactosidase activity is proportional to interaction strength between the two proteins being tested. Experiments were performed in triplicate. (b) Schematic of RpoE and its domains fused to α subunit used in bac2-hybrid assay.

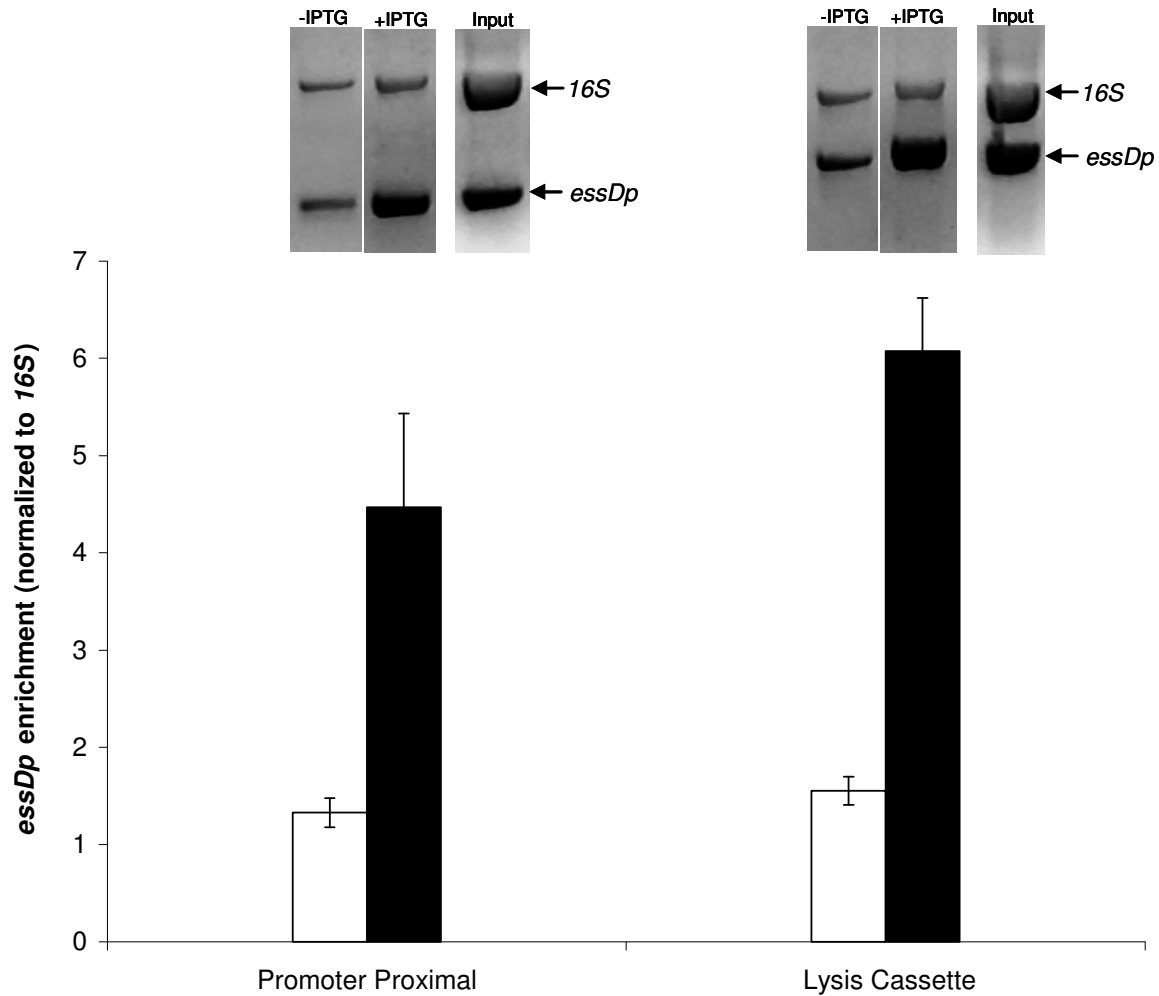


**Figure 3.6.**  $Q^{DLP12}$  localizes to the same protein complex as  $\beta$  subunit. Cells were grown for 12hrs in the presence of 1mM IPTG to induce  $Q\text{-His}^{DLP12}$  overexpression, pelleted, crosslinked, lysed, and subjected to denaturing  $Ni^{2+}$  affinity chromatography.  $Q\text{-His}^{DLP12}$  was pulled down, along with associated proteins. Purified fraction was either boiled to reverse crosslinks (B) or not (N) prior to running on SDS PAGE gel, transferring to PVDF, and immunoblotting with anti-His or anti- $\beta$  antibody to detect  $Q^{DLP12}$  or  $\beta$  subunit, respectively. Arrows indicate  $Q^{DLP12}$  and  $\beta$ .

$Q^{DLP12}$  binds to *essDp* and DLP12 lysis cassette *in vivo*

It is known that  $Q^\lambda$  becomes stably incorporated into the RNAP holoenzyme during transcription of the lysis genes, so we hypothesized that  $Q^{DLP12}$  would function similarly. We performed an affinity column pulldown assay and purified the crosslinked DNA from bacteria with or without  $Q\text{-His}^{DLP12}$  overexpression. PCR analysis indicated that over expression of  $Q\text{-His}^{DLP12}$  led to a 3.5 fold increase in *essDp* proximal to the transcription start site and a 4 fold enrichment downstream of the start sight (Fig. 3.7).

This suggests that Q-His<sup>DLP12</sup> is present in the transcription complex during expression of the DLP12 lysis genes.



**Figure 3.7.** PCR quantification after chromatin pulldown using Ni<sup>2+</sup> beads demonstrated that Q overexpression increased Q<sup>DLP12</sup> enrichment both proximal to the *essDp* transcription start site (Promoter Proximal) and downstream within the DLP12 lysis genes (Lysis Cassette). Cells were cultured at 30°C in low-salt LB shaking for 12hr either without (-IPTG) or with (+IPTG) Q overexpression. Cells were crosslinked and lysed and DNA was recovered from the proteins that were pulled down. PCR analysis was employed to determine relative enrichment of *essDp* and lysis cassette DNA by comparison with *16S rRNA*. Input: total DNA control. Gel bands were quantified using ImageJ software. Experiments were performed in triplicate (images shown from one representative replicate).

### 3.5 Discussion

Our work demonstrates that the alternative sigma factor RpoE is a positive regulator of the DLP12 lysis genes (Figure 3.1). In  $\lambda$  phage,  $Q^\lambda$  is stably incorporated into the RNAP holoenzyme through multiple interactions with the core subunits as well as specific regions of RpoD (Nickels, Roberts et al. 2002; Deighan and Hochschild 2007; Deighan, Diez et al. 2008). These interactions with RpoD are believed to destabilize RpoD-DNA interactions and allow the paused core RNAP complex to release from the pause site, at which point the Q-modified RNAP complex can move past the terminator and continue transcription. In  $\lambda$  phage, RpoD is recruited to  $P_R'$  for transcription of the lysis genes, but in DLP12 our data suggest that RpoE performs the analogous function. This was confirmed via an *in vitro* transcription assay which demonstrated that purified RpoE was sufficient to permit core RNAP to transcribe the *essDp* template (Figure 3.2).

We identified a putative RpoE -35 region within *essDp* that is essential for proper expression of the lysis genes (Figure 3.3), consistent with other RpoE-driven promoters (Rhodius and Mutalik 2010), but found that the mutations in specific residues in the -10 region were not sufficient to block RpoE-mediated transcription from the promoter (Dombroski, Walter et al. 1993).  $Q^{DLP12}$  overexpression in -35 mutants resulted in reduced lysis cassette expression levels, suggesting that excess  $Q^{DLP12}$  was not able to compensate for poor RpoE binding (Figure 3.4). This led us to hypothesize that *essDp* regulation might be dependent upon interaction between  $Q^{DLP12}$  and RpoE.

Based on those published observations and our data, we postulated that RpoE was interacting with  $Q^{DLP12}$  in an analogous manner to that of RpoD and  $Q^\lambda$ . As expected, the bacterial 2-hybrid assay revealed a significant interaction between RpoE and  $Q^{DLP12}$  *in*

*vivo* (Figure 3.5). Furthermore, the  $\beta$  subunit of RNAP co-purified with Q-His<sup>DLP12</sup> when the latter was pulled down from crosslinked cell lysates (3.6), strongly suggesting that the two transcription factors are functioning in concert to transcribe the DLP12 lysis genes. Amplification of the DNA recovered from the same pulldown assay verified that Q<sup>DLP12</sup> remains stably bound to RNAP core during transcription of the lysis genes and is not just associating at the promoter (Figure 3.7).

These data make a compelling case for cooption and domestication of the native lambdoid regulatory mechanism by *E. coli* and adds to the short list of sigma factors (RpoD and RpoS) known to promote the transcription of lambdoid lysis cassettes (Nakamura, Kurihara et al. 1979; Nickels, Roberts et al. 2002; Roucourt and Lavigne 2009).

Prophage genes have been shown to impact bacterial physiology in various ways including altering growth rate (Wang 2010), evading host immune responses (*bor and lom*) (Barondess and Beckwith 1995; Vica Pacheco S 1997), altering endogenous mutation rates (Chikova and Schaaper 2006; Pal, Macia et al. 2007), increasing resistance to antibiotics and biocides (Wang, Kim et al. 2010), and altering biofilm formation (Webb, Lau et al. 2004; Rice, Tan et al. 2009; Wang, Kim et al. 2010). While biofilm formation has been shown to be affected by the production of functional filamentous prophage Pf1 and Pf4 in *Pseudomonas aeruginosa* (Webb, Lau et al. 2004; Rice, Tan et al. 2009), the mechanisms whereby defective prophages affect biofilm formation in *E. coli* are not well understood (Wang, Kim et al. 2010).

We previously showed that the putative antiterminator Q<sup>DLP12</sup> was important for expression of the DLP12 lysis genes and biofilm formation in a curli-overexpressing *E.*

*coli* strain (Rueggeberg, Toba et al. 2013). However, the exact mechanism through which the lysis cassette was expressed remained to be elucidated. In this work we provide both *in vivo* and *in vitro* evidence that the alternative sigma factor RpoE targets the -35 region of *essDp* and works with Q<sup>DLP12</sup> and the  $\beta$  subunit of RNAP to transcribe the lysis genes. This suggests that *E.coli* has not only co-opted this important phage regulatory mechanism, but that it has also domesticated it by placing it under the control of the tightly regulated sigma factor RpoE. Although the evolutionary driving force behind this process remains unclear, this level of domestication contributes to the evidence that defective prophage, which were long considered genetic baggage (Casjens 2003) are important for host fitness (Wang *et. al* 2010).

## **LITERATURE CITED**

- Barondess, J. J. and J. Beckwith (1995). "bor gene of phage lambda, involved in serum resistance, encodes a widely conserved outer membrane lipoprotein." J Bacteriol **177**(5): 1247-53.
- Berry, J., Summer, E.J., Struck, D.K., and Young, R. (2008). "The final step in the phage infection cycle: the Rz and Rz1 lysis proteins link the inner and outer membranes." Mol Microbiol **70**: 341-351.
- Boyd, E. F., B. M. Davis, et al. (2001). "Bacteriophage-bacteriophage interactions in the evolution of pathogenic bacteria." Trends in Microbiology **9**(3): 137-144.
- Brussow, H., C. Canchaya, et al. (2004). "Phages and the evolution of bacterial pathogens: From genomic rearrangements to lysogenic conversion." Microbiology and Molecular Biology Reviews **68**(3): 560-+.
- Canchaya, C., G. Fournous, et al. (2004). "The impact of prophages on bacterial chromosomes." Molecular Microbiology **53**(1): 9-18.
- Casjens, S. (2003). "Prophages and bacterial genomics: what have we learned so far? ." Mol Microbiol **49**: 277-300.
- Chikova, A. K. and R. M. Schaaper (2006). "Mutator and antimutator effects of the bacteriophage P1 hot gene product." J Bacteriol **188**(16): 5831-8.
- Deighan, P., C. M. Diez, et al. (2008). "The bacteriophage lambda Q antiterminator protein contacts the beta-flap domain of RNA polymerase." Proc Natl Acad Sci U S A **105**(40): 15305-10.
- Deighan, P. and A. Hochschild (2007). "The bacteriophage lambda Q anti-terminator protein regulates late gene expression as a stable component of the transcription elongation complex." Molecular Microbiology **63**(3): 911-920.
- Dombroski, A. J., W. A. Walter, et al. (1993). "Amino-terminal amino acids modulate sigma-factor DNA-binding activity." Genes Dev **7**(12A): 2446-55.
- Dove, S. L. and A. Hochschild (2004). "A bacterial two-hybrid system based on transcription activation." Methods Mol Biol **261**: 231-46.
- Edlin, G., R. C. Tait, et al. (1984). "A Bacteriophage-Lambda Cohesive Ends (Cos) DNA Fragment Enhances the Fitness of Plasmid-Containing Bacteria Growing in Energy-Limited Chemostats." Bio-Technology **2**(3): 251-254.
- Forde, S. E., J. N. Thompson, et al. (2008). "Coevolution drives temporal changes in fitness and diversity across environments in a bacteria-bacteriophage interaction." Evolution **62**(8): 1830-9.
- Guo, H. C., M. Kainz, et al. (1991). "Characterization of the Late-Genes Regulatory Region of Phage-21." Journal of Bacteriology **173**(4): 1554-1560.
- Guo, J. S. and J. W. Roberts (2004). "DNA binding regions of Q proteins of phages lambda and phi 80." Journal of Bacteriology **186**(11): 3599-3608.
- Hu, J. C., M. G. Kornacker, et al. (2000). "Escherichia coli one- and two-hybrid systems for the analysis and identification of protein-protein interactions." Methods **20**(1): 80-94.
- Lindsey, D. F., D. A. Mullin, et al. (1989). "Characterization of the cryptic lambdoid prophage DLP12 of Escherichia coli and overlap of the DLP12 integrase gene with the tRNA gene argU." J Bacteriol **171**(11): 6197-205.

- Nakamura, Y., T. Kurihara, et al. (1979). "Sigma subunit of Escherichia coli RNA polymerase affects the function of lambda N gene." Proc Natl Acad Sci U S A **76**(9): 4593-7.
- Nickels, B. E. (2009). "Genetic assays to define and characterize protein-protein interactions involved in gene regulation." Methods **47**(1): 53-62.
- Nickels, B. E., C. W. Roberts, et al. (2002). "The sigma(70) subunit of RNA polymerase is contacted by the lambda Q antiterminator during early elongation." Molecular Cell **10**(3): 611-622.
- O'Brien, A. D., G. D. LaVeck, et al. (1982). "Production of Shigella dysenteriae type 1-like cytotoxin by Escherichia coli." J Infect Dis **146**(6): 763-9.
- Oppenheim, A. B., O. Kobiler, et al. (2005). "Switches in bacteriophage lambda development." Annu Rev Genet **39**: 409-29.
- Pal, C., M. D. Macia, et al. (2007). "Coevolution with viruses drives the evolution of bacterial mutation rates." Nature **450**(7172): 1079-81.
- Rhodium, V. A., W. C. Suh, et al. (2006). "Conserved and variable functions of the sigmaE stress response in related genomes." PLoS Biol **4**(1): e2.
- Rice, S. A., C. H. Tan, et al. (2009). "The biofilm life cycle and virulence of Pseudomonas aeruginosa are dependent on a filamentous prophage." ISME J **3**(3): 271-82.
- Roberts, J. W., W. Yarnell, et al. (1998). "Antitermination by bacteriophage lambda Q protein." Cold Spring Harbor Symposia on Quantitative Biology **63**: 319-325.
- Roucourt, B. and R. Lavigne (2009). "The role of interactions between phage and bacterial proteins within the infected cell: a diverse and puzzling interactome." Environ Microbiol **11**(11): 2789-805.
- Rueggeberg, K. G., F. A. Toba, et al. (2013). "A Q-like transcription factor regulates biofilm development in Escherichia coli by controlling expression of the DLP12 lysis cassette." Microbiology **159**(Pt 4): 691-700.
- Salazar, O., Ansejo JA (2007). "Enzymatic lysis of microbial cells." Biotechnol Lett **29**(7): 985-94.
- Schmieger, H. and P. Schicklmaier (1999). "Transduction of multiple drug resistance of Salmonella enterica serovar typhimurium DT104." Fems Microbiology Letters **170**(1): 251-256.
- Strockbine, N. A., M. P. Jackson, et al. (1988). "Cloning and Sequencing of the Genes for Shiga Toxin from Shigella-Dysenteriae Type-1." Journal of Bacteriology **170**(3): 1116-1122.
- Sukchawalit, R., P. Vattanaviboon, et al. (1999). "Construction and characterization of regulated L-arabinose-inducible broad host range expression vectors in Xanthomonas." FEMS Microbiol Lett **181**(2): 217-23.
- Tenover, F. C. (2006). "Mechanisms of antimicrobial resistance in bacteria." American Journal of Medicine **119**(6): S3-S10.
- Toba, F. A., M. G. Thompson, et al. (2011). "Role of DLP12 lysis genes in Escherichia coli biofilm formation." Microbiology **157**(Pt 6): 1640-50.
- Vica Pacheco S, G. G. O., Paniagua Contreras GL (1997). "The lom gene of bacteriophage lambda is involved in Escherichia coli K12 adhesion to human buccal epithelial cells." FEMS Microbiol Lett(156): 129-132.



- Vidal, O., R. Longin, et al. (1998). "Isolation of an Escherichia coli K-12 mutant strain able to form biofilms on inert surfaces: involvement of a new ompR allele that increases curli expression." J Bacteriol **180**(9): 2442-9.
- Wang, X., Y. Kim, et al. (2010). "Cryptic prophages help bacteria cope with adverse environments." Nat Commun **1**(9): 147.
- Webb, J. S., M. Lau, et al. (2004). "Bacteriophage and phenotypic variation in Pseudomonas aeruginosa biofilm development." J Bacteriol **186**(23): 8066-73.
- Young, I., I. Wang, et al. (2000). "Phages will out: strategies of host cell lysis." Trends Microbiol **8**(3): 120-8.
- Zhou, Y., T. Shi, et al. (2006). "Evidence that the promoter can influence assembly of antitermination complexes at downstream RNA sites." Journal of Bacteriology **188**(6): 2222-2232.

## CHAPTER 4

### GlcNAc-6P IMPACTS CURLI EXPRESSION BY MODULATING NagC AND ALTERING H-NS LEVELS IN DLP12 LYSIS CASSETTE MUTANTS

#### 4.1 ABSTRACT

Proper expression of the DLP12 lysis cassette (*essD*, *ybcT*, *rzpD/rzoD*) is required in certain *Escherichia coli* strains for appropriate curli expression and biofilm formation. The action of these lysis genes causes a change in the abundance of peptidoglycan breakdown products. N-acetylglucosamine-6-phosphate (GlcNAc-6P), a breakdown product peptidoglycan, has been shown to influence curli expression in *E. coli*. We hypothesized that the mutants lacking a functional lysis cassette must be sensing subtle changes in the levels of GlcNAc-6P through a transcription factor signaling pathway and in turn, regulating expression from the curli promoter (*csgD/B*). In this work, we show that the reduced curli and biofilm defect found in the lysis cassette mutants can be rescued by deleting either *ampG* or *nagK*, which encode proteins that required for GlcNAc-6P production from peptidoglycan degradation products.. In addition, we report on the role of the global regulator, H-NS, in mediating the GlcNAc-specific effect on curli production. Mutants lacking *hns* were resistant to GlcNAc-induced curli reduction. Lastly, we describe a novel role for NagC in regulating intracellular H-NS levels through transcriptional repression. A closer look at H-NS levels in DLP12 mutants showed a 3 fold increase over WT that was restored to WT levels when NagC was overexpressed. Furthermore, NagC overexpression was sufficient to restore curli expression in the DLP12 mutants. We propose that GlcNAc-6P is acting as a global effector, influencing various cellular processes, including curli production, through the direct regulation of H-

NS levels via NagC. This work provides novel evidence for the role of NagC, as a GlcNAc-6P-responsive global super regulator, which transduces cell wall status to downstream transcription pathways.

## 4.2 INTRODUCTION

Intracellular accumulation of N-acetylglucosamine-6-phosphate (GlcNAc-6-P) has been shown to cause a significant drop in curli production in *E. coli* (Barnhart, Lynem et al. 2006). Curli fibers promote that attachment of some *E. coli* strains to abiotic or biotic surfaces at the initial adhesion stage of biofilm formation (Fink, Black et al. 2012; Lee, Kim et al. 2012). The regulation of curli fiber expression is quite complex, involving over a dozen transcription factors that bind to the promoter region of *csgD*, the curli master regulator, and at least five small RNAs (sRNA) which target the 5' UTR of the *csgD* transcript (Holmqvist, Reimegard et al. ; Jorgensen, Nielsen et al. ; Mika, Busse et al. ; Boehm and Vogel 2012; Thomason, Fontaine et al. 2012). CsgD expression results in increased production of CsgA and CsgB, the curli subunit and nucleator protein, respectively. CsgD also induces expression of its own operon, which includes the curli accessory and secretion proteins CsgE, F, G (Barnhart and Chapman 2006; Barnhart, Lynem et al. 2006).

Barnhart et al., found that accumulation of GlcNAc-6-P in a  $\Delta nagA$  mutant was accompanied by a significant decline in transcription from both *csgD* and *csgB* promoters, suggesting that the negative effect on curli expression is through the action of at least one transcription factor at the divergently transcribed curli promoter. They provided clear evidence that the GlcNAc-6-P impact on curli production was not directly

mediated by NagC, which regulates transcription of the genes encoding GlcNAc-6-P degradation and import, and had been shown to directly down regulate expression of fimbrial genes. The mechanism whereby GlcNAc-6-P affects transcription from the curli promoter remains unresolved.

Though much of the work detailing the function of the *nag* regulons (*nagE*, *nagBAC*) has focused on its role in the metabolism of exogenous GlcNAc (Holmes and Russell 1972; Rolls and Shuster 1972; Alvarez-Anorve, Calcagno et al. 2005), this amino sugar is the central product of peptidoglycan recycling and NagE has recently been implicated in peptidoglycan turnover (Plumbridge 2009). *Escherichia coli* turns over 50% of its peptidoglycan per generation during normal cell growth (Cheng, Li et al. 2000). This is a tightly coordinated process whereby newly synthesized glycan strands are systematically crosslinked into the cell wall as the preexisting strands are cleaved out (Holtje 1998; Scheffers and Pinho 2005). Removal of the original glycan strands occurs through the action of multiple lytic transglycosylases, carboxypeptidases, and amidases which hydrolyze the heteropolymer into *N*-acetylglucosaminyl- $\beta$ -1,4-anhydro-*N*-acetylmuramic acid (GlcNAc-anhMurNAc)-peptide monomers, disaccharide monomers, and peptides (Shockman and Holtje, 1994; Holtje 1998; Ize, Stanley et al. 2003). The vast majority of breakdown products (>92%) are reutilized to generate new peptidoglycan precursors (Fig. 1) (Holtje, 1998; Park, 1993). The GlcNAc-anhMurNAc breakdown products are transported into the cytoplasm by the cytoplasmic membrane protein, AmpG, where they can be further processed by cytosolic proteins such as NagZ, AmpD, and NagK (Uehara and Park 2004; Chahboune, Decaffmeyer et al. 2005). NagK,

a kinase that is specific for GlcNAc, completes the conversion of GlcNAc from GlcNAc-anhMurNAc-peptides to GlcNAc-6-P (Uehara and Park 2004).

We previously noted that deleting the genes encoding peptidoglycan degradation (the lysis cassette *essD:S*, *ybcS:R*, and *ybcT:Rz*) or their regulator (*ybcQ: Q*) from the Defective Prophage found at the 12<sup>th</sup> minute of *E. coli*'s chromosome (DLP12) caused a dramatic reduction in curli production (Toba et al., 2011, Rueggeberg et al., 2013). We were able to rescue the production of curli in these mutants by deleting *nagK* which suggested that the loss of curli production may have been due to alterations in peptidoglycan recycling and changes in the intracellular level of GlcNAc-6-P.

In this work we demonstrate that the loss of curli production in the mutants can also be rescued by deletion of either *nagK* or *ampG*. Given the connection to intracellular GlcNAc-6P levels, we identified regulators differentially binding the *csg* promoter in extracts of cells grown with or without GlcNAc. We found that H-NS bound the curli promoter only in extracts of cells grown with GlcNAc and confirmed a GlcNAc-specific role for H-NS *in vivo*. A previously published *in silico* analysis predicted a NagC binding site upstream of the H-NS promoter (Oberto 2010), suggesting a possible link between GlcNAc levels and H-NS. Western blot analysis confirmed that H-NS was upregulated in  $\Delta Q$  and was restored to wild type levels by ectopic expression of *nagC*. The latter also restored curli production in  $\Delta Q$ . This work provides genetic evidence that the DLP12 lysis cassette affects intracellular GlcNAc-6-P concentrations and mediates curli regulation indirectly via NagC's effect on H-NS levels. These results implicate GlcNAc-6-P as an important global transcriptional effector and suggest a novel role for NagC as a

global transcriptional regulator, providing a mechanistic framework for linking the integrity of the bacterial cell wall with global regulatory changes.

### **4.3 MATERIALS AND METHODS**

#### Media and Reagents

Bacterial cultures were prepared on low-salt lysogeny broth (LB<sub>ls</sub>; 10 g tryptone, 5 g yeast extract, and 5 g NaCl / L media) liquid and plate media supplemented with antibiotics, when appropriate (ampicillin (Amp): 150 µg / mL, kanamycin (Kan): 50 µg / mL, tetracycline (Tet): 10 µg / mL). LB<sub>ls</sub> media containing N-acetylglucosamine (GlcNAc) was made by adding filter sterilized (0.22 µm) GlcNAc to autoclaved LB<sub>ls</sub>. All relevant reagents were prepared using milliQ water.

#### Strain Generation

*E. coli* strain PHL628 is an MG1655 derivative with an *ompR234* mutation (Vidal et al., 1998). PHL 628 deletion mutants were constructed by allele replacement using a λ-RED strategy described elsewhere (Datsenko and Wanner 2000). Briefly, a chloramphenicol (Cm) interrupted version of the gene of interest was created using PCR-mediated ligation (Choi and Schweizer 2005). 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 λ-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 chloramphenicol. After confirmed recombination via PCR, the Cm marker was removed using FLP-recombinase from plasmid pCP20 (Datsenko and Wanner 2000). All plasmid vectors are temperature sensitive and were cured from the cells by growth at 43°C. The markerless knockout mutants were confirmed by PCR and sequencing. *E. coli* BW25113 deletion strains were obtained from the Keio collection (see appendix) and cultured in LB-Kan. The deletion strains were resolved of their kanamycin resistance marker by first introducing the pCP20 plasmid. P1 phage transduction was then used to transfer the Kan-linked *ompR234* allele to BW25113 strains.. Reporter plasmids bearing *lux* transcriptional fusions to the promoters of *csgB* and *csgD* (pNLP55 and pNLP56, respectively) (Price and Raivio, 2009) and a plasmid with a *csgBp-GFP* promoter fusion (Toba et al., 2011) were transformed into cells and were selected for with the appropriate antibiotic. (See Table 4.1)

#### *In vivo* Reporter Assay of Curli Transcription

After having been electroporated with the appropriate plasmid, strains were grown for 24 hours in 2 mL of LB<sub>1s</sub> + antibiotic at 30°C with shaking at 150 rpm. After an incubation period of 24 hours, a 1/100 dilution of this bacterial culture was made in 1 mL of fresh LB<sub>1s</sub>, with or without 0.25% GlcNAc added. Quadruplicate 200 µL aliquots of this mixture were pipetted into separate wells of a 96-well plate. The 96-well plates

were incubated at 30°C with shaking at ~150 rpm; and the luminescence / OD<sub>600</sub>, was recorded at the designated time periods over 24 hrs using a Synergy HT Plate reader.

**Table 4.1.** Strains and Plasmids used in this study

<b>Strain or Plasmid Name:</b>	<b>Insert:</b>	<b>Resistance:</b>	<b>Source:</b>
pNLP10	(empty) fused with <i>lux</i>	Tetracycline	Price and Raivio, 2009
pcsgBp-luciferase (pNLP55)	<i>csgBACp</i> fused with <i>lux</i>	Tetracycline	Price and Raivio, 2009
pcsgDp-luciferase (pNLP56)	<i>csgDEFGp</i> fused with <i>lux</i>	Tetracycline	Price and Raivio, 2009
pJBA110	(empty) fused with <i>gfp</i>	Ampicillin	Toba et al. (2011)
pcsgBp-GFP	<i>csgBACp</i> fused with <i>gfp</i>	Ampicillin	Toba et al. (2011)
pNTR	<i>lacZp</i> fused with (empty)	Ampicillin	Mobile Plasmid Collection
pNTR-HNS	<i>lacZp</i> fused with <i>hns</i>	Ampicillin	Mobile Plasmid Collection
pNTR-nagC	<i>lacZp</i> fused to <i>nagC</i>	Ampicillin	Mobile Plasmid Collection
<i>E. coli</i> PHL 628		Kanamycin	Vidal et al. (1998)
<i>E. coli</i> PHL 628 $\Delta S$		Kanamycin	Toba et al. (2011)
<i>E. coli</i> PHL 628 $\Delta R$		Kanamycin	Toba et al. (2011)
<i>E. coli</i> PHL 628 $\Delta Rz$		Kanamycin	Toba et al. (2011)
<i>E. coli</i> PHL 628 $\Delta Q$		Kanamycin	Rueggeberg et al (2013)
<i>E. coli</i> BW25113		None	Culture Collection
<i>E. coli</i> BW25113 $\Delta hns$		Kanamycin	Keio Collection
<i>E. coli</i> PHL 628 $\Delta S \Delta nagK$		Kanamycin	Toba et al. (2011)
<i>E. coli</i> PHL 628 $\Delta R \Delta nagK$		Kanamycin	Toba et al. (2011)
<i>E. coli</i> PHL 628 $\Delta Rz \Delta nagK$		Kanamycin	Toba et al. (2011)
<i>E. coli</i> PHL 628 $\Delta S \Delta ampG$		Kanamycin	This Study
<i>E. coli</i> PHL 628 $\Delta R \Delta ampG$		Kanamycin	This Study
<i>E. coli</i> PHL 628 $\Delta Rz \Delta ampG$		Kanamycin	This Study

### Biotin-Streptavidin Bead Pulldown Assay

A biotin-labeled PCR product was used as bait for cytoplasmic proteins which were later analyzed by LC/MS. *Escherichia coli* strains BW25113 and 628 protein extracts were prepared by inoculating 500 mL of LB<sub>1s</sub>, with or without 0.25% GlcNAc, with 5 mL of an overnight culture incubated at 30°C with shaking at 150 rpm. These samples were incubated at 30°C with shaking at 150 rpm for a 16 hour period, after which the cells were collected via centrifugation (~8,000g for 10 min) and stored at -20°C. The cells were later resuspended in Sonication Buffer [50 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 0.5 mM EDTA] supplemented with 1 mg ml<sup>-1</sup> lysozyme. These cell



suspensions were incubated at room temperature for 30 min and were then disrupted by sonication (50% on / 50% off intervals of 1 min duration, until the solution was transparent). Low-speed centrifugation (3000g for 30 min at 4°C) and subsequent higher-speed centrifugation (8000g for 10 min at 4°C) were used to separate the soluble proteins from cellular debris. 750 µg of Dynabeads MyOne Streptavidin C1 were washed (3x) using Binding Buffer [50 mM Tris-HCl (pH 8.0), 0.5mM EDTA, 100 mM NaCl, and 0.05 mM Triton X-100] and magnetic separation, in which a magnet was used to sequester the beads while the solution was pipetted and disposed of. These beads were then incubated with 5 µg of biotin-labeled csgBAC-csgDEFG intergenic region PCR product. Unbound DNA was removed by subsequent (3x) washes and magnetic separation. The DNA-bound beads were allowed to equilibrate briefly with Binding Buffer and then also with the protein extract, supplemented with 100 µg ml<sup>-1</sup> herring sperm DNA, for 4 hours at room temp with gentle rotation. The unbound protein was removed by magnetic separation and subsequent (3x) washes. Proteins bound to the beads were eluted with 30 µL of 1M NaCl. The eluted proteins were separated and visualized on a 12% SDS-PAGE gel. After confirming the presence of distinct protein bands, the protein samples were sent to the Cornell Biotechnology Center for LC-MS analysis.

### Western Blot Analysis

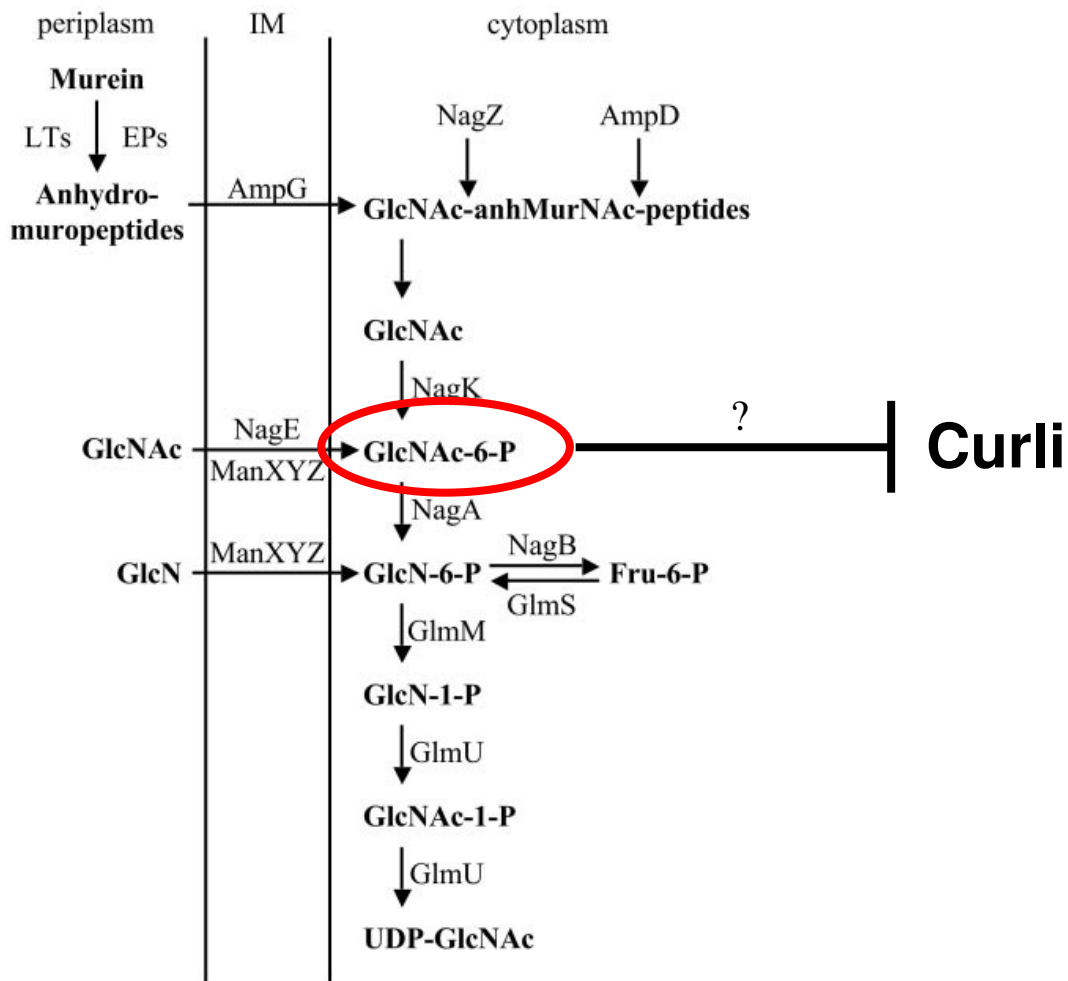
Proteins were transferred via a wet transfer procedure on polyvinylidene difluoride membranes (MilliPore), blocked for 1hr at room temp with 5% milk powder

(w/v) in PBST (phosphate buffered saline (PBS), 0.1% Tween20), and incubated with polyclonal rabbit anti-H-NS antibody (C. Gross) 1:5,000 overnight at 4°C. Membranes were incubated with goat anti-rabbit HRP (Thermo) 1:250 for 1hr at room temp. HRP signal was detected with chemiluminescence solution (ECL, Pierce). Quantitation of immunoblot was performed using ImageJ.

#### **4.4 RESULTS**

##### Deletion of *ampG* and *nagK* rescues curli expression in the DLP12 lysis cassette mutants

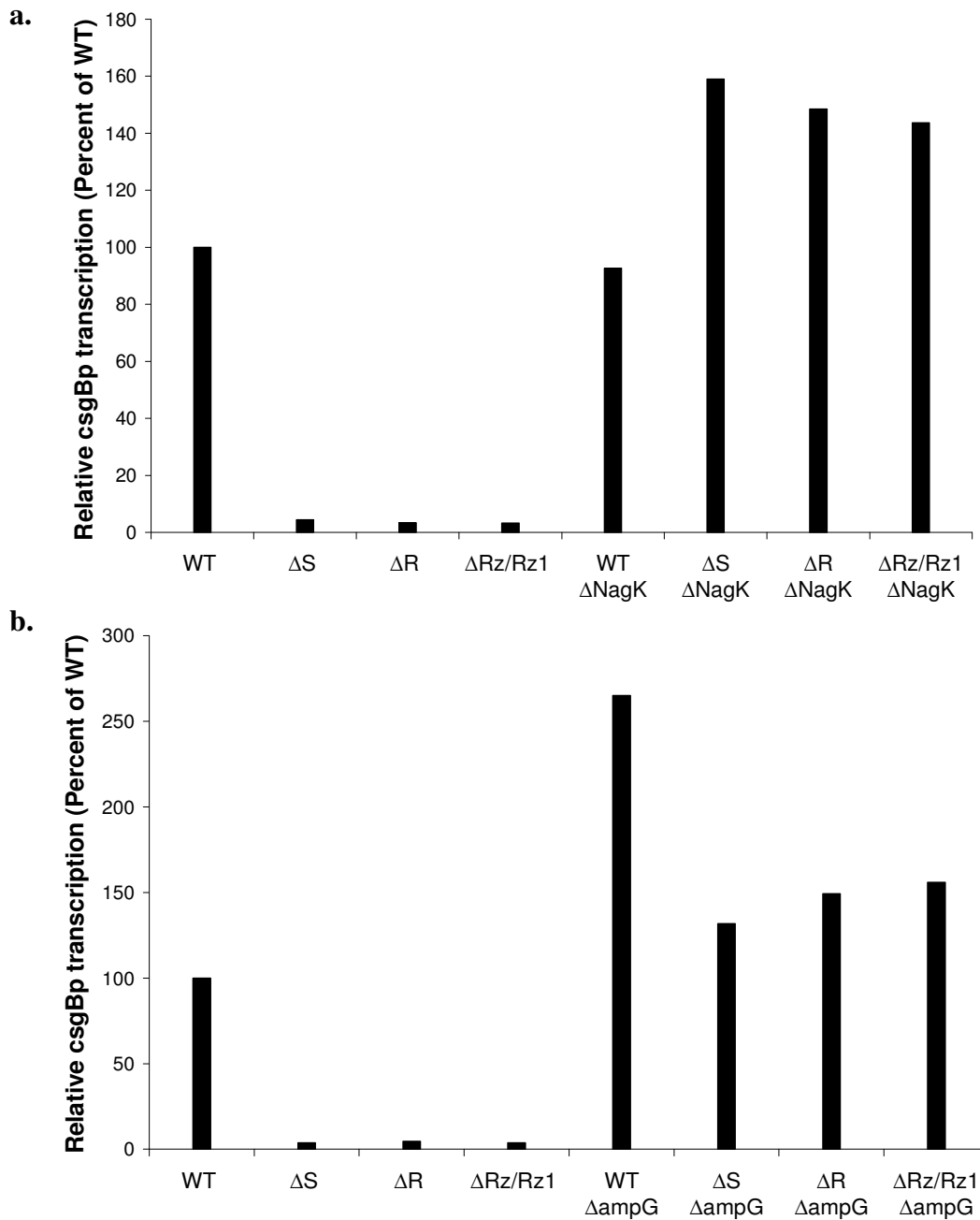
Peptidoglycan recycling is the major source of intracellular GlcNAc during normal cell growth (Fig. 4.1) (Barnhart, Lynem et al. 2006). Given the confirmed peptidoglycan degradation capacity of the DLP12 lysis cassette (Srividhya and Krishnaswamy 2007), we hypothesized that its loss might be affecting curli transcription by altering intracellular GlcNAc levels. Deletion of *ampG* and *nagK* was therefore undertaken to determine what effects decreased peptidoglycan recycling and intracellular GlcNAc6P levels, respectively, would have on curli transcription in the  $\Delta S$  and  $\Delta R$  mutants. A *csgBp-GFP* reporter construct present in the strains revealed that deletion of either *nagK* which encodes the GlcNAc kinase, or *ampG* which encodes the GlcNAc-anhMurNAc-tetra peptide transporter, rescued the defective curli phenotype of the  $\Delta S$  and  $\Delta R$  mutants, restoring curli transcription to near WT levels (Fig. 4.2a and b).



**Figure 4.1.** Schematic of peptidoglycan recycling in a gram negative cell. Increased GlcNAc-6P levels result in decreased curli production, through unknown mechanism (Barnhart et al. 2006).

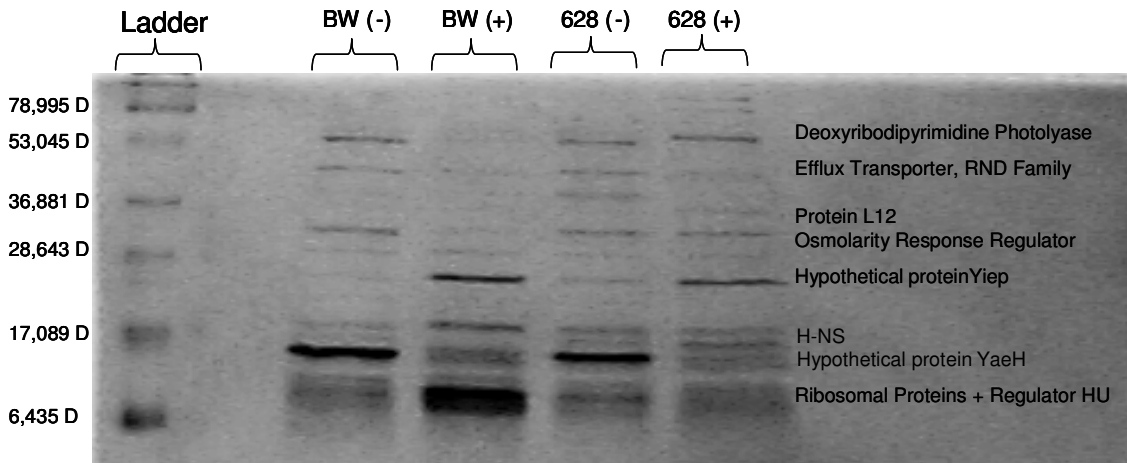
#### GlcNAc treatment alters activator/repressor binding at the *csgD-B* intergenic region

Since previous genetic efforts to identify the regulator responsible for mediating the effect of GlcNAc on curli transcription were unsuccessful (Barnhardt and Chapman 2006) we attempted an alternative biochemical approach to identifying proteins that bound the *csgD-B* intergenic region in a GlcNAc-specific manner. Streptavidin pull-downs of the biotin labeled intergenic region incubated with GlcNAc-exposed cell



**Figure 4.2.** NagK and AmpG are involved in the curli expression defect found in the DLP12 lysis cassette mutants. (a) Deletion of *nagK* restores curli production in the DLP12 lysis cassette knockouts to WT levels (chart modified from Toba et al. 2011). (b) Deletion of *ampG* also restores curli production in the DLP12 lysis cassette knockouts. Cells bearing the reporter vector *pJBA110* carrying the *csgB* promoter (*csgBp*) fused to a short lived GFP were studied for gene expression. Experiments were done in quadruplicate.

extracts contained several proteins that were either enriched or reduced in abundance as compared to those of untreated control (Fig. 4.3). H-NS, in particular was undetectable in the pulldown from untreated cells, but was enriched to 20% molar concentration in the pulldown from GlcNAc-treated cells as determined by nanoLC/MS (Table 4.2).



**Figure 4.3.** Profile of proteins binding the *csgDB* promoter region *in vitro*. Cells (Strain BW 25113 and PHL 628) were grown in 500 mL of either LB (-) or LB with 0.25% GlcNAc (+) for 16 hrs before being harvested by centrifugation. SDS-PAGE gel of proteins eluted from biotin labeled promoter recovered with streptavidin Dynabeads (.75 mg beads / rxn) after it had been incubated with 150 mg protein (30 mg / mL).

### GlcNAc's effect on *csgD* transcription is mediated in part by H-NS

H-NS has recently been shown to be a negative regulator of curli transcription (Ogasawara, Yamada et al. 2010), however, a link to GlcNAc levels has never been identified. To test the hypothesis that H-NS mediates the effect of intracellular GlcNAc levels on curli expression we compared luminescence production from the *csgDp-luxCDABE* and *csgBp-luxCDABE* reporter plasmids in the wild type and H-NS mutant, both in the presence and absence of GlcNAc. Remarkably, knocking out H-NS

**Table 4.2.** NanoLC/MS identification of proteins associated with *csgD*-B intergenic region.

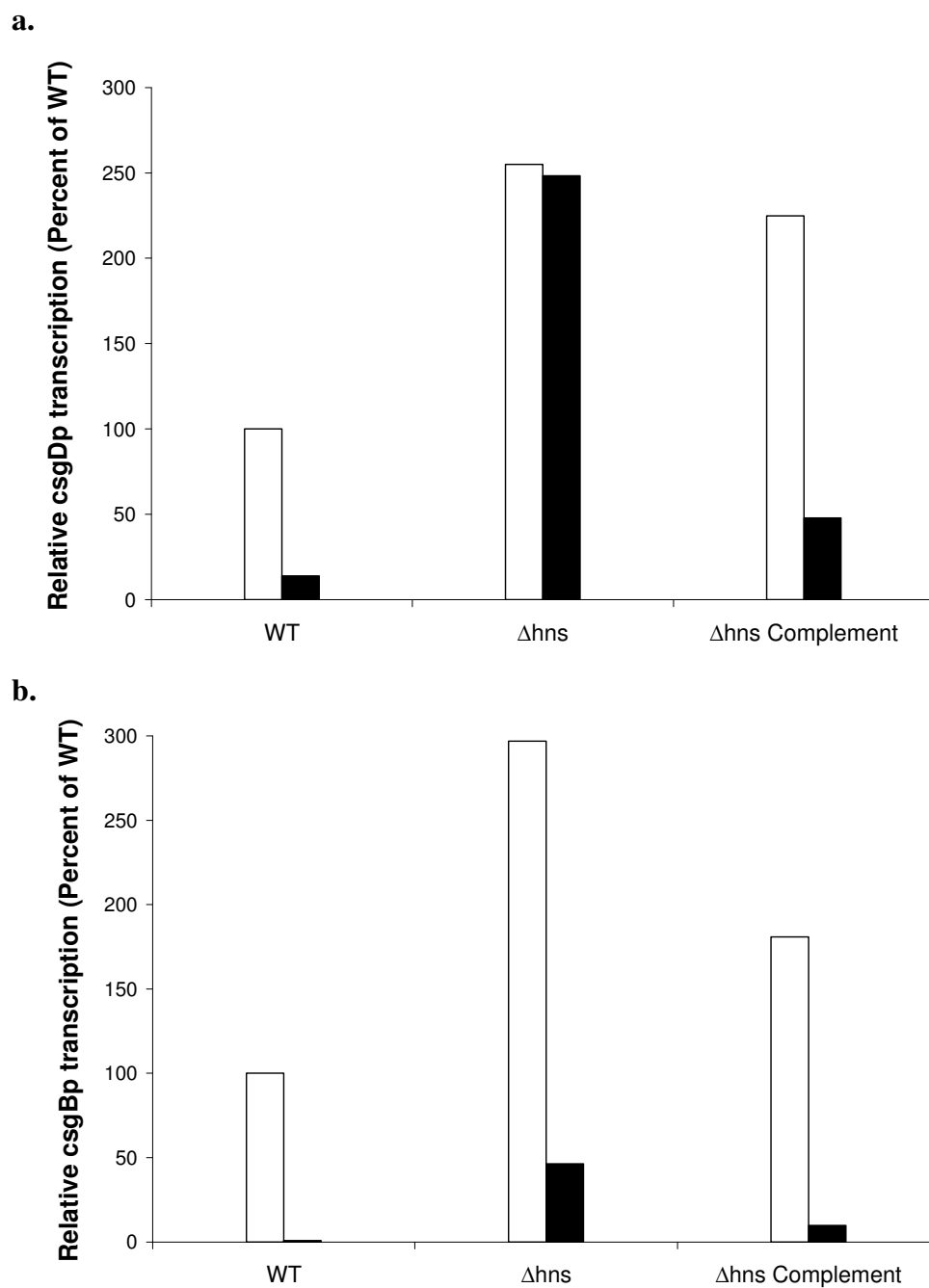
<b>Biotin-Streptavidin Pull-down Protein Identification</b>		
	<b>GlcNAc (-)</b>	<b>GlcNAc (+)</b>
<b><u>Name:</u></b>	<b><u>Molar %:</u></b>	<b><u>Molar %:</u></b>
• YaeH Hypothetical Protein Z0175	35.9%	4.27%
• IHF Integration Host Factor Subunit Alpha	2.64%	Not Detected
• YhiX (aka GadX) Chain A, of DNA-binding Transcriptional Dual Regulator	2.11%	Not Detected
• HisI	1.32%	Not Detected
• OmpR	1.05%	Not Detected
• HupA Transcriptional Regulator HU subunit alpha	Not Detected	3.07%
• YieP Hypothetical Protein Z5258	Not Detected	1.11%
• H-NS	Not Detected	19.88%

completely eliminated the effect of GlcNAc exposure on *csgD* transcription (Fig. 4.4a).

Deletion of H-NS also abrogated much of the GlcNAc effect on *csgB* transcription, bringing it to 50% of the level observed in the untreated WT cells; a 150 fold increase from WT cells exposed to GlcNAc (Fig. 4.4b). As expected based on previously published work (Ogasawara, Yamada et al. 2010), untreated  $\Delta hns$  cells had significantly increased *csgDp* transcription levels when compared to untreated WT cells (Fig. 4.4a).

#### NagC overexpression represses H-NS expression and rescues curli expression defect in DLP12 lysis cassette mutants

Despite these observations, there is no published report detailing a mechanistic link between peptidoglycan recycling, intracellular GlcNAc6-P levels and H-NS.



**Figure 4.4.** GlcNAc affects *csgD/B* transcription through H-NS. Cells bearing the reporter plasmid *pNLP10* carrying *lux* transcriptional fusions to the promoters of (a) *csgD* and (b) *csgB* were studied for gene expression. *csgD/Bp* transcription was normalized to that of WT cells. White bars: no GlcNAc added, Black bars: 0.25% GlcNAc added. Experiments were done in quadruplicate.

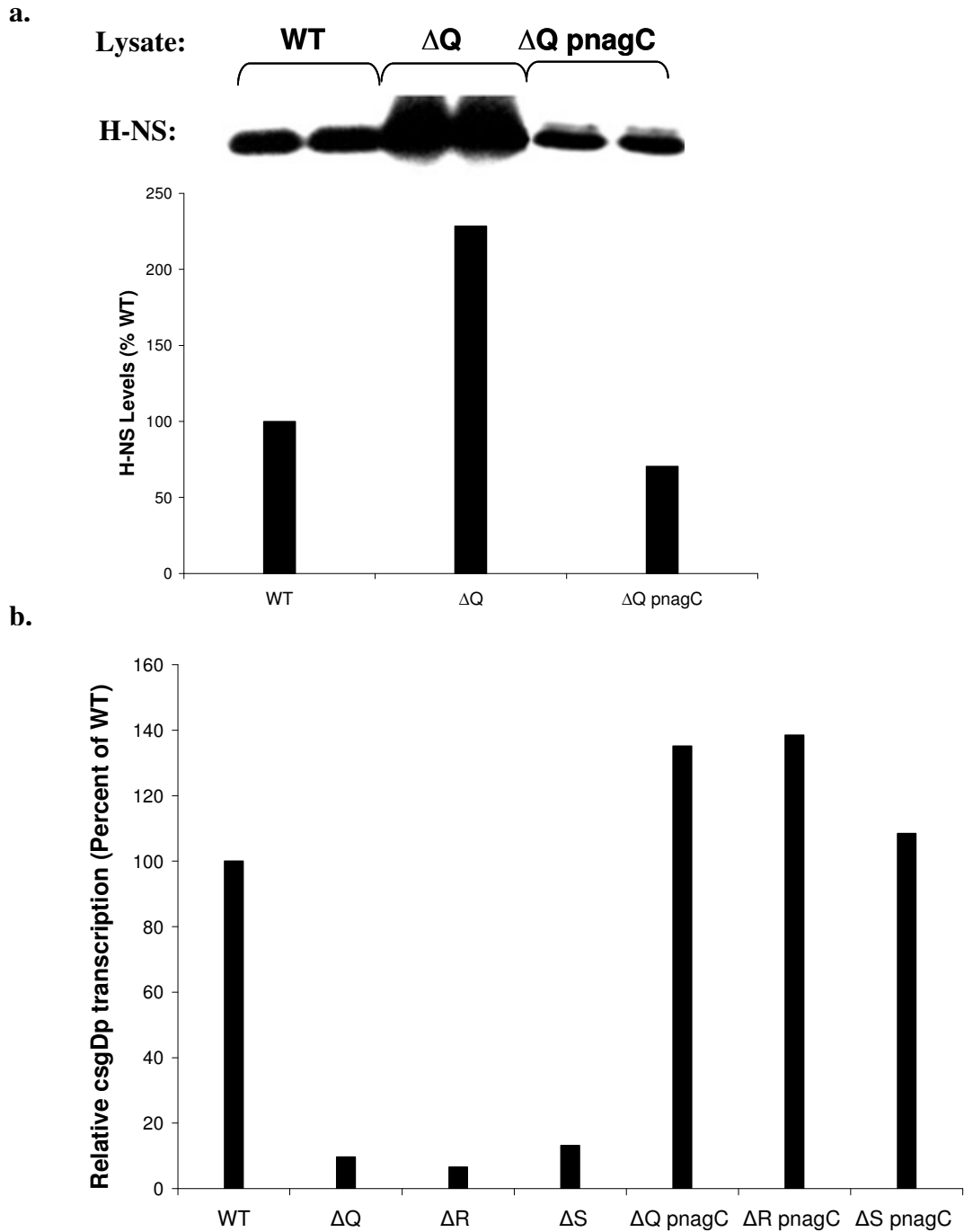
However, given that GlcNAc-6-P is the allosteric effector of NagC, we hypothesized that there might be a link between NagC and H-NS levels. NagC is a negative regulator of the *nag* operon and is released from its own and numerous other promoters when it is bound by GlcNAc6-P. Recently, an *in silico* analysis identified a putative NagC binding site in the H-NS promoter, *hnsP*. This suggested to us that NagC may act as a negative regulator of H-NS expression. We tested this hypothesis by measuring H-NS levels in the wild type,  $\Delta Q$ , and  $\Delta Q$  pNagC. As expected,  $\Delta Q$  had approximately 3 times higher levels of H-NS than the wild type. Basal expression of NagC from the *lac* promoter of pNTR-NagC, however, was sufficient to reduce H-NS below wild type levels, indicating that NagC is negatively regulating expression of H-NS (Fig. 4.5a).

In light of these data, we postulated that NagC overexpression should also rescue curli production in the DLP12 lysis cassette. We therefore overexpressed NagC in the  $\Delta Q$ ,  $\Delta S$  and  $\Delta R$  mutants and measured curli transcription. Basal expression of NagC completely restored curli production in the  $\Delta Q$ ,  $\Delta S$  and  $\Delta R$  mutants (Fig. 4.5b).

## 4.5 DISCUSSION

This study elucidates the mechanism by which the action of the lysis cassette of a defective prophage (DLP12) impacts the expression of curli fibers in *E. coli* and provides important new insights into transcriptional control of the global regulator H-NS. We showed that mutations known to affect the accumulation of GlcNAc-6P in *E. coli* (*ampG* or *nagK*) are sufficient to restore curli expression in DLP12 lysis mutants to wild type levels (Fig. 4.2). This suggests that the DLP12 lysis cassette is impacting curli expression indirectly, by modulating the abundance of intracellular GlcNAc-6P. It was previously





**Figure 4.5.** (a) NagC overexpression represses H-NS expression in DLP12 lysis cassette mutants. Cells were cultured for 16hrs at 30°C in low salt LB prior to lysis and western blot analysis of cell lysate using anti-H-NS polyclonal antibodies. Top: immunoblot of H-NS levels. Bottom: Quantification of relative intracellular H-NS levels using ImageJ software. Experiments were performed in duplicate. (b) NagC overexpression rescues curli expression defect in DLP12 lysis cassette mutants. Cells bearing the reporter plasmid *pNLP10* carrying *lux* transcriptional fusions to the *csgD* promoter (*csgDp*) were studied for gene expression. Experiments were done in quadruplicate.

shown that deletion of *nagA*, a GlcNAc-6P deacetylase, resulted in an increase in GlcNAc-6P levels, which in turn prevented expression of curli fibers (Barnhart, Lynem et al. 2006), yet the mechanism of action of GlcNAc-6P remained elusive. To elucidate the role of GlcNAc-6P we performed promoter pulldown assays and found H-NS to be the most abundant DNA binding protein recovered from the extracts of cells grown in the presence of GlcNAc. We also found H-NS to be responsible for mediating the effects of elevated GlcNAc-6P on curli expression *in vivo* (Fig 4.4).

Although H-NS had previously been reported to down regulate curli production there have been no reports providing a mechanistic description of the intracellular signals responsible for this phenomenon. H-NS is a global transcriptional regulator that controls expression of 5% of *E. coli* genes by actively binding to A/T rich stretches of DNA at promoters and within genes, inducing bending (Azam and Ishihama 1999; Hommais, Krin et al. 2001). In this work we have shown that mutations known to increase intracellular GlcNAc-6P cause a subsequent increase in H-NS abundance and binding at the curli promoter.

H-NS binding at the *csgD* promoter is known to be affected by the relative abundance of interacting protein partners and DNA binding competitors such as integration host factor (IHF) or to changes in the amount of H-NS itself (Ogasawara, Yamada et al. 2010). In attempting to identify a link between GlcNAc and H-NS we found *in silico* predictions for a potential NagC binding site in the H-NS promoter (Oberto 2010) on Ecocyc (Keseler, Collado-Vides et al. 2010). We confirmed the role of this putative NagC binding site by overexpressing NagC in the DLP12 lysis cassette mutants and showed that ectopic expression of NagC was sufficient to rescue curli

production in  $\Delta Q$  (Fig 4.5b). As expected, overexpression of NagC also restored intracellular H-NS to wild type levels, strongly suggesting that NagC is a negative regulator of H-NS expression (Fig 4.5a). These results suggest that loss of the DLP12 lysis genes increases intracellular GlcNAc-6P, derepressing NagC, and thereby derepressing the down regulation of H-NS. The associated increase in H-NS explains the observed repression of curli production in the DLP12 mutants.

Taken together, however, our results have a much broader implication; they suggest that GlcNAc-6P is a major signaling molecule in the cell, reporting on cell wall status via NagC. We therefore suggest a novel role for NagC as a global super regulator which can modulate H-NS levels in response to GlcNAc-6P, a sensitive indicator of cell wall integrity.

## LITERATURE CITED

**Alvarez-Anorve, L. I., Calcagno, M. L. & Plumbridge, J. (2005).** Why does *Escherichia coli* grow more slowly on glucosamine than on N-acetylglucosamine? Effects of enzyme levels and allosteric activation of GlcN6P deaminase (NagB) on growth rates. *Journal of Bacteriology* **187**, 2974-2982.

**Azam, T. A. & Ishihama, A. (1999).** Twelve species of the nucleoid-associated protein from *Escherichia coli*. Sequence recognition specificity and DNA binding affinity. *J Biol Chem* **274**, 33105-33113.

**Barnhart, M. M. & Chapman, M. R. (2006).** Curli biogenesis and function. *Annu Rev Microbiol* **60**, 131-147.

**Barnhart, M. M., Lynem, J. & Chapman, M. R. (2006).** GlcNAc-6P levels modulate the expression of Curli fibers by *Escherichia coli*. *J Bacteriol* **188**, 5212-5219.

**Boehm, A. & Vogel, J. (2012).** The *csgD* mRNA as a hub for signal integration via multiple small RNAs. *Mol Microbiol* **84**, 1-5.

**Chahboune, A., Decaffmeyer, M., Brasseur, R. & Joris, B. (2005).** Membrane topology of the *Escherichia coli* AmpG permease required for recycling of cell wall anhydromuropeptides and AmpC beta-lactamase induction. *Antimicrob Agents Chemother* **49**, 1145-1149.

**Cheng, Q., Li, H., Merdek, K. & Park, J. T. (2000).** Molecular characterization of the beta-N-acetylglucosaminidase of *Escherichia coli* and its role in cell wall recycling. *J Bacteriol* **182**, 4836-4840.

**Choi, K. H. & Schweizer, H. P. (2005).** An improved method for rapid generation of unmarked *Pseudomonas aeruginosa* deletion mutants. *BMC Microbiol* **5**, 30.

**Datsenko, K. A. & Wanner, B. L. (2000).** One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci U S A* **97**, 6640-6645.

**Fink, R. C., Black, E. P., Hou, Z., Sugawara, M., Sadowsky, M. J. & Diez-Gonzalez, F. (2012).** Transcriptional responses of *Escherichia coli* K-12 and O157:H7 associated with lettuce leaves. *Appl Environ Microbiol* **78**, 1752-1764.

**Holmes, R. P. & Russell, R. R. B. (1972).** Mutations Affecting Amino Sugar Metabolism in *Escherichia-Coli* K-12. *Journal of Bacteriology* **11**, 290-&.

**Holmqvist, E., Reimegard, J., Sterk, M., Grantcharova, N., Romling, U. & Wagner, E. G.** Two antisense RNAs target the transcriptional regulator CsgD to inhibit curli synthesis. *EMBO J* **29**, 1840-1850.

- Holtje, J. V. (1998).** Growth of the stress-bearing and shape-maintaining murein sacculus of *Escherichia coli*. *Microbiol Mol Biol Rev* **62**, 181-203.
- Hommais, F., Krin, E., Laurent-Winter, C., Soutourina, O., Malpertuy, A., Le Caer, J. P., Danchin, A. & Bertin, P. (2001).** Large-scale monitoring of pleiotropic regulation of gene expression by the prokaryotic nucleoid-associated protein, H-NS. *Mol Microbiol* **40**, 20-36.
- Ize, B., Stanley, N. R., Buchanan, G. & Palmer, T. (2003).** Role of the *Escherichia coli* Tat pathway in outer membrane integrity. *Mol Microbiol* **48**, 1183-1193.
- Jorgensen, M. G., Nielsen, J. S., Boysen, A., Franch, T., Moller-Jensen, J. & Valentin-Hansen, P.** Small regulatory RNAs control the multi-cellular adhesive lifestyle of *Escherichia coli*. *Mol Microbiol* **84**, 36-50.
- Keseler, I. M., Collado-Vides, J., Santos-Zavaleta, A. & other authors (2010).** EcoCyc: a comprehensive database of *Escherichia coli* biology. *Nucleic Acids Res* **39**, D583-590.
- Lee, J. H., Kim, Y. G., Cho, M. H., Wood, T. K. & Lee, J. (2012).** Transcriptomic analysis for genetic mechanisms of the factors related to biofilm formation in *Escherichia coli* O157:H7. *Curr Microbiol* **62**, 1321-1330.
- Mika, F., Busse, S., Possling, A., Berkholz, J., Tschowri, N., Sommerfeldt, N., Pruteanu, M. & Hengge, R.** Targeting of *csgD* by the small regulatory RNA RprA links stationary phase, biofilm formation and cell envelope stress in *Escherichia coli*. *Mol Microbiol* **84**, 51-65.
- Oberto, J. (2010).** FITBAR: a web tool for the robust prediction of prokaryotic regulons. *BMC Bioinformatics* **11**, 554.
- Ogasawara, H., Yamada, K., Kori, A., Yamamoto, K. & Ishihama, A. (2010).** Regulation of the *Escherichia coli* *csgD* promoter: interplay between five transcription factors. *Microbiology* **156**, 2470-2483.
- Plumbridge, J. (2009).** An alternative route for recycling of N-acetylglucosamine from peptidoglycan involves the N-acetylglucosamine phosphotransferase system in *Escherichia coli*. *J Bacteriol* **191**, 5641-5647.
- Rolls, J. P. & Shuster, C. W. (1972).** Amino Sugar Assimilation by *Escherichia-Coli*. *Journal of Bacteriology* **112**, 894-&.
- Scheffers, D. J. & Pinho, M. G. (2005).** Bacterial cell wall synthesis: new insights from localization studies. *Microbiol Mol Biol Rev* **69**, 585-607.

**Srividhya, K. V. & Krishnaswamy, S. (2007).** Subclassification and targeted characterization of prophage-encoded two-component cell lysis cassette. *J Biosci* **32**, 979-990.

**Thomason, M. K., Fontaine, F., De Lay, N. & Storz, G. (2012).** A small RNA that regulates motility and biofilm formation in response to changes in nutrient availability in *Escherichia coli*. *Mol Microbiol* **84**, 17-35.

**Uehara, T. & Park, J. T. (2004).** The N-acetyl-D-glucosamine kinase of *Escherichia coli* and its role in murein recycling. *J Bacteriol* **186**, 7273-7279.

## CHAPTER 5

### CONCLUSION: THE LINK BETWEEN CELL WALL STRESS AND BIOFILM FORMATION

In enteric bacteria, envelope stress is sensed through various two component systems, notably: OmpR/EnvZ, CpxA/R, and Rcs. These systems have been shown to regulate curli gene expression in *E. coli*. OmpR/EnvZ has a powerful positive effect on curli expression. OmpR/EnvZ senses changes in osmolarity and regulates the porins OmpC and OmpF. EnvZ, receives a signal which it transfers to OmpR, the response regulator that can directly influence gene transcription (Pratt, Hsing et al. 1996).

Activation of the CpxA/R two component system occurs due to increased envelope stress or due to misfolding of periplasmic proteins. The CpxR response regulator can then upregulate various periplasmic proteases and chaperones to enable the cell to adjust to the stressor (Hung, Raivio et al. 2001). Activated CpxR negatively regulates curli transcription (Barnhart and Chapman 2006). The Rcs phosphorelay system becomes activated under conditions of outer membrane stress (excess temperature, overexpression of membrane protein, and altered osmolarity) resulting in the synthesis of colanic acid capsule, cell division, periplasmic proteins, motility, and a small RNA (Clarke 2010). Like CpxR, the Rcs response regulator (RcsB) represses curli expression (Barnhart and Chapman 2006). Interestingly, the Rcs system is required for biofilm formation, which suggests that curli expression is transient and only required during the initial stages of biofilm attachment (Barnhart and Chapman 2006).

In addition to the two component systems, envelope stress is also sensed by the extracytoplasmic sigma factor, RpoE. RpoE is a minor sigma factor, specializing in

responses to the effects of heat shock and other stresses on membrane and periplasmic proteins (Rouviere, De Las Penas et al. 1995). Induction of the RpoE regulon occurs when high temperature or another stressor causes an accumulation of misfolded outer membrane proteins (OMPs). These OMP proteins interact with the protease DegS, enabling it to free RpoE from the inner membrane through cleavage of RseA. The free RpoE subunit can then promote expression of itself and additional factors such as the heat shock sigma factor, RpoH and the periplasmic protease, DegP (Hiratsu, Amemura et al. 1995). These actions can lead to a cascade of events, such as degradation of misfolded proteins via proteases and assisted folding of nascent proteins by chaperones (Raina, Missiakas et al. 1995). These examples only represent a subset of the signaling systems that *E. coli* utilizes to adapt to changes in the surrounding environment, illustrating the complexity of the physiology of the bacterial cell. It is important to recognize that altered curli formation is only a minor outcome that arises when cells experience membrane stress; as such, it seems logical to also study other cellular changes that might occur under these conditions.

This work provides evidence for the role that phage genes play in altering the lifestyle choice of the bacterial host. We describe the regulation of the lysis genes in a defective lambdoid prophage (DLP12), showing that a Q like protein ( $Q^{DLP12}$ ) retains its function as a transcriptional activator for these lysis genes. In addition we present confirmation that the transcription process has been co-opted into the regulon of the alternative extracytoplasmic stress response sigma factor (RpoE). We show that  $Q^{DLP12}$ -mediated upregulation of the lysis cassette is dependent on RpoE binding at the promoter region and that there is a physical interaction between  $Q^{DLP12}$  and RpoE. It appears that



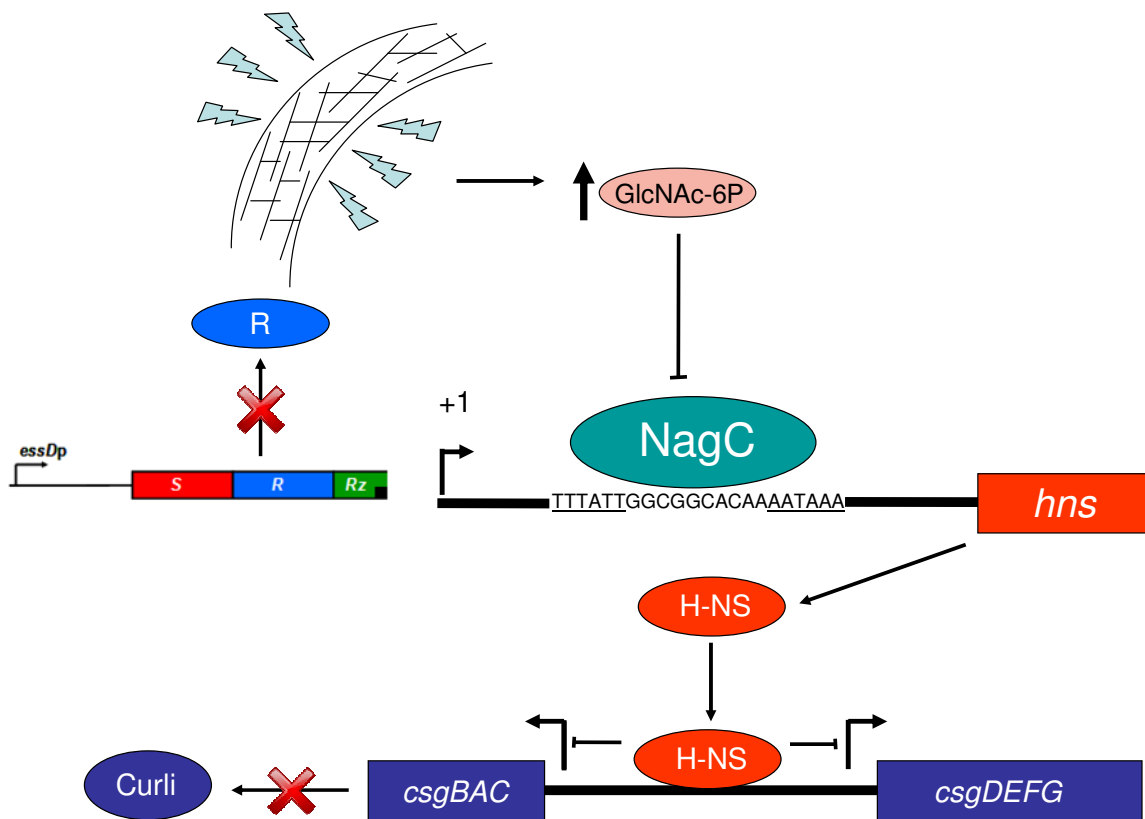
DLP12, due to mutations in the genes encoding excision, has been domesticated by the bacteria. We proceed to elucidate the mechanism by which expression of the DLP12 lysis genes impacts production of curli fibers and subsequent biofilm formation in *Escherichia coli*. The lysis gene products, when overexpressed, cause hydrolysis of the peptidoglycan sacculus and concomitant host lysis; however, it seems that controlled expression of these potentially lethal genes is required for *E. coli* to express curli at sufficient levels for biofilm formation. In the final section of this thesis, we provide preliminary evidence describing how changes in the abundance of peptidoglycan breakdown products are sensed by the cell and translated into signals that influence various cellular processes, including transcription of the genes that encode curli.

We show, through genetic means, that the DLP12 lysis gene mutants likely produce increased intracellular GlcNAc-6P levels, which result in diminished curli production. This corroborates previous work that describes GlcNAc-6P as a negative effector of curli transcription (Barnhart, Lynem et al. 2006). We discovered that elevated GlcNAc-6P levels cause an increase in levels of the global regulator H-NS, which directly binds the curli promoter, inhibiting transcription. Indeed, exposure to high levels of GlcNAc, a GlcNAc-6P precursor, resulted in H-NS enrichment at the curli promoter. Subsequent analysis of DLP12 mutants defective for lysis cassette expression revealed that H-NS levels were significantly elevated when compared to WT. These data provide the link between increased abundance of GlcNAc-6P and increased H-NS levels in the cell. We investigated possible avenues for GlcNAc-6P modulation of H-NS levels and found *in silico* predictions of a putative NagC binding site at the *hns* promoter (Oberto 2010).

NagC functions as a transcriptional regulator, binding directly to promoter DNA. GlcNAc-6P negatively influences NagC's ability to bind DNA through direct interaction with an allosteric site on NagC. We hypothesized that the increased GlcNAc-6P levels would derepress NagC from the *hns* promoter and result in increased H-NS expression. We postulated that overexpressing NagC ectopically would down regulate H-NS expression and consequently restore curli production in the DLP12 lysis gene mutants. Under NagC overexpression, mutants defective for lysis gene expression exhibited WT levels of H-NS in the cell. In addition, overexpression of NagC in the DLP12 lysis gene mutants restored curli expression. These results support our claim that GlcNAc-6P is regulating H-NS abundance via modulation of NagC and ultimately impacting curli transcription. Taken together, our results provide evidence that GlcNAc-6P is a novel global effector, serving as a signal for bacterial cell wall integrity and influencing downstream signal transduction pathways regulated by H-NS. Also, we propose a novel role for NagC as a global super regulator which senses intracellular levels of GlcNAc-6P and in turn, alters expression of H-NS to allow the cell to adapt to the cell wall stress.

Further work needs to be done to verify the direct binding of NagC to the *hns* promoter (*hnsP*) and to prove that this interaction is required for controlled H-NS expression in the face of fluctuating GlcNAc-6P levels. This can be addressed *in vitro* via electrophoretic gel mobility shift assays with purified NagC and *hnsP*. By including small unlabeled competitor probes or through site directed mutagenesis, we can locate the exact binding region within the promoter. In addition, we can employ *in vivo* chromatin precipitation (ChIP) to show enrichment of NagC at *hnsP* under low GlcNAc-6P conditions and a resulting decrease in enrichment of NagC under elevated GlcNAc-6P

conditions. This would enable us to link the degree of NagC binding at *hnsP*, with intracellular H-NS levels and curli expression. Furthermore, we can utilize a  $\Delta nagC$  strain to underscore the importance of NagC in controlling H-NS levels within the cell. We would expect to see increased H-NS levels in the  $\Delta nagC$  mutant when analyzed via Western blotting. Lastly, we can utilize a *nagCp-gfp* promoter fusion construct to measure *in vivo* NagC expression in the DLP12 lysis cassette mutants and in bacteria with high levels of GlcNAc-6P. This would reveal a potential decrease to the NagC pool that might occur in response to increased intracellular GlcNAc-6P.



**Figure 5.1.** Absence of DLP12 lysis products results in cell wall stress, which causes increased intracellular GlcNAc-6P. GlcNAc-6P interacts with NagC and prevents binding of NagC to *hns* promoter, allowing for H-NS expression. H-NS binds to divergent curli promoter region, repressing curli expression.

## **LITERATURE CITED**

- Barnhart, M. M. and M. R. Chapman (2006). "Curli biogenesis and function." Annu Rev Microbiol **60**: 131-47.
- Barnhart, M. M., J. Lynem, et al. (2006). "GlcNAc-6P levels modulate the expression of Curli fibers by Escherichia coli." J Bacteriol **188**(14): 5212-9.
- Clarke, D. J. (2010). "The Rcs phosphorelay: more than just a two-component pathway." Future Microbiology **5**(8): 1173-1184.
- Hiratsu, K., M. Amemura, et al. (1995). "The RpoE Gene of Escherichia-Coli, Which Encodes Sigma(E), Is Essential for Bacterial-Growth at High-Temperature." Journal of Bacteriology **177**(10): 2918-2922.
- Hung, D. L., T. L. Raivio, et al. (2001). "Cpx signaling pathway monitors biogenesis and affects assembly and expression of P pili." EMBO J **20**(7): 1508-18.
- Oberto, J. (2010). "FITBAR: a web tool for the robust prediction of prokaryotic regulons." BMC Bioinformatics **11**: 554.
- Pratt, L. A., W. Hsing, et al. (1996). "From acids to osmZ: multiple factors influence synthesis of the OmpF and OmpC porins in Escherichia coli." Mol Microbiol **20**(5): 911-7.
- Raina, S., D. Missiakas, et al. (1995). "The rpoE gene encoding the sigma E (sigma 24) heat shock sigma factor of Escherichia coli." EMBO J **14**(5): 1043-55.
- Rhodium, V. A. and V. K. Mutalik (2010). "Predicting strength and function for promoters of the Escherichia coli alternative sigma factor, epsilon(E)." Proceedings of the National Academy of Sciences of the United States of America **107**(7): 2854-2859.
- Rouviere, P. E., A. De Las Penas, et al. (1995). "rpoE, the gene encoding the second heat-shock sigma factor, sigma E, in Escherichia coli." EMBO J **14**(5): 1032-42.