POST-TRANSCRIPTIONAL REGULATION OF VIRULENCE GENES BY GLMY AND GLMZ IN ENTEROHEMORRHAGIC *E. COLI*

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POST-TRANSCRIPTIONAL REGULATION OF VIRULENCE GENES BY GLMY AND GLMZ IN ENTEROHEMORRHAGIC *E. COLI*

by

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POST-TRANSCRIPTIONAL REGULATION OF VIRULENCE GENES
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The University of Texas Southwestern Medical Center at Dallas, 2013

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Enterohemorrhagic *E. coli* O157:H7 (EHEC) is a major cause of foodborne illness and hemolytic uremic syndrome (HUS) throughout the world. One of the major virulence factors in this pathogen is a type III secretion system (T3SS) encoded by the locus of enterocyte effacement (LEE). EHEC uses this proteinaceous needle to inject effector proteins into host cells to hijack various host cellular processes, as well as the translocation and insertion of the translocated intimin receptor (Tir) into the host cell membrane. The bacterial adhesin intimin then binds to Tir, allowing EHEC to tightly adhere to the host cell's membrane (109). Tir also indirectly recruits another bacterial effector EspFu, which induces actin polymerization. This causes the formation of the characteristic pedestal that cups the bacterial cell. This process from the assembly of the needle apparatus to the formation of the pedestal must be tightly regulated both transcriptionally and post-transcriptionally.

The two two-component systems QseEF and QseBC have been previously shown to regulate various virulence genes. We established that these systems both regulate the transcription of the small RNA (sRNA) *glmY*. GlmY is known to stabilize another sRNA, GlmZ, which activates the translation of glucosamine synthetase (GlmS) (72). Here we show that GlmY and GlmZ are also important players in the post-transcriptional regulation of virulence genes in EHEC.

The transcription factor QseF is required for the expression of EspFu and thus pedestal formation. This defect can be complemented by overexpression of either GlmY or GlmZ and is not at the transcriptional level. Instead, the expression of *espFu* requires a processing event that is QseF dependent. We have shown that GlmZ also post-transcriptionally regulates two of the operons of the LEE, *LEE4* and *LEE5*. Both of these operons are transcribed from a single promoter, but there is a processing event that separates the first gene of the operon from the rest that requires the endoribonuclease RNase E. Overexpression of either sRNA results in the downregulation of the latter fragment of both the *LEE4* and *LEE5* operons. In the case of *LEE4*, this is through direct binding of GlmZ to a region within the *LEE4* mRNA.

We also investigated the global role of GlmY and GlmZ in EHEC through microarrays and RNA sequencing of the knockout strains. Aside from the LEE, GlmZ also regulates curli which are used to facilitate bacteria attachment to host cells. These data show that GlmZ has been co-opted into being an important regulator of virulence genes in EHEC.

TABLE OF CONTENTS

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LIST OF FIGURES

LIST OF TABLES

LIST OF DEFINITIONS

86-24	Enterohemorrhagic *E. coli* wild-type strain 86-24
Eae	Intimin
E. coli	*Escherichia coli*
EHEC	Enterohemorrhagic *E. coli*
EPEC	Enteropathogenic *E. coli*
EspA	*E. coli* secretion protein A
FAS	Fluorescent actin staining
GlmS	Glucosamine synthetase
GlmY	Regulator of glucosamine synthetase Y
GlmZ	Regulator of glucosamine synthetase Z
HUS	Hemolytic uremic syndrome
LEE	Locus of Enterocyte Effacement
MC4100	*E. coli* K12 strain MC4100
MG1655	*E. coil* K12 strain MG1655
mRNA	Messenger RNA
RpoA	RNA polymerase subunit α
sRNA	Small RNA
Stx	Shiga toxin
Tir	Translocated intimin receptor

CHAPTER ONE

Literature Review

HISTORY

Escherichia coli, a member of the Enterobacteriaceae family in the γ-Proteobacteria phylum, was first isolated by the German bacteriologist Theodor Escherich in 1885 (61). *E. coli* is a Gram negative, motile, facultative anaerobe rod that is typically found as a commensal in the mammalian colon. It has been used as one of the primary model organisms and is used extensively in molecular biology and biotechnology.

Even though many strains are harmless or even beneficial to the host, others have acquired different virulence factors through horizontal gene transfer that allow them to cause a wide array of different diseases in humans (102). One of these, enterohemorrhagic *E. coli*, or EHEC, was first identified as a human pathogen in 1982 following an outbreak of food poisoning in which 47 people were hospitalized with a nonfebrile illness characterized by bloody diarrhea. The outbreak was linked to the consumption of undercooked hamburger meat from McDonald's restaurants in Oregon and Michigan and the causative agent was found to be a noninvasive and nontoxigenic variant *of E. coli* with the serotype O157:H7, with the O referring the O chain of the lipopolysaccharide (LPS) and H the flagellar antigen (188). This dissertation focuses on studying the EHEC strain 86-24 which was isolated from a patient in 1986 (3). Since its initial discovery, EHEC has been recognized as a major cause of hemorrhagic colitis throughout the world.

EPIDEMIOLOGY AND CLINICAL PRESENTATION

EHEC is a foodborne pathogen that is normally spread through the consumption of contaminated meat or produce as well as many other foods such as cider, juice, or milk (34, 73, 132, 141). The reservoirs are ruminants such as cattle, deer, sheep, or goats which are asymptomatically infected with the organism, and direct human infection from these animals such as from petting zoos has also been reported (32, 115, 253). EHEC has a low infectious dose of 10-100 cfus which contributes to its ability to cause large outbreaks (12). The CDC estimates there are 96,000 EHEC O157 infections every year in the US and 170,000 non-O157 serotypes with an average of 30 deaths (198). In 2012, there were three outbreaks of EHEC, one of which was caused by O157 that was linked to the consumption of organic spinach (Figure 1.1). In this outbreak, 33 people were known to be infected with 46% being hospitalized and 2 cases progressing to hemolytic uremic syndrome (HUS) with 0 deaths.

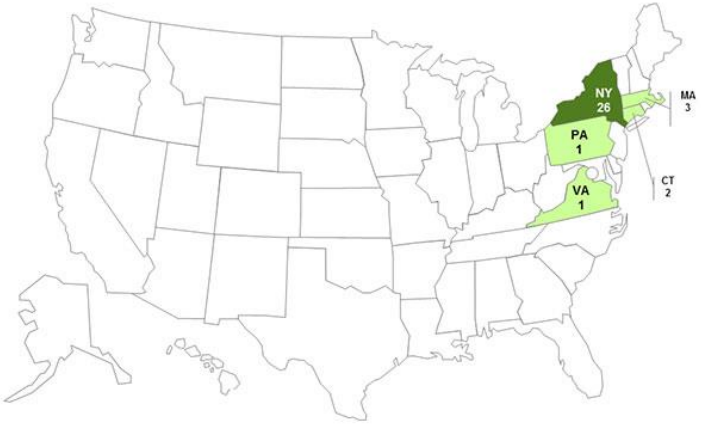

Figure 1.1: Epimeology of 2012 EHEC Outbreak. Map showing the cases per state of the 2012 EHEC O157 outbreak. Image courtesy of the CDC website

In a typical case symptoms begin within 3-8 days following consumption of contaminated food or drink starting with severe abdominal cramps and watery diarrhea, 50% of the patients experience extreme nausea and vomiting. Within 1-5 days later this progresses to bloody diarrhea in most patients which usually lasts 2-4 days after which the patient typically recovers (16, 199). While the actual infection is resolved within a week from the onset of symptoms, 2-14 days later 5 to 10% of cases develop hemolytic uremic syndrome (HUS). (40, 179) (Figure 1.2). HUS is characterized by hemolytic anemia, thrombocytopenia and renal failure, leading to death in 5% of the cases (173). Even if the patient survives HUS, there are many sequelae, with 50% experiencing chronic renal or neurological problems (199, 211).

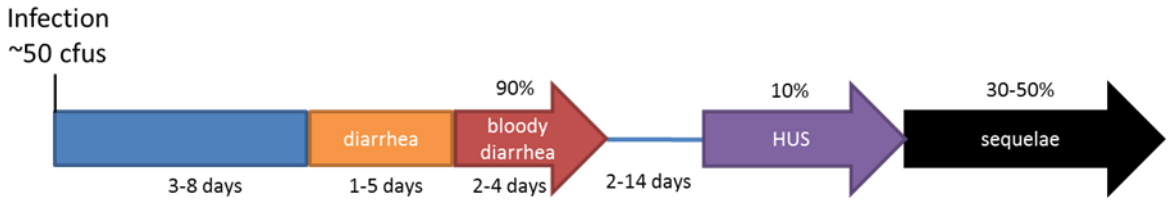

Figure 1.2: Course of EHEC infection. Diagram showing the time course of EHEC infection and probability of advancing to the next stage

Clinical diagnosis of EHEC is typically performed by plating stool samples from patients on sorbitol-MacConkey agar. EHEC, unlike other varieties of *E. coli*, is unable to ferment sorbitol, and will not turn red on this media (129). Isolates can also be confirmed by ELISA using anti-O antigen or anti-Shiga toxin antibodies or by PCR (169). Recommended treatment for EHEC infection is largely supportive and consists of providing fluids and electrolytes. The use of antibiotics is controversial, and has been linked to a greater risk of

HUS due to the increased expression of Shiga toxin (168). The treatment for HUS largely consists of managing the anemia, hypertension, and the loss of kidney function through dialysis.

VIRULENCE FACTORS

Locus of Enterocyte Effacement and the EHEC Pedestal

When EHEC infects its host, the bacterium attaches to an epithelial cell through an attaching and effacing (AE) lesion where it rearranges the host's cytoskeleton, which leads to the effacement of the microvilli and the formation of a characteristic pedestal structure that cups the bacterial cell (Figure 1.3).

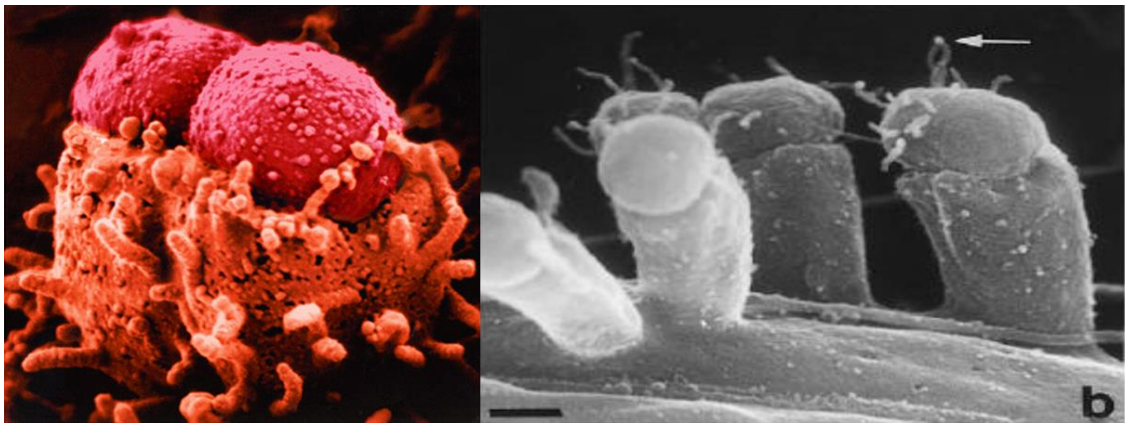

Figure 1.3: EHEC Pedestal. Scanning electron micrographs of an EHEC (left) and the closely related EPEC (right) pedestals on epithelial cells. (102, 116).

Most of the genes responsible for this phenotype are located in a 35kb pathogenicity island called the locus of enterocyte effacement (LEE) (Figure 1.4) (133). The LEE contains five major operons, *LEE1-5*, that encode a type three secretion system (T3SS), as well as many of

its regulators and several effector proteins (27, 58, 136). The first operon (*LEE1*) contains *ler*, which encodes the master regulator of the LEE (136). Additionally, *LEE1* along with *LEE3* encode the EscRSTUV inner membrane complex of the T3SS which is assembled in a sec-dependent manner. *LEE2* encodes the outer membrane secretin EscC and the perisplasm-spanning protein EscJ (44, 67). The *LEE4* operon encodes the needle protein EscF (255). However, unlike most other T3SS, this needle is then extended through the addition of an EspA filament (*LEE4*) that is up to 1μm in length (45, 116, 167). This filament structure is then capped with the EspDB translocon pore that inserts into the host cell membrane (Figure 1.4) (92, 117).

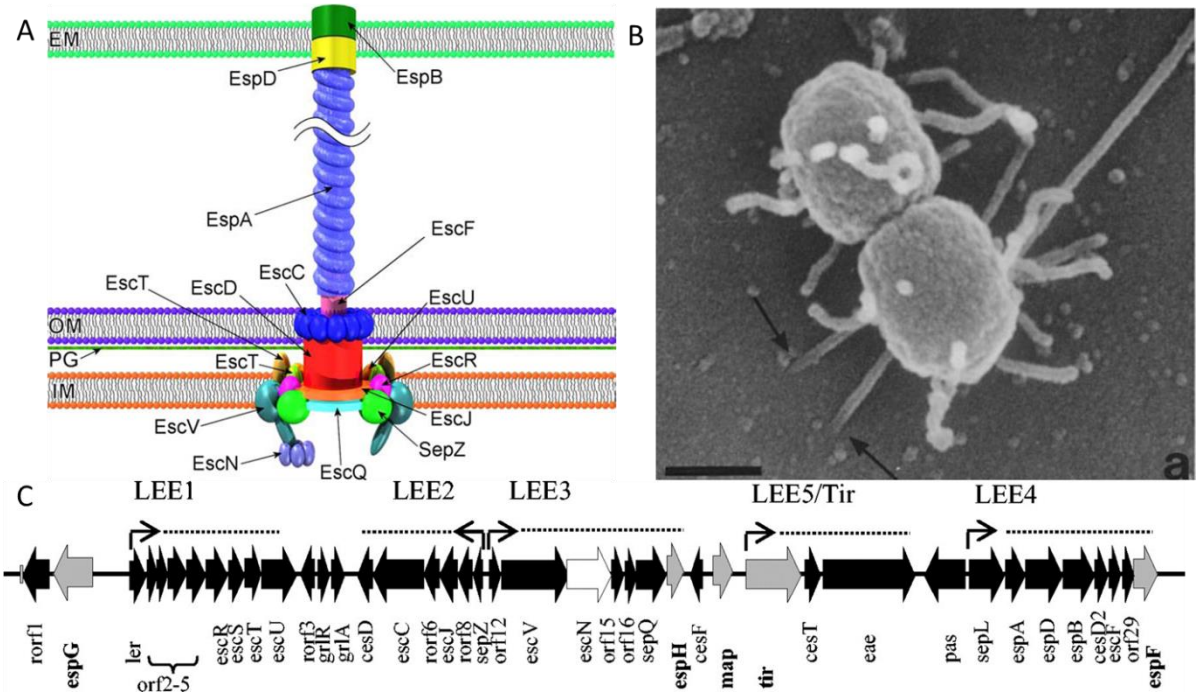

Figure 1.4: LEE T3SS. Cartoon diagram of the structure of the LEE needle apparatus (A) (167). Scanning electron micrograph showing the EspA filament extending from an EPEC cell (B) (116). Schematic of the LEE pathogenicity island (C) (190).

In addition to the structural proteins of the LEE, there are other proteins involved in the formation and function of the needle apparatus. EscN encoded by the *LEE3* operon is the ATPase that unfolds the effector and translocon proteins so that they can then pass through the needle and filament (7). SepD (*LEE2*) and SepL (*LEE4*) together regulate the formation of the EspADB translocon and the hierarchy of effector secretion. SepL directly binds to the effector Tir and is required for the formation of the translocon (48, 158, 248). The LEE also contains six effector proteins. EspG destabilizes the Golgi apparatus and affects microtubule assembly (39, 83, 203, 233). EspH inhibits Rho-GEFs and is involved in rearrangement of the host cytoskeleton (256). Map is a Rho-GEF mimic and is a member of the WxxxE family of effectors (4, 162). The exact function of EspF is unknown, but it has been implicated in the depletion of host mismatch repair proteins and the induction of apoptosis of infected cells (127, 264). The effector EspZ promotes host cell survival by interacting with CD98 in addition to regulating the translocation of other effectors (17, 192, 206). The *LEE5* operon encodes Tir, the translocated intimin receptor and the intimin adhesin (58). In addition to these effectors the LEE also contains various chaperones for many of the proteins secreted through the T3SS, including CesT for Tir and Map (42, 55), CesF for EspF(57), CesD and CesD2 for EspD and EspB (153, 244) and CesAB for EspA and EspB (43).

AE lesion formation requires the T3SS, as well as two effectors Tir and EspFu, and the adhesin intimin, encoded by the *eae* gene in *LEE5*. This outer membrane protein is secreted through the sec system and has been shown to be necessary for colonization (51, 52, 87). The effector Tir is able to insert itself into the host cell membrane where it can interact

with intimin on the bacterial cell, allowing the bacterium to tightly adhere to epithelial cells (109). Tir then recruits the host protein insulin receptor tyrosine kinase substrate (IRTKS) which then recruits the non-LEE encoded effector EspFu/TccP (28, 66, 239). EspFu then recruits N-WASP and the actin nucleating complex Arp2/3 which leads to the polymerization of actin beneath the bacteria cell (Figure 1.5).

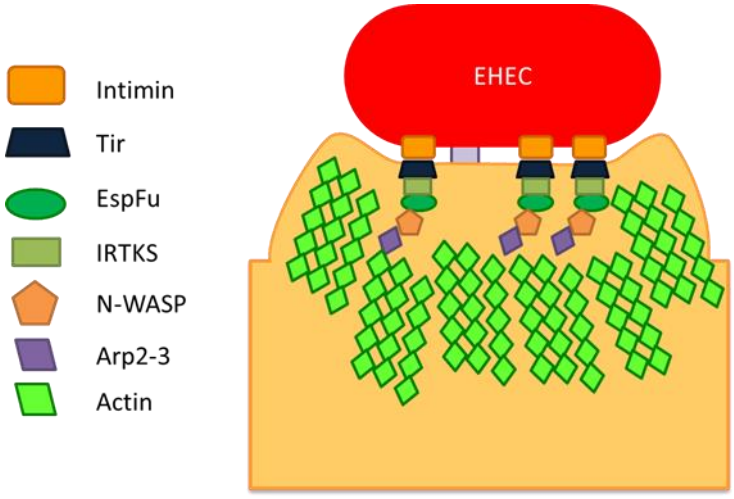

Figure 1.5: Cartoon diagram of the proteins required to form the AE lesion beneath EHEC

Regulation of the LEE

The LEE is tightly regulated at multiple levels. The entire pathogenicity island is extremely AT-rich and is repressed by the global regulator H-NS (227). Ler, the *LEE1* encoded master regulator, antagonizes this repression and acts as a positive regulator of the rest of the LEE (27). There are several known regulators of *ler* transcription, including Cra, KdpE (155), QseA (108, 209), EutR (126), Fis (69), GadE (99), Hha (208), EivF (263), and IHF (63). GrlA and GrlR are two other LEE encoded regulators of the LEE that activate and

repress LEE expression, respectively (11, 94, 97, 194). The global regulator of stationary phase in *E. coli*, the sigma factor RpoS, is also known to act as a repressor of the LEE (50).

There is significant post-transcriptional control of the LEE as well. Mutants of the RNA chaperone Hfq have differential expression of virtually every virulence gene, although whether the effect is positive or negative depends on the strain. In 86-24, the strain used in this study, Hfq is required for the expression of virulence genes with the knockout having a severe defect in their expression (81, 107, 205). Several known regulators of the LEE are under post-transcriptional control. RpoS is tightly regulated post-transcriptionally by three different small RNAs (sRNAs), RprA, DsrA, and OxyS (186). The *grlRA* operon is under post-transcriptional control by some yet unknown sRNA. Additionally, the *LEE4* operon undergoes a RNase E dependent processing event with the first gene, SepL, being processed off the rest of the operon (123). The RNA binding protein and global regulator CsrA has also been reported to regulate *LEE1* and *LEE4* (19). This high level of regulation at every level allows for tight spatial and temporal control of LEE expression.

Shiga Toxin

The primary cause of morbidity and mortality from EHEC infection is renal failure following HUS, and the virulence factor responsible for this is Shiga toxin (Stx) (103). This toxin has two major subtypes, Stx1 and Stx2 that are sero-specific. In EHEC, these toxins are encoded in prophages with some strains being capable of carrying multiple varieties

(223). Stx2 is 1000 times more cytotoxic than Stx1 and patients infected with strains encoding this toxin are more likely to develop HUS (164).

Stx is a classic AB exotoxin. The A subunit is the enzymatically active portion, while the B subunit acts by the binding to the toxin receptor. Stx has a 1:5 stoichiometric ratio of A:B subunits (Figure 1.6). The B subunit binds to the glycolipid receptor globotriaosylceramide (Gb3), and causes the toxin to be internalized by clathrin-dependent endocytosis (111, 120). Gb3 is abundant on kidney cells, which leads to them being targeted by Shiga toxin, causing their death and HUS. Once internalized the A subunit acts as an N-glycosidase that specifically cleaves an adenine from the 18S rRNA resulting in a shutdown of protein translation followed by cell death (59, 197).

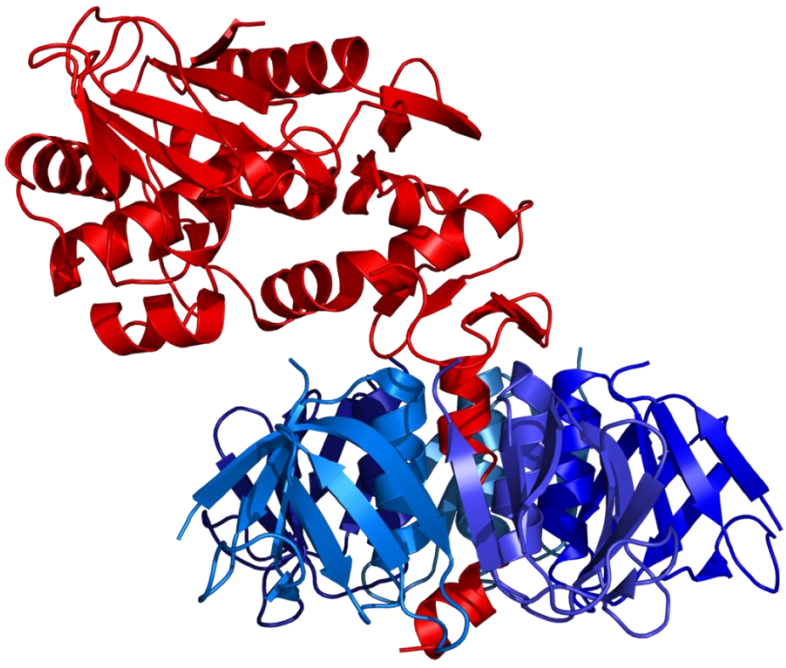

Figure 1.6: Crystal structure of Shiga toxin 2. Ribbon diagram of Stx2. The A subunit is red and the five B subunits are shown blue. (62).

The *stxAB* genes are located in the late genes of a λ prophage. These genes are not expressed under normal conditions due to the phage cI repressor that maintains the phage in a lysogenic state (151, 174, 177). If the bacterial cell encounters extreme stresses that cause DNA damage, the SOS response is triggered (178). Damage to the chromosome produces ssDNA that activates the RecA protease. RecA then is able to bind to the repressor LexA, leading to its self-cleavage and promoting the expression of DNA repair enzymes (121). RecA also cleaves the phage-encoded repressor cI (191), resulting in the prophage entering its lytic cycle that leads to the production and release of Shiga toxin (237). Treatment with various kinds of antibiotics such as beta-lactams or fluoroquinolones is capable of inducing the SOS response in *E. coli*, triggering the lytic cycle of a λ phage (105). Hence the use of antibiotics during an EHEC infection is controversial, and is believed to increase the risk of HUS (195).

Other Virulence Factors

In addition to the LEE and the previously mentioned effector EspFu, EHEC contains at least 40 other effectors (231). Many of these, including EspFu, are encoded within cryptic prophages. This suggests that many of them were recently acquired though horizontal gene transfer and there is potentially even greater diversity of effectors in the *E. coli* pangenome (231). Fourteen of these effectors belong to the NleG of E3 ubiquitin ligases, although their targets are not yet known (258). The effector NleA disrupts tight junctions, and has been

shown to be important for virulence in the related attaching and effacing pathogen *Citrobacter rodentium* (75, 112, 148, 228).

EHEC also contains the 93kb F-like pO157 plasmid (24). This plasmid encodes several known virulence factors including a hemolysin (15, 200), a catalase (23), a type II secretion system (201), ToxB (226), a metalloprotease StcE (118), and a serine protease called EspP (22). The EHEC LPS O-antigen is required for full virulence in various animal models and likely protects the bacteria from the host (104, 144).

In addition to the tight adherence provided by the Tir-Intimin interaction, there are many other potential adherence factors whose role in virulence is largely unknown. EHEC produces curli encoded by the *csg* genes. Curli have been shown to mediate attachment to abiotic surfaces, as well as increase attachment to epithelial cells *in vitro* (113, 170). EHEC also encodes a type IV pilus called the hemorrhagic coli pili (HCP) that binds to host extracellular matrix proteins, and contributes to biofilm formation by mediating bacterial-bacterial interaction (259). There are many other potential fimbriae and adhesins in the EHEC genome; however, most of these are not expressed under any known laboratory condition and many are likely not functional (124).

ANIMAL MODELS

There are several animal models that have been used to study EHEC pathogenesis, including mice, rabbits, chickens, dogs, pigs, cows, ferrets, macaques and baboons (137). One of the most useful of these is the infant rabbit model, where 3-day-old rabbits are

infected. These animals are subsequently colonized and experience diarrhea, colonic inflammation and death (166).

There are several different varieties of mouse models such as conventional, antibiotic treated, or germ-free, however, these are of limited utility in studying EHEC infection. While the mice are susceptible to Shiga toxin, EHEC does not form attaching and effacing lesions in the mouse intestines or cause hemorrhagic colitis (53, 243). There is a closely related organism *Citrobacter rodentium* that is a natural mouse pathogen. This organism also contains the LEE and is capable of forming AE lesions on epithelial cells in the mouse colon. C3H/HeJ mice are highly susceptible to infection and will die after oral challenge with *Citrobacter* (254). Because of the ease of care for mice, *Citrobacter* provides a useful proxy to study EHEC pathogenesis *in vivo*. The genetic tractability of mice also allows for the study of the host immune response to an AE lesion forming pathogen.

INTERKINGDOM SIGNALING

History

Cell to cell signaling in bacteria was first described in the marine organism V*ibrio fischeri* (84, 150). This bacterium is a symbiont of the Hawaiian bobtail squid, where it resides in a special light organ. It is able to mask the squid's silhouette at night by producing light, thus protecting it from predators. *V. fischeri* governs its production of light through quorum sensing. The signal, an acyl homoserine lactone (AHL) called autoinducer 1, is produced by the LuxI enzyme. AI-1 diffuses into the environment, and when it reaches a

critical threshold, it is able to bind to the LuxR transcription factor that then activates the luciferase genes required for light production (60). This system ensures light is only produced by the bacteria at high population densities such as those in the squid's light organ at night.

Since its discovery, homologues of the LuxRI system have been found in many other species of bacteria. Different species produce AHLs of varying acyl-chain length in order to create specificity towards their particular LuxR receptor (252). The human pathogen *Pseudomonas aeruginosa* has two complete LuxRI systems (LasRI and RhlRI), and an orphan LuxR (QscR) (64, 215). These systems have been shown to regulate adhesion, biofilm formation, and virulence (193, 215). *E. coli* and *Salmonella enterica* have an orphan LuxR homologue SdiA, but they do not have a homologue for the LuxI synthase (2, 140). Orphan systems such as this have been shown to be involved in interspecies communication (64).

A second quorum sensing system called AI-2 was discovered as a LuxRI independent system that regulates bioluminescence in *Vibrio harveyi* (13). AI-2 is a furanosyl borate diester in *V. harveyi* and a tetrahydroxytetrahydrofuran in *E. coli* and *Salmonella* that is produced as a byproduct of the activated methyl cycle and is produced by the enzyme encoded by the *luxS* gene (143, 224). In *E. coli* and *Salmonella*, AI-2 has been shown to regulate its uptake system encoded in the *lsr* operon (225). Investigation into the role of AI-2 signaling has been complicated by the important role of LuxS in cell metabolism, and the presence of the sRNA *micA* which is adjacent to the *luxS* locus (114, 171).

AI-3 and the Qse two-component systems

AI-3, a third quorum sensing signal, is produced by various bacterial species including *E. coli*, *Salmonella*, *Citrobacter*, *Klebsiella*, *Enterobacter*, *Bacteriodes*, and *Bifidobacterium*. Little is known about this signaling molecule, but it is distinct from AI-1 and AI-2. In EHEC the AI-3 system has been shown to regulate expression of the LEE and the flagella (246, 247), and its receptor is QseC (37). In addition to sensing this bacterial signaling molecule, QseC is also able to sense the host hormones epinephrine and norepinephrine (37).

QseC is a histidine kinase encoded in an operon with its cognate response regulator QseB. It responds to AI-3, epinephrine, and norepinephrine by increasing its autophosphorylation followed by transferring of the phosphate to a response regulator. QseC is a promiscuous kinase, and is capable of phosphorylating three response regulators, its cognate QseB, KdpE, and QseF (90). QseB regulates the expression of the flagella regulon by binding to the promoter of the master regulator *flhDC*, with phosphorylated QseB activating transcription while unphosphorylated QseB acts as a repressor. KdpE activates the transcription of *ler* by working in concert with the metabolite regulator Cra (Figure 1.7) (155).

In addition to QseC, there is another histidine kinase that senses epinephrine, QseE, which is encoded in the *qseEGFglnB* operon. QseE is also capable of sensing sulfate and phosphate and is only known to phosphorylate its cognate response regulator, QseF (182).

This operon also contains the outer membrane lipoprotein QseG, the PII nitrogen regulating protein GlnB, and QseE's cognate response regulator QseF (182, 183). QseF is a promiscuous response regulator and is capable of being phosphorylated by multiple histidine kinases, including BaeS, EnvZ, RstB, UhpB, as well as QseC, and QseE (Figure 1.7) (261).

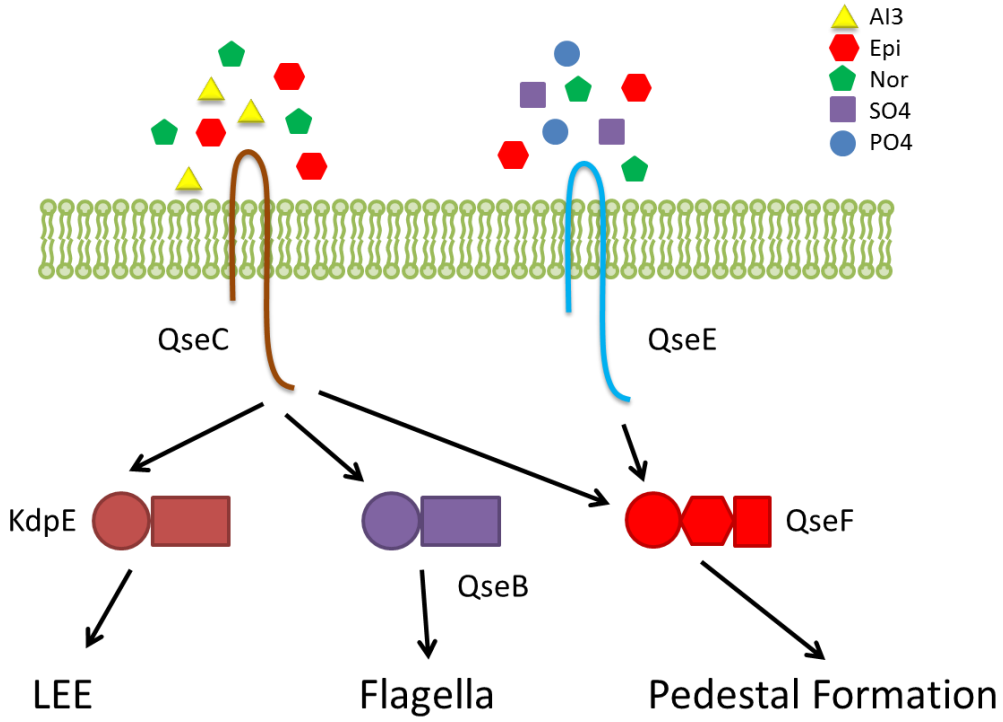

Figure 1.7: Diagram of the Qse cascade in EHEC. QseC senses AI-3, epinephrine, and norepinephrine and is capable of phosphorylating three response regulators KdpE, QseB, and QseF. QseE senses epinephrine, sulfate, and phosphate and can phosphorylate QseF. KdpE activates transcription of the LEE, QseB regulates the flagella, and QseF is required for pesestal formation

QseF

QseF is a σ^{54}-dependent transcriptional activator. This family of transcription factors is a distinct class that recruits the alternative σ^{54} RNA polymerase holoenzyme (139). In *E.*

coli, σ^{54} regulated genes are canonically involved in nitrogen assimilation, however it has also been implicated in the regulation of virulence in EHEC and other organisms (5, 82, 185, 189). The σ^{54} promoter sequence contains conserved -24/-12 regions where the RNA polymerase binds, distinct from the -35/-10 sequences of the σ^{70} housekeeping RNA polymerase (229). Unlike the other sigma subunits of RNA polymerase, σ^{54} RNA polymerase lacks an ATPase, and is unable to form the DNA open complex and initiate transcription, which results in all σ^{54} promoters being by default 'off.' Consequently, σ^{54}-dependent transcriptional activators, or bacterial enhancer binding proteins (bEBP), contain an AAA+ ATPase domain in order to melt the DNA helix and initiate transcription after they recruit the σ^{54} RNA polymerase. These transcription factors form a hexameric ring that binds to DNA further upstream than traditional bacterial transcription factors, up to 800 bp from the transcription start site. This distance means the DNA must loop for the transcription factor to interact with the RNA polymerase (26, 33).

Initial work found that QseF is required for the expression of the effector EspFu. Mutants are unable to form pedestals on HeLa cells and this could be complemented by the expression of EspFu *in trans* from a plasmid (183). This regulation is not direct as EspFu does not have a σ^{54} promoter and QseF does not bind to the *espFu* promoter region. The intermediate factor in this regulation is not known. However, QseF was later reported to directly activate the transcription of the gene encoding the small RNA *glmY* located upstream of the *qseEGFglnB* operon (184).

RNA REGULATION IN BACTERIA

History and Early Examples

RNAs have long been known to be important regulators, and before eukaryotic microRNAs (miRNAa) and small interfering RNAs (siRNAa) were discovered, examples of regulatory RNAs were known in bacteria. The first such example was RNAI encoded in ColE1 replicon plasmids. Another RNA, RNAII, serves as a primer that initiates replication of the plasmid. RNAI base pairs with RNAII, preventing it from binding to the plasmid DNA thus halting its replication (222, 232). RNAI is also negatively regulated by the plasmid encoded protein PcnB, which polyadenylates the RNA leading to its degradation (85, 260). The second discovered example came from the transposon Tn10 where a RNA encoded within the transposon blocks the translation of the transposase mRNA (214). The first chromosomally encoded regulatory RNA discovered was *micF*, which blocks the translation of the porin *ompF* (145). Since these early discoveries many more sRNAs have been reported that can be categorized into several major groups.

Riboswitches and cis-acting elements

The first major variety of RNA regulators are those that are encoded within the mRNA they regulate. The classic *trp* operon attenuator belongs to this class. This operon encodes the genes for tryptophan synthesis and a leader peptide. When tryptophan levels in the cell are high, charged trp tRNAs are common, leading to translation of the leader peptide. Since transcription and translation are coupled, the active translation of the mRNA allows for

a hairpin to form which halts transcription. When tryptophan levels are low, translation of the leader peptide stalls, and the unprotected reading frame prevents the formation of the hairpin allowing transcription of the tryptophan synthesis genes to continue (18). Similar attenuation systems exist in other amino acid synthesis genes (138).

T-boxes, a related but distinct system, exist in Gram-positive bacteria where they are typically used to regulate amino acid synthesis and aminoacyl tRNA synthetase. In this system, uncharged tRNAs directly bind to the 5' UTR and prevent the formation of the anti-terminator, while charged tRNAs cannot bind, terminating transcription of the operon (Figure 1.8) (76).

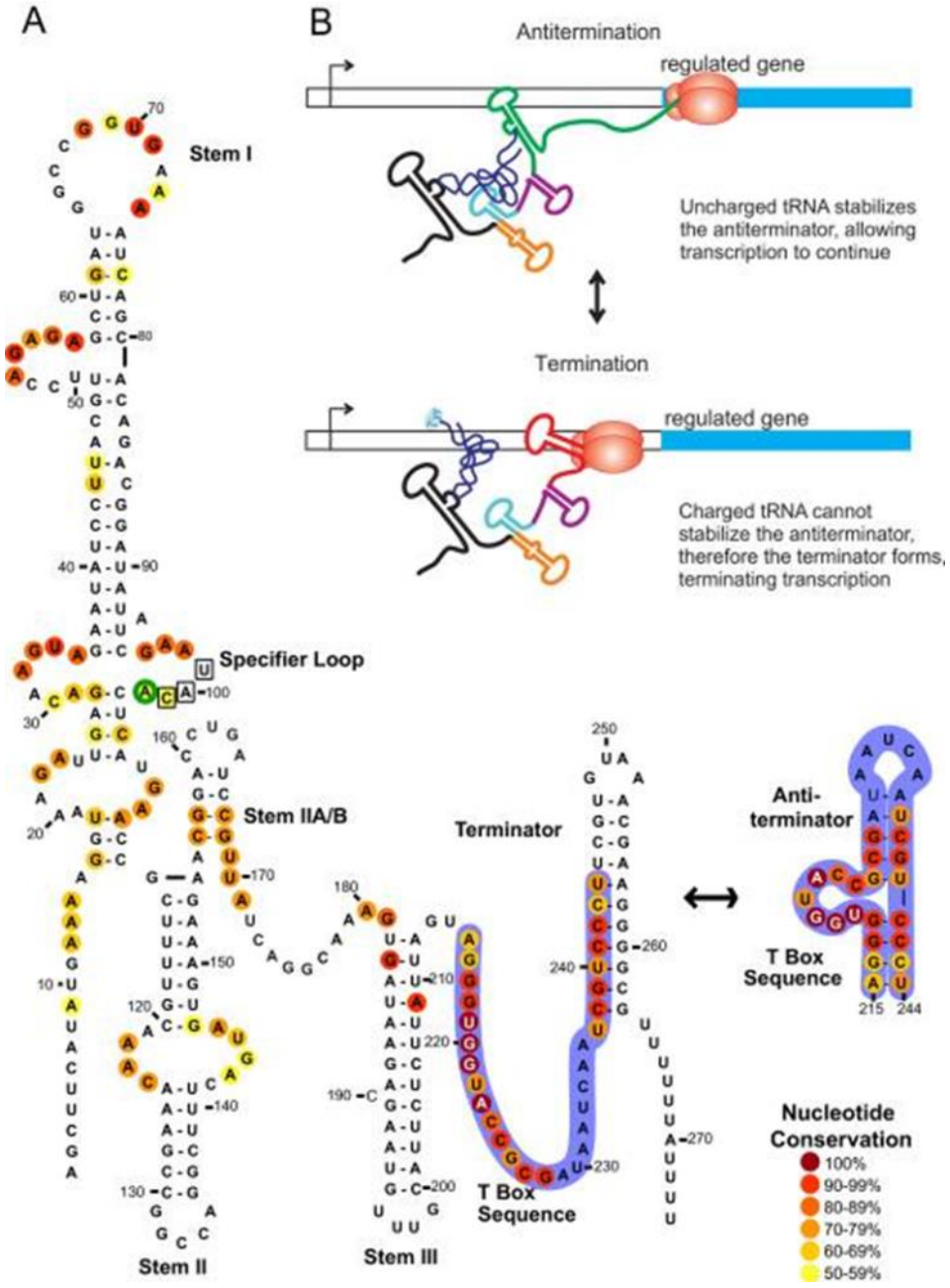

Figure 1.8: T-Box. Diagram showing the secondary structure of a T-Box element (A). The T-box element that forms the terminator is shown in blue. Upon binding to uncharged tRNAs the anti-terminator is stabilized, allowing transcription to continue (B) (76).

RNA thermometers are a fairly simple way to regulate gene expression. At low temperatures, a hairpin structure forms, blocking the ribosome binding site (RBS). When the bacterium is exposed to higher temperatures, this hairpin melts, revealing the RBS and allowing translation. This system is often used to regulate heat shock proteins and virulence genes (149).

Riboswitches are generally 5' UTRs that are capable of binding small molecules, although that term can also apply to the previously described systems. A riboswitch consists of two elements, an aptamer region and the expression platform. The aptamer region is the highly conserved ligand binding region that transmits the environmental signal to the expression platform through allosteric changes that alter base pairing. Most riboswitches modulate translation by controlling access to the ribosome binding site; however, a few regulate transcription through the formation of a terminator loop (128). Riboswitches are modular, with the same aptamer region being capable of controlling different expression platforms. For example, the cobalamin riboswitch that senses the coenzyme B12 acts as a transcriptional terminator of the *btuB* operon in Gram-positive bacteria, and translational activator of the *cob* genes in Gram-negatives (Figure 1.9) (128).

The RNA structures are capable of performing other regulatory functions as well. The *glmS* riboswitch found in Gram-positives acts in an autocatalytic manner. Upon sensing its ligand, glucosamine, the aptamer domain activates the ribozyme expression platform which cleaves the downstream open reading frame, which encodes glucosamine synthetase.

There are potentially many more functions of cis-encoded RNA elements that have not yet been discovered (251).

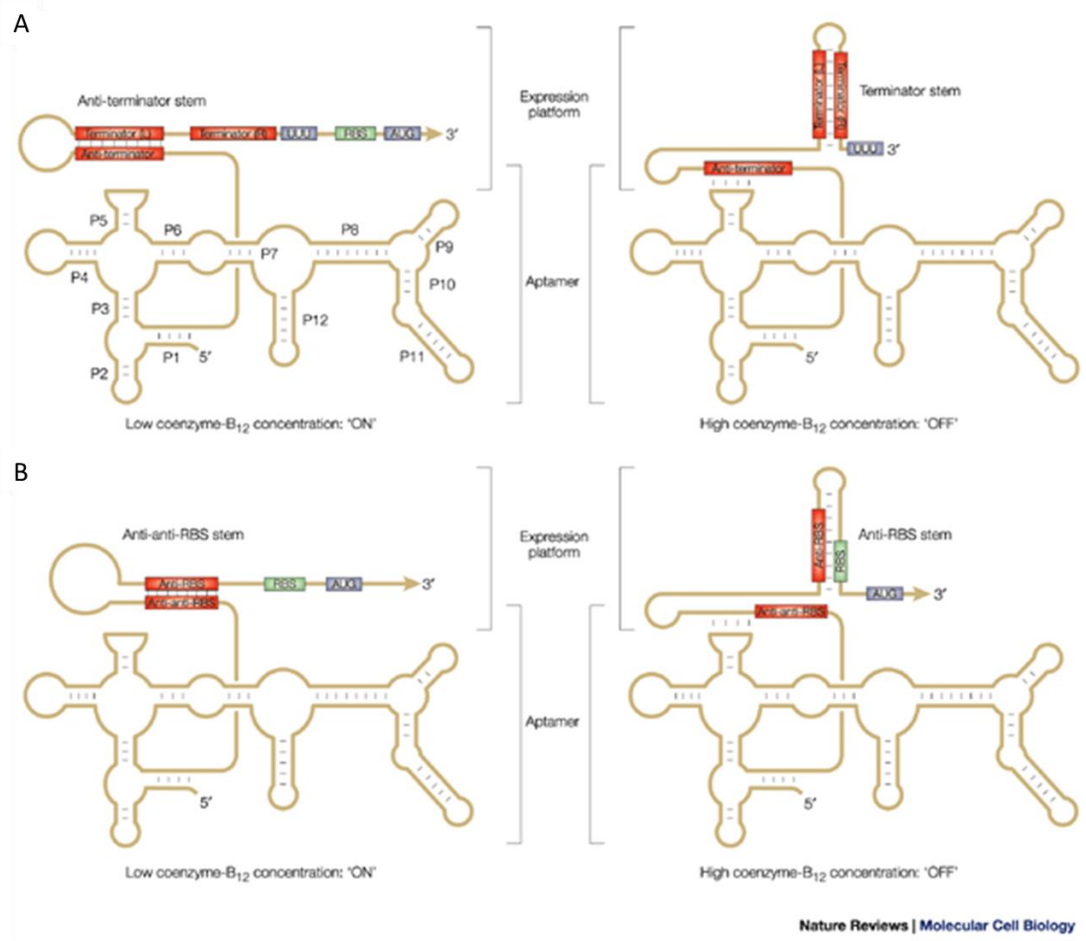

Figure 1.9: B12 Riboswitch. Diagram showing the cobalamin riboswitch and its different expression platforms. In (A) the sensing of coenzyme-B_{12} by the riboswitches causes the antiterminator to bind to the aptamer sensing domain, allowing the terminator stem to form, halting transcription. In (B), the binding of the coenzyme-B_{12} breaks the anti-anti-RBS stem loop, allowing the anti-RBS stem to form and halting translation (128).

Cis-encoded sRNAs

Other mechanisms of RNA regulation in bacteria use a small RNA (sRNA) encoded separately from its target mRNA as the regulatory element. One class of these is cis-encoded RNAs, often called antisense RNAs, where the sRNA and target RNA are encoded in the same region from opposite strands. This results in a sRNA with perfect complementarity to its target. This system is common on plasmid, phages, mobile elements and toxin-antitoxin systems, such as the previously described ColE1 origin and Tn10 systems (Figure 1.10).

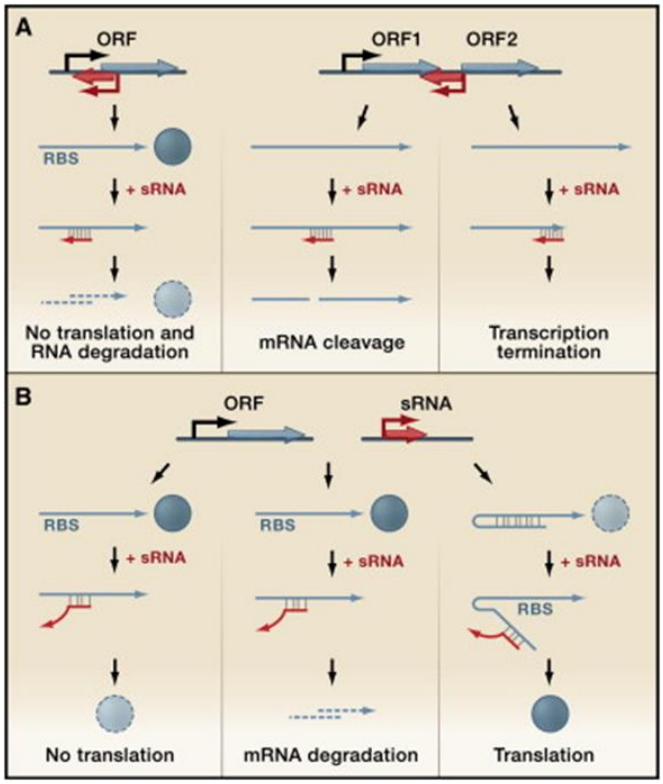

Figure 1.10: sRNA regulation. Examples of possible outcomes of cis-encoded sRNAs (A). The sRNA can block translation which leads to mRNA degradation, bind to the mRNA and cause cleavage, or stop transcription by binding to the terminator. In trans-encoded (B), the sRNA can bind to the RBS and block translation, recruit nucleases to destabilize the mRNA, or bind to the anti-RBS loop and activate translation (251).

Cis-encoded RNAs are also capable of regulating entire operons. The *gad* genes in *E. coli* are responsible for acid resistance, and two of the regulators are encoded in the *gadXW* operon. The intergenic region contains a promoter on the reverse strand that controls transcription of the *gadY* sRNA. GadY binds to the complementary sequences in the intergenic region with an overlap into the *gadX* coding region. This leads to a processing event that separates the ORFs of the two genes. This stabilizes the *gadX* transcript, which encodes the transcription factor that activates the *gad* acid resistance genes (Figure 1.11) (161, 234). These sRNAs are less well charactizerized and are much more difficult to identify through standard RNA sequencing unless steps are taken to detect the directionality of reads.

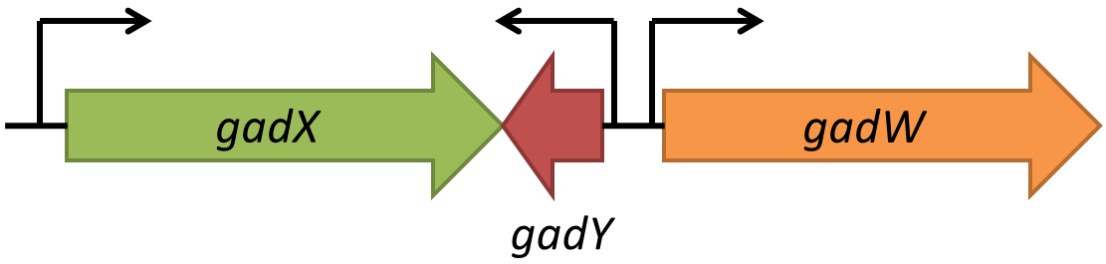

Figure 1.11: GadY. Diagram of the gadXW operon. The *gadW* gene also has its own promoter. The GadY sRNA is encoded within the intergenic region of this operon and binds to this region, leading to the cleavage of this operon. The resultant monocistronic *gadX* mRNA is more stable.

Trans-encoded sRNAs

Trans-encoded sRNAs are encoded in different loci than the genes they regulate. They lack perfect complementarity and are considered to function in a roughly analogous

manner to eukaryotic miRNAs (3). In Gram-negative bacteria these sRNAs typically require the RNA chaperon Hfq to mediate binding to their target RNA. There are likely other, as of yet unidentified RNA binding proteins as well.

The majority of known examples of trans-encoded sRNAs regulate their targets in a negative manner. The most common mechanism is for the sRNA to bind to the 5' UTR of a gene and directly block the target transcript's ribosome binding site, which is sufficient to downregulate gene expression (147). However, *in vivo*, the blocking of translation typically leads to the degradation of the target mRNA. Without the ribosomes coating the mRNA during translation, it is vulnerable to degradation by RNases. Additionally, Hfq is capable of binding the endoribonuclease RNase E, the major ribonuclease in *E. coli* and other γ-Proteobacteria, which can then directly degrade the target RNA (47). The secondary ribonuclease RNase III can also be recruited to target mRNAs (160). This ensures that the translation inhibition by sRNAs is irreversible. There are also known cases where the sRNA binds directly to the coding region of the mRNA to destabilize it directly by recruiting an endonuclease. Little is understood about how this mechanism works since the ribosome's helicase activity under other circumstances would be sufficient to remove the sRNA (172). In contrast the positively regulating sRNAs act by breaking the anti-RBS hairpin. While less common, there are still many examples of this form of regulation known (80, 175).

These trans-acting sRNAs are often involved in complex regulatory circuits. A single sRNA is capable of regulating multiple targets, such the *Salmonella* sRNA GvcB, which downregulates seven different ABC transporters (207). The same gene is also capable of

being regulated by multiple sRNAs, often in different manners. In *E. coli*, translation of the stationary phase sigma factor RpoS is blocked by the oxidative stress regulated sRNA OxyS. At the same time, translation is activated by DsrA at low temperatures, and RprA in response to cell surface stress (186). In most cases, the same region of the sRNA interacts with all its targets in a manner analogous to the seed region of eukaryotic sRNAs (257), however there are a few unpublished reports of sRNAs that have multiple target interacting regions.

Other Regulators

There are other regulatory RNAs in bacteria that do not easily fit into these classification schemes. CsrA is an RNA binding protein that binds to conserved GGA motifs in the 5' UTRs of various RNAs to block translation and destabilize them. CsrA has been shown to regulate several different processes including carbon metabolism, biofilm formation, and motility (230). In EHEC, CsrA is able to bind to *LEE1* and *LEE4* and has been shown to regulate virulence in other organisms as well (19, 125). The two sRNAs CsrB and CsrC contain repeated GGA motifs, and are able to act as negative CsrA regulators by sequestering the CsrA protein (9).

The *E. coli* 6S RNA structurally mimics the transcription open complex and binds to the σ^{70} RNA polymerase, preventing it from binding DNA and activating transcription (249). It is highly expressed during stationary phase where it is capable of binding a significant proportion of the σ^{70} holoenzyme, acting as a global repressor of housekeeping genes. However, it is unable to effectively bind to the alternate sigma factor containing RNA

polymerases, including σ^S (235). The 6S RNA also functions as a template for transcription. The resultant 14-20nt product RNA (pRNA) is highly produced during the outgrowth from stationary phase and is involved in the release of 6S from the σ^{70}-RNA polymerase (250). The 6S RNA effectively acts as a switch, allowing the bacterial cell to temporarily shut off a large portion of its transcriptome. Additionally, some sRNAs also encode for peptides or small proteins in addition to their role as RNA mediators (184). There are likely other mechanisms of post-transcriptional regulation that have not yet been discovered.

GLMY AND GLMZ

Glucosamine metabolism

GlmS, glucosamine synthetase, catalyzes the conversion of fructose-6-phosphate to glucosamine-6-phosphate (Figure 1.11). Glucosamine is central to cell metabolism and is absolutely required for bacteria to grow. Barring an exogenous source of glucosamine, GlmS is an essential gene in bacteria. In *E. coli* and other enterobacteria, *glmS* is encoded in the *glmUS* operon. GlmU is a bifunctional enzyme that catalyzes two subsequent steps in glucosamine utilization, the acetylation of glucosamine-1-phosphate to N-acetylglucosamine-1-phosphate, and the transfer of uridine to create UDP-GlcNAC, which serves as the precursor for LPS and cell wall biogenesis (68).

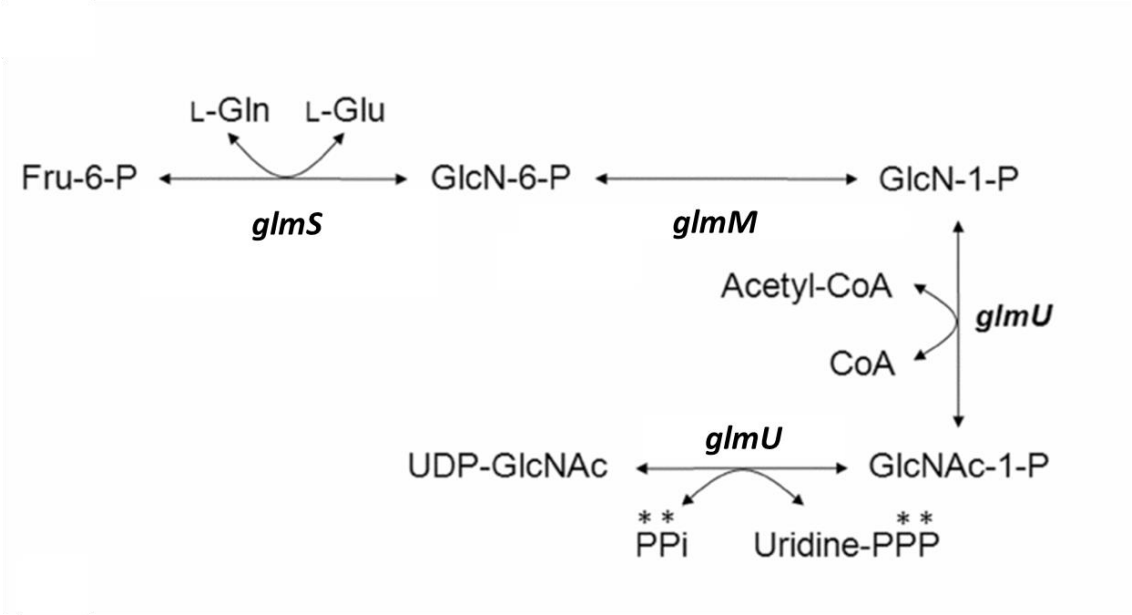

Figure 1.12: Glucosamine pathway. Diagram showing the synthesis of glucosamine from Fru-6-P to UDP-GlcNAc and the enzymes involved. Adapted from Yang, *et al* (262).

Regulation of GlmS

While transcription of *glmU* and *glmS* are permanently linked, the cell does not have equal need for these two enzymes. GlmU is always needed for recycling of the cell wall; however, GlmS is not needed when exogenous glucosamine is available. To solve this problem, the cell regulates *glmS* post-transcriptionally. The *glmUS* operon undergoes an RNase E mediated processing event which separates the *glmU* and *glmS* transcripts. The resultant *glmS* transcript is not only susceptible to polyadenylation and degradation, but its translation is inhibited by a hairpin loop blocking the RBS (98). GlmZ, an Hfq dependent trans-encoded sRNA, binds to *glmS* and relieves this hairpin allowing for its translation, while having no effect on the expression of *glmU* (Figure 1.13) (238).

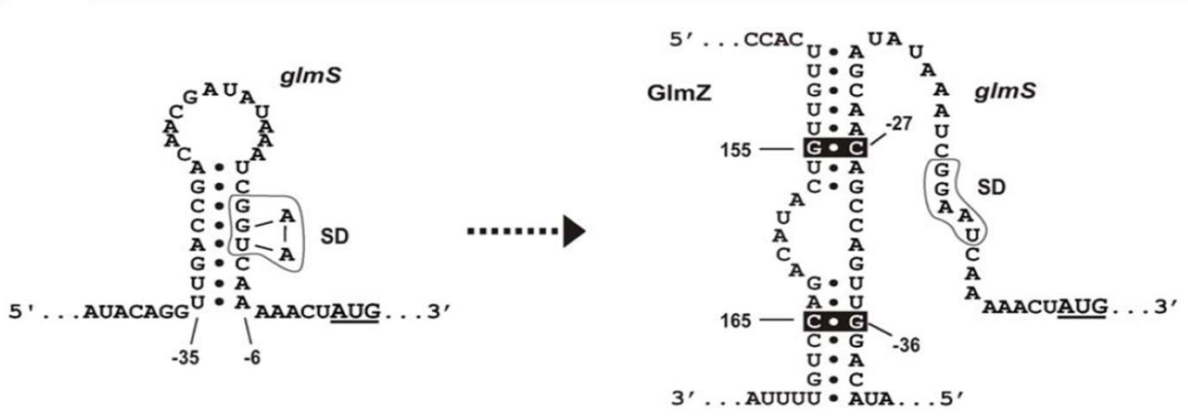

Figure 1.13: GlmS-GlmZ interaction. GlmS hairpin (left) and GlmS and bound to GlmZ in its translationally active state (right) (238)

GlmY and GlmZ

GlmZ is a 207nt sRNA. There is currently nothing known about its regulation at the transcriptional level, but it is known to be heavily regulated post-transcriptionally. The protein RapZ binds to GlmZ and recruits RNase E to cleave GlmZ towards its 3' end. The resultant 153nt transcript is missing the *glmS* binding region and is unstable (72). Another sRNA, GlmY, has significant sequence homology to GlmZ and nearly identical secondary structure (Figure 1.14). This Hfq-independent sRNA acts as a molecular mimic and binds to RapZ, displacing GlmZ and preventing its processing and degradation (71).

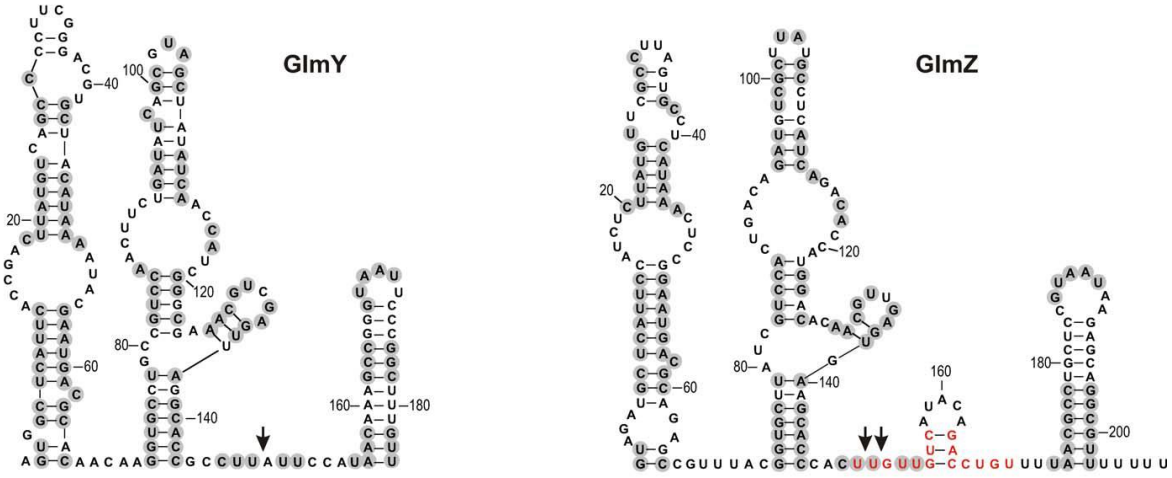

Figure 1.14: GlmY and GlmZ. Secondary structure of GlmY and GlmZ. Arrows indicate known cleavage sites and red lettering indicates the *glmS* binding site (238).

This anti-adapter RNA GlmY is also regulated post-transcriptionally; it is polyadenylated by the poly(A) polymerase PcnB (100). In bacteria, polyadenylation acts as a degradation signal (146), which results in GlmY having a short half-life. GlmY also undergoes a processing event which does not affect its stability or binding to RapZ. Transcription of *glmY* is controlled by two overlapping promoters, a σ^{70} and a σ^{54}. The σ^{54} promoter is activated by QseF. In a *qseF*, mutant *glmY* is not expressed due to the σ^{54} holoenzyme acting as a repressor, suggesting that QseF is the only σ^{54}-dependent activator of *glmY* (184).

This GlmY-GlmZ cascade is also controlled through a glucosamine-6-phosphate feedback mechanism. Upon depletion of GlcN6P, GlmY accumulates in the cell. This is through an unknown mechanism, as transcription of *glmY* is not dependent on levels of GlcN6P (71, 100, 184).

CHAPTER TWO

Overall Objective and Synopsis

Enterohemorrhagic *E. coli* is a major cause of hemorrhagic colitis and hemolytic uremic syndrome (102). The dangers associated with the induction of Shiga toxin due to antibiotic treatment limits the available treatments to palliative care (199). Understanding the regulation of virulence in this organism could lead to better treatments. The key role of cell signaling in the regulation of virulence has made it an interesting target for drug discovery, however, not much is known about the downstream effects of this signaling pathway.

Previous work has shown that the response regulator QseF is required for the expression of *espFu* and thus pedestal formation; however, this regulation was also shown to be indirect and there must be another, unknown factor involved (182). Another investigator subsequently identified QseF as a regulator of the gene encoding the sRNA *glmY* (184). We hypothesize that *glmY* or *glmZ* are the missing element in the regulation of *espFu* and are potentially involved in post-transcriptional regulation of other virulence genes in EHEC. To investigate this, we overexpressed both sRNAs and constructed knockouts of both genes. We showed that *glmY* and *glmZ* are capable of complementing the pedestal formation defect of the *qseF* mutant and are involved in the regulation of *espFu*. We also determined through northern blots that both sRNAs regulate the *LEE4* and *LEE5* operons and determined through site directed mutagenesis that GlmZ directly binds the *LEE4* transcript. We also determined that QseB regulates the transcription of *glmY*. Microarray analysis and whole RNA

sequencing of total RNA from these mutants was also performed. We assessed their whole transcriptome and determined that *glmY* and *glmZ* also regulate the expression of curli, which are involved in host cell attachment (113).

In these studies we show that the Qse cell signaling pathway of EHEC regulates the *glmY* and *glmZ* sRNA cascade, which is intimately involved in the post-transcriptional regulation of the LEE and other virulence genes. This level of regulation is likely important for the temporal regulation of the assembly of the type three secretion system and timing of the bacterial infection.

CHAPTER THREE

Methodology

STRAINS, PLASMIDS, AND GROWTH CONDITIONS

All bacteria strains, plasmids, and primers used in this study are listed on Table 1-3. Bacteria were maintained and grown in Luria Bertani (LB) or low glucose Dulbecco's Modified Eagle's Medium (DMEM) at 37ºC and 250rpm. When appropriate, media were supplemented, with 50μg/ml streptomycin, 50μg/ml kanamycin, 50μg/ml chloramphenicol, 100μg/ml ampicillin, or 50μg/ml tetracycline. 0.2% arabinose was used to induce pBAD expression vectors.

RECOMBINANT DNA TECHNIQUES

Standard protocols were used for PCR amplification, plasmid purification, restriction enzyme digestion, ligation and transformation (196). Primers were designed using the IDT Oligoanalyzer 3.1 and GenScript Real-time PCR Primer Design. Nonpolar mutants were created using the lambda red recombination system (46). In brief, the plasmid pKD3 was used as a template, primer pairs glmYREDF and glmYREDR for *glmY*, glmZREDF and glmZREDR for *glmZ,* and yhbJREDF and yhbJREDR for *rapZ,* were used to amplify PCR products that were then gel-purified (Qiagen). This was then used as a template for another round of PCR amplification and PCR purified (Qiagen). The strains Δ*qseC*, wild type and later Δ*glmY* were transformed with the temperature sensitive helper plasmid pKD46. Resistant transformants were then streaked onto LB plates and grown overnight (ON) at

30ºC. Bacteria on the plate were inoculated into a flask of LB with 25mM arabinose and grown to an OD_{600} 0.5 at 30ºC and transformed with the PCR products. The electroporated cells were then recovered in SOC media overnight at room temperature, plated on chloramphenicol supplemented LB plates and incubated overnight at 37ºC. Resultant colonies were screened for ampicillin sensitivity and chloramphenicol resistance and checked for proper insertion via PCR. The chloramphenicol cassette was then resolved by electroporating deletion candidates with the resolvase plasmid pCP20, grown at 37ºC for 5 hours then screening resulting colonies for sensitivity for both ampicillin and chloramphenicol. Final verification of gene deletion was performed by PCR amplification and sequencing.

Plasmids for mutant complementation, β-galactosidase assays and protein expression were constructed by amplifying the coding regions from the EHEC strain 86-24 using Phusion polymerase (NEB), digesting with appropriate restriction enzymes and ligating with T4-ligase (NEB). The sRNAs *glmY* and *glmZ*, were cloned into pBAD33 using glmY33F and glmY33R, and glmZ33F and glmz33R, respectively, to create pCG30 and pCG301. *LEE4* and *LEE5* were cloned into pACYC177 using lee4F and lee4R, and lee5F and lee5R to created pCG85 and pCG87. Site-directed mutagenesis was performed using the QuickChange II Site-Directed Mutagenesis Kit (Stratagene). The primers glmZglmSF and glmZglmSR were used to created glmZ* in pCG102, glmZlee4F and glmZlee4R were used to create glmZ-QC in pCG77, and lee4QCF and lee4QCR were used to created lee4-QC in pCG89.

The *espJ-espFu* region was amplified using espJFuF and EspJFuR and cloned into PCR BUNT TOPO (Invitrogen) to create pCG92. Deletion mutants were created using sewing PCR (210) and this plasmid as a template. EspFuNoUTRF and EspFuNoUTRR created EspJFu1, EspFu2F and EspFu2R created EspJFu2, EspFu3F and EspFu3R created EspJFu3, and EspJF and EspJFuR created EspFu4. These PCR products were put into PCR BLUNT TOPO to generate pCG93, pCG94, pCG95, and pCG96, respectively. These including espJ-Fu were then subcloned into pACYC177 to generate pCG97, pCG98, pCG99, pCG100, and pCG101.

The *espJ-espFu* constructs were created by first cloning the region from the *espJ* promoter to *espFu* with a FLAG tag added via a primer and inserting it into the Zero Blunt TOPO vector (Invitrogen). The deletion mutants were then generated through sewing PCR. Proper cloning of all plasmids was confirmed by sequencing.

RNA PURIFICATION

Cultures grown overnight aerobically at 37ºC in LB were diluted 1:100 into DMEM and grown in triplicate to an OD_{600} of 1.0 or grown for 6 hours at 37ºC and 5% CO_2, and then pelleted and suspended in Trizol (Invitrogen). The Ribopure Bacteria isolation kit (Ambion) was then used to extract RNA from these biological replicates according to manufacturer's protocols except for two modifications: Trizol was used instead of RNAwiz, and the cells were not disrupted by vortexing with beads. Samples were DNase I treated to

manufacturer's specifications and the concentration of RNA was determined with a Nanodrop.

NORTHERN BLOT

Bacteria were grown aerobically in low glucose DMEM supplemented with 0.2% arabinose at 37ºC to an OD_{600} of 1.0 from a 1:100 dilution of an overnight grown in LB. RNA was extracted and 5µg of each sample was run on a 1% formaldehyde agarose gel and transferred overnight to a Zeta-Probe Membrane (Bio-Rad). RNA probes were created by amplifying a segment of the gene of interest with the T7 promoter and *in vitro* transcribing using the Maxiscript T7 kit (Ambion) with radiolabeled α-UTP. An *espA* probe was created with espAF and espAR, *sepL* probe with sepLF and sepLR, *tir* with tirF and tirR, *eae* with eaeF and eaeR, *espFu* with espFuF and espFuR, and *glmS* with glmSF and glmSR. The oligo probe for the 5S endogenous control was labeled with γ-ATP using T4 polynucleotide kinase (NEB). The membranes were then hybridized ON using Ultrahyb (Ambion) at 68ºC for the RNA probes and 37ºC for the oligo probes. The membranes were washed and exposed to a phosphorimager screen overnight and then visualized with a STORM scanner (GE Healthcare).

QUANTITATIVE REAL TIME POLYMERASE CHAIN REACTION (qRT-PCR)

Quantification of RNA transcription was performed as described previously (246). Extracted RNA was diluted to a concentration of 50ng/µl and mixed with Sybr Green,

validated primers (Table 3), RNase inhibitor and reverse transcriptase (AB). The mix was used in a one-step reaction utilizing an ABI 7500 Fast sequence detection system. Data was normalized to endogenous *rpoA* levels and analyzed using the comparative critical threshold (CT) method. Data was presented as fold changes over wt levels. The error bars represent the standard deviations of the ΔΔCT value. The Student's unpaired t test was used to determine statistical significance, with a p-value of ≤0.05 being considered significant.

REPORTER ASSAYS

Transcriptional reporters were constructed using the plasmid pRS551 (213). The *glmY* and *glmZ* promoters were cloned into this plasmid with glmYtxnF and glmYtxnR, and glmZtxnF and glmZtxnR to created pCG48 and pCG49. The *espFu* promoter in pCG50 was created previously (155).

The translation fusion vector pCG56 was created by cloning lacZ from the *E. coli* strain MG1655 into pBAD24 using the primers lacZ24F and lacZ24R. The translation reporter vectors were created as follows: pCG59: glmStslF and glmStslR for GlmS, pCG60: tirtslF and tirtslR, pCG61: cesTtslF and cesTtslR, pCG62: eaetslF and eaetslR, pCG63: sepLtslF and sepLtslR, pCG64: espAtslF and espAtslR, pCG65: espDtslF and espDtslR pCG66: espBtslF and espBtslR, pCG57: EspJFuF and EspJFuR.

Transcriptional and translational β-galactosidase assays were performed using the same protocol. Bacteria containing the reporter plasmid were grown ON in LB with the appropriate antibiotic and then diluted 1:100 into clear DMEM supplemented with 0.2%

arabinose and grown to an OD_{600} of 0.8 . These were then assayed for β-galactosidase activity using ONPG as previously described (142).

For the experiments with the GlmS::LacZ reporter plasmid, bacteria were grown ON in LB with 0.2% arabinose and assayed as before with the OD_{420} reading normalized using specific activity.

OPERON ANALYSIS BY RTPCR

Wildtype and Δ*qseF* strains were grown to an OD_{600} 1.0 in low glucose DMEM and RNA was extracted as described. RNA was converted to cDNA using Superscript II (Invitrogen) and EspFuR (Table 3), according to the manufacturer's specification. cDNA was then amplified by Phusion (NEB) with the primers EspF1 and EspFuR for the larger transcript and EspFu2 and EspFuR for the smaller transcript and run on a agarose gel and visualized with ethidium bromide.

PROTEIN PURIFICATION

The pQE30 plasmid containing QseB was previously constructed (90). This plasmid was transformed into the *E.coli* strain BL-21(DE3) (Invitrogen). Resulting transformants were grown to OD_{600} 0.5 at 37ºC in LB, then induced by adding IPTG to a final concentration of 0.5mM and growing for 3 hours at 37ºC.

QseF was cloned into pBAD myc/his A using qseFBADF and qseFBADR generating pCG23. This plasmid was then transformed into TOP10 cells (Invitrogen). Resulting

transformants were grown to OD_{600} 0.5 at 37ºC in LB, then induced by adding arabinose to a final concentration of 0.2% and growing for 3 hours at 37ºC.

Bacteria were lysed in an Emulsiflex in a lysis buffer [50mM sodium phosphate, 300mM NaC, 20mM imidazole, pH 8.0] and nickel columns (Qiagen) were used to purify both proteins from cleared lysates. The nickel beats were washed 5x with lysis buffer and eluted in elution buffer [50mM sodium phosphate, 300mM NaC, 250mM imidazole, pH 8.0]. Purity was verified by Coomasie and Western blot, while the protein concentration was quantified by Nanodrop and the Biorad protein assay.

ELECTROPHORETIC MOBILITY SHIFT ASSAYS (EMSA)

EMSAs were used to determine binding of purified QseF or QseB to promoter sequences. The assays were performed as previously described (108). Briefly, defined regions of the promoter from 496 bp upstream of the *qseE* translation start site to 4nt in the coding region (Table 3) were amplified by PCR, purified, quantified and end-labeled using radiolabeled γ-ATP (Perkin Elmer) and T4 polynucleotide kinase (NEB) according to manufacturer's instructions. The radiolabeled probes were then repurified to remove unincorporated ATP. EMSAs were performed by adding increasing amounts of purified recombinant protein to 2ng labeled probe in binding buffer [60nM HEPES pH 7.5, 5mM EDTA, 3mM DTT, 300mM KCl, 25mM MgCl2, 50ng polydIdC, 500µg/ml BSA (NEB)] (38). Response regulators were phosphorylated through the addition of lithium acetyl phosphate. The reactions had a final volume of 40µl and were incubated for 20 min at room

temperature then loaded on a 6% polyacrylamide gel after addition of a 5% ficol DNA loading buffer. The gel was run at 180V for 6hrs or 50V overnight, dried and exposed on a film.

WESTERN BLOTS

Bacteria were grown overnight in LB and then diluted 1:100 into low glucose DMEM and grown to an OD OD_{600} of 1.0. The pellets were then lysed under denaturing conditions using Buffer C (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8M urea, pH 6.3) and run on a 12% SDS-PAGE and transferred to a PVDF membrane. The membrane was probed with an α-FLAG antibody (Sigma) or α-RpoA antibody (Santa Cruz Biotechnology) as the endogenous control with a HRP-conjugated secondary antibody that was visualized with ECL (GE Bioscience) and exposed to film.

CELL CULTURE AND FLUORESCENT ACTIN STAINING

HeLa cells were maintained in high glucose DMEM supplemented with 10% FBS and pen-strep glutamine and grown at 37ºC and 5% CO2. Cells were split into a 12 well plate, grown to confluency, washed and given low glucose DMEM supplemented with 10% fetal bovine sera (FBS), and then infected with bacteria grown ON in LB statically at 37ºC at a 1:100 dilution. After 3hrs of infection the media was removed and replaced and the infection continued for 3 more hours. The bacteria were then washed, fixed in formaldehyde, permeabilized with 0.2% Triton X-100, strained with FITC-phalloidin, and propidium iodide

as previously described (194). Cells were then visualized by fluorescent microscopy. Statistics were then performed by randomly imaging different fields and counting the first 100 cells while recording the number of bacteria attached to each one. The Student's t-test was used to determine significance.

LIVE-CELL IMAGING

The Lifeact::GFP expressing cell line was created using the Flip-In System (Invitrogen). HeLa cells were transfected with pLacZ::Zeocin using Fugene 6 (Promega) to create FRT sites in the genome. Cells were then selected with 100µg/ml zeocin. Resistant foci were grown up and assayed by Southern blot against *lacZ* for single insertions, and then β-galactosidase assays were performed to measure expression of the inserted locus. High expressing single insertions were then transfected with the flippase helper plasmid pOD44 and Lifeact::GFP cloned into the pFRT plasmid. These transfected cells were then selected in 100µg/ml hygromycin and resistant foci were visualized under fluorescent microscopy to measure levels of Lifeact::GFP expression. These cells were then maintained in 50µg/ml hygromycin. When they are split before infection hygromycin is not added.

EHEC were transformed with the mCherry expressing plasmid pDP151 and grown ON at 37ºC statically in LB. The Lifeact::GFP cell's media was replaced with low low glucose DMEM supplemented with 10% FBS and infected with a 1:100 dilution of the ON. The infection was allowed to continue for 2hrs at 37ºC and 5% CO_2, then the cells are

washed 3 times with DMEM and visualized by live cell imaging with a Zeiss scope. Images were taken every 2 minutes for 2 hours.

TEMPERATURE SENSITIVE RNASE E

The rne^{wt} strain NL3433 and rne^{ts} NL3431 strain (8) were transformed with either the p*LEE4* or p*LEE5* plasmids and pGlmZ. Both were grown ON in LB at 30ºC in the appropriate antibiotics and then grown to an OD_{600} of 1.0 from a 1:100 dilution in LB at 35ºC. Once they reached this point, they were shifted to the nonpermissive temperature 43ºC for 15 minutes and then RNA was extracted from the samples and used on northern blots as previously indicated.

CONGO RED PLATES

Curli formation was detected by growing bacteria on Congo red plates. 250ml of media was prepared using 2.5g tryptone, 1.25g of yeast extract, and 3.75g agar. Before pouring, Congo red dye was added to a final concentration of 40µg/ml and Coomassie brilliant blue to a final concentration of 20µg/ml. 5µl of each strain of bacteria were spotted onto the plates in triplicate. The plate was incubated at 30ºC overnight and then pictures were taken with a color camera.

MICROARRAY GLOBAL ANALYSIS

Microarray global analysis of the transcriptome of wt, Δ*glmY*, Δ*glmZ*, and Δ*rpoS* strains of EHEC were performed on extracted RNA according to manufacturer's instructions outlined in the Affymetrix Gene Expression Technical Manual. RNA was extracted as previously described and used as a template for reverse transcription to cDNA. This DNA was then fragmented with DNase I, labeled and hybridized to the *E.coli* Genome GeneChip 2.0. The Genechips contain over 10,000 probe sets directed towards 20366 genes from four different strains of *E.coli*: the K-12 laboratory strain MG1655, the O157:H7 EHEC strain EDL933, the O157:H7 EHEC strain Sakai and the uropathogenic strain CFT073.

The results were gathered from scanning the chips and the data was normalized using the MAS5 method. Expression between chips was compared using the using the Affymetrix GeneChip Operating Software v1.4. (93).

RNA SEQUENCING

RNA sequencing was performed twice with different protocols. The first time RNA from wt, Δ*glmY*, and Δ*glmZ* strains were treated with Microb Express (Ambion) to remove rRNA. Samples were then processed with the TruSeq Total RNA sequencing kit (Illumina). In summary, RNA is fragmented, converted to cDNA, ligated to adaptors, amplified, and run on an Illumina sequencing machine using paired-end sequencing.

In the second run, RNA was extracted from wt, Δ*glmY*, Δ*glmZ*, and Δ*hfq* grown statically at 37ºC. Bacterial mRNA was selectively amplified with the Ovation RNA-Seq System (Nugene) and then processed with the Encore RNA Complete RNA-seq Library

system (Nugene). A paired-end sequencing run was then performed on an Illumina sequencing machine.

The data were analyzed by different methods. Raw reads matched to the EDL933 reference sequence using Bowtie 2 and subsequently compared using the R Bioconductor module DESeq (6). Alternatively the raw data were aligned and analyzed with Rockhopper (131). For visualization purposes, the data were also aligned with Bowtie, sorted and indexed with Samtools, then visualized with BamView in Artemis (31, 119).

Table 3.1: Strains

Strain name	Genotype	Citation
86-24	wild type EHEC O157:H7	(74)
NR02	Δ*qseF* 86-24	(183)
MC474	Δ*qseB* 86-24	(90)
CG13	Δ*espFu* 86-24	This study
CG06	Δ*glmY* 86-24	This study
CG07	Δ*glmZ* 86-24	This study
N3431	*rne-3071* ts RNaseE	(8)
N3433	rne^{+}	(8)
MC4100	araD139Δ(argF-lac)U169 rpsL150 relA1 fblB3501 deoC1 ptsF25 rbsR	(212)

Table 3.2: Plasmids

Plasmid name	Contents	Citation
pRS551	*lacZ* reporter gene fusion vector	(213)
pBAD24	Cloning vector	(77)
pBAD33	Cloning vector	(77)
pACYC177	Cloning vector	New England Biolabs
Zero Blunt TOPO	Cloning vector	Invitrogen
pFRT/*lac*Zeo	Plasmid for inserting FRT site	Invitrogen
pOG44	Expresses the Flp recombinase	Invitrogen
pcDNA5/FRT	Cloning region flanked by FRT sites	Invitrogen
pKD3	FRT flanked Cm^{R} cassette	(46)
pKD46	λ red recombinase plasmid	(46)
pCP20	ts flippase plasmid	(46)
pCG48	*glmY*::lacZ in pRS551	This study
pCG49	*glmZ*::lacZ in pRS551	This study
pCG50	*espFu*::lacZ in pRS551	(155)
pCG56	LacZ in pBAD24	This study
pCG57	EspFu::LacZ in pCG56	This study
pCG92	EspJ-EspFu::FLAG in TOPO	This study
pCG93	EspJ-EspFu::FLAG deletion 1	This study
pCG94	EspJ-EspFu::FLAG deletion 2	This study
pCG95	EspJ-EspFu::FLAG deletion 3	This study
pCG96	EspJ-EspFu::FLAG deletion 4	This study
pCG30	*glmY* in pBAD33	This study
pCG31	*glmZ* in pBAD33	This study
pCG102	*glmZ** in pBAD33	This study
pCG59	GlmS::LacZ in pCG56	This study
pCG60	Tir::LacZ in pCG56	This study

pCG61	CesT::LacZ in pCG56	This study
pCG62	Eae::LacZ in pCG56	This study
pCG63	SepL::LacZ in pCG56	This study
pCG64	EspA::LacZ in pCG56	This study
pCG65	EspD::LacZ in pCG56	This study
pCG66	EspB::LacZ in pCG56	This study
pCG74	*LEE4* in pACYC177	This study
pCG89	*LEE4*QC in pACYC177	This study
pCG77	GlmZQC in pBAD33	This study

Table 3.3: Primers

Oligo name	**Sequence**
Lambda Red	
glmYREDF	CCTCAAAGCAACGTATAATCAGTACGTTACCAAACTATTTTCTTTATTGGGTGTAGGCTGGAGCTGCTTC
glmYREDR	GAGGGGAAGTTCAGATACAACAAAGCCGGGAATTACCCGGCTTTGTTATGGAATCATATGAATATCCTCCTTAG
glmZREDF	CTCAGGAAGCAAAGAGGATTACAGAATTATCTCATAACAAGTGTTAAGGGTGTAGGCTGGAGCTGCTTC
glmZREDR	GTAGTTCCTTCTCACCCGGAGGCAAGCACCTCCGGGACCTTCCTGACACATAACATATGAATATCCTCCTTAG
EspFuREDF	GGACATAAGAGACATACAACACAGGAGGAATGGCTGTTTTTTAATAGTGCGTGTAGGCTGGAGCTGCTTC
EspFuREDR	AATAACCGGTAACTGTCAGGTCAGAGCTAATATAGGTAATTATATTATAACATATGAATATCCTCCTTAG
Plasmid	
glmYtxnF	CAT GAATTC CAA CTT CGG GAG CTT TTT TG
glmYtxnR	CAT GGATCC AAG TCG GTG AAT GAG CC
glmZtxnF	CAT GAATTC TGA TAT GTA AGG CAG GTT TAT TAT ATC
glmZtxnR	CAT GGATCC GAG ATG GAA TGA GCA TCT AC
LacZF	CAT CTGCAG ACC ATG ATT ACG GAT TCA C
LacZR	GTA AAGCTT TTA TTT TTG ACA CCA GAC CA
EspFutslF	CAT GGTACC CCG CAT GAA ACC TTT CTT AGC
EspFutslR	GTA CTGCAG TGG AAA AAG TGA AGA AAC ATT GTT AA
glmStslF	CAT GGTACC TTC TGG CCG GGT AAT CC
glmStslR	GTA CTGCAG CGC ATC AAC AAC GGC CAG
tirtslF	CAT GGTACC GAT TGT TAT CTA TTT ACA TAA AAT AAA AAG GAG ATA TTT
tirtslR	GTA CTGCAG CCC TGC ACC GTC GGT TTG
cesTtslF	CAT GGTACC GGA ATA ACA TTG CTG CAG GAT G
cesTtslR	GTA GTCGAC AGA ACA CAA TCT GTT TTC ATT AAA TGA AAT AG
eaetslF	CAT GGTACC AGA TAA AAT CCG ATC TAT TAA TAT AAT TTA TTT CTC ATT C
eaetslR	GTA CTGCAG AAA CAA TCC TAA ACC AGC ACT AAG C
sepLtslF	CAT GGTACC GTC TAA GAA TAG AGT AGA AAG GAA GCT G
sepLtslR	GTA CTGCAG CGT TAA TTG CTG AGA TTC TAA TTC AAA ATC TAA TG
espAtslF	CAT GGTACC ATT ATT AAT GAT TGG TAA AGT TAT CGA CTA TAA GG
espAtslR	GTA CTGCAG ATC CTT CGA CAT ATT ACC TAA GTC ATA GAT
espDtslF	CAT GGTACC GCA ATC TCT ACA ATA TAG AAC TAT TTC AGC
espDtslR	GTA CTGCAG ACC CGT TTC AGA TTG AGT AAT ACC
espBtslF	CAT GGTACC TCC GAT TCA GCA CGG GTA AAT AG
espBtslR	GTA CTGCAG AGC AGA TGC GGC AAC TGC
EspJFuF	CAT GGTACC CAA TAA ATC TCG TCA CTG AGC TCA AT
EspJFuR	CTA CTGCAG TTTATCATCATCATCTTTATAATCCGAGCGCTTAGATGTATTAATGCC
EspFuNoUTR1R	TTA CCT TAT AAG TAA ATA TGT CAA TAC CTT
EspFuNoUTR2F	AAGGTATTGACATAT TTACTTATAAGGTAA

EspFu2F	AAGGTATTGACATAT GCATGAAACCTTTCTTAGC
EspFu2R	GCT AAG AAA GGT TTC ATG CAT ATG TCA ATA CCT T
EspFu3F	GTTTTTTAATAGTGCTTTGAATTCC TTACTTATAAGGTAA
EspFu3R	TTA CCT TAT AAG TAA GGA ATT CAA AGC ACT ATT AAA AAA C
LEE4F	CAT ggatcc GGG GAA AGC AAA ACG TAA CTG
LEE4R	GTA gacgtc CCG GGA TGA AGC CATCTAAG
glmZQCF	GTTTACGGTGCTTAT GGTCCACTGA G AGATGTCGCTTATGC
glmZQCR	GCA TAA GCG ACA TCT CTC AGT GGA CCA TAA GCA CCG TAA AC
LEE4QCF	GATGCGGTAAAGGCTTTGGAGGACTCTATGGATCATGAGACGAGTCAGTTATTGTACAG
LEE4QCR	CTG TAC AAT AAC TGA CTC GTC TCA TGA TCC ATA GAG TCC TCC AAA GCC TTT ACC GCA TC
EMSA Probe	
glmYPromK12F	GCGGCATTAAGCACCAACCACTAT
glmYPromK12R	ATATCAGAAGTTGGACGGCAGGCA
glmYPromEHECF	CGCCGCATCCGGCATTAAATACAA
glmYPromEHECR	ATATCAGAAGTTGGACGGCAGGCA
glmYPromEPECF	AATCACGACAATGGGTGGTTTGCC
glmYPromEPECR	TGTTACGGAATAAGGCGGTGCCTA
Northern Probe	
EspFuProbeF	CAACCGTCAACCGCAATATTACAG
EspFuProbeR	TAATACGACTCACTATAGGG ATGTTCAGCCATACTCCGGgcagg
sepLprobeF	GCCTGGGATATCGCAAAGGTAG
sepLprobeR	TAATACGACTCACTATAGGG AATTCAATCTTAACAAACTTAGATTGC
espAprobeF	GGTAATATGTCGAAGGATGAGG
espAprobeR	TAATACGACTCACTATAGGG CAC GTC TTG AGG AAG TTT GGC
espDprobeF	GGGTACTTCTGGTATTACTCAATC
espDprobeR	TAATACGACTCACTATAGGG GAG AGA GAT AGG GAT AGC TTG TG
espBprobeF	CTGCTTACTGATGGTAAGGTTG
espBprobeR	TAATACGACTCACTATAGGG GTT CAT TGC TGC AAA AGA ACC
tirProbeF	GGCGGCGTCTGAGATAAC
tirProbeR	TAATACGACTCACTATAGGG CCA CGG CCT CCA GTA AAT AC
cesTprobeF	GGTATTGGATCTATTTCATTTAATG
cesTprobeR	TAATACGACTCACTATAGGG GGA AAC GTA ACG CTA AC
eaeProbeF	CCGGACGTGACCAAAAGC
eaeProbeR	TAATACGACTCACTATAGGG GTA ATA AGA AGT CCA GTG AAC TAC CG
5Sprobe	CTACGGCGTTTCACTTCTGAGTTC
Stable Cell Line	
LifeactF	CAT AAG CTT CAC CAT GGG TGT TG
LifeactR	GTA GCGGCCGC TTA CTT GTA CAG CTC GTC CAT G
qPCR	
rpoA_F	GCGCTCATCTTCTTCCGAAT
rpoA_R	CGCGGTCGTGGTTATGTG
ler_F	CGACCAGGTCTGCCC
ler_R	GCGCGGAACTCATC
espA_F	TCAGAATCGCAGCCTGAAAA
espA_R	CGAAGGATGAGGTGGTTAAGCT
eae_F	GCTGGCCTTGGTTTGATCA
eae_R	GCGGAGATGACTTCAGCACTT
stx2a_F	ACCCCACCGGGCAGTT
stx2a_R	GGTCAAAACGCGCCTGATA
EscF1F	GCGATTCTGTGCCAGAGTTACTT
EscF1R	CCGCAAACTGCAACTCTAACAT
EspF1F	TGGAATTAGTAACGCTGCTTCTACA
EspF1R	CCCCCGCAGAGCTCACT

CHAPTER FOUR

Post-transcriptional Control of Microbial-Induced Rearrangement of Host Cell Actin

INTRODUCTION

Exploitation of the host cytoskeleton by bacterial pathogens is an essential feature of bacterial-host associations. Actin remodeling promotes bacterial invasion of non-phagocytic cells, survival within cells, cell-to-cell spread and locomotion, and colonization at the interface of the host epithelium (30, 65, 88). Although there is vigorous investigation of the cell biology underlying these bacterially-mediated cytoskeleton modifications, knowledge of the plasticity and dynamics of the bacterial signaling networks that regulate expression of genes necessary for these phenotypes in lacking. Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 is a major food-borne pathogen and causative agent of hemorrhagic colitis and hemolytic uremic syndrome (HUS). The mortality associated with EHEC infections stems from the production and release of a potent Shiga toxin by these bacteria. Shiga toxin induces cell death in endothelial cells, primarily in the urinary tract, causing HUS (102). EHEC attaches to epithelial cells of the intestinal lining and forms an attaching and effacing (AE) lesion. These lesions are characterized by the effacement of microvilli and the formation of a pedestal-like structure beneath each individual bacterium (220). To induce pedestal formation on epithelial cells, EHEC employs a type three secretion system (T3SS), a needle-like structure that translocates bacterial effectors directly within host cells. This T3SS together with several other genes necessary for AE lesion formation are encoded within a chromosomal pathogenicity island named the locus of enterocyte effacement (LEE) (95,

133). The LEE region contains five major operons: *LEE1-5* (55, 58, 136), which encode the components of the T3SS (95), an adhesin (intimin) (96) and its receptor Tir, which is itself translocated through the T3SS to the host cell, where upon its insertion in the cell membrane it serves as a receptor for the bacterial adhesin intimin (109) and other effector proteins (56, 101, 110, 134, 236). The LEE encoded T3SS also translocates effector proteins encoded outside of the LEE region, including EspFu/TccP, which is important for efficient AE lesion formation (28, 49, 66, 231).

Expression of the LEE and *espFu* genes is governed through complex multi-layered signaling cascades in response to many environmental cues including human hormones (epinephrine [Epi] and norepinephrine [NE]), bacterial small signaling molecules (autoinducer-3 [AI-3], indole, acyl homoserine lactones), carbon and nitrogen sources, and stress responses among others. This regulation occurs both at the transcriptional and post-transcriptional levels (19, 20, 91, 106, 107, 123, 135, 156, 165, 205, 218). In bacteria, there are many different mechanisms of post-transcriptional regulation of genes (221). One of the more abundant classes is *trans*-acting small RNAs (sRNAs). These sRNAs require the RNA chaperone Hfq and act by directly binding to mRNAs at the ribosome binding site (RBS) to repress translation, cause direct degradation of the mRNA by recruitment of nucleases, or activate translation by relieving a hairpin that blocks the RBS. While genes of the LEE are known to be post-transcriptionally regulated, no sRNAs responsible for this have been identified to date.

The AI-3/epinephrine (Epi)/norepinephrine (NE) inter-kingdom signaling cascade activates expression of virulence genes in EHEC (36, 217, 218, 245). The host hormones Epi/NE are specifically sensed by two membrane-bound histidine sensor kinases (HKs): QseC and QseE, which are the first bacterial adrenergic receptors identified (35, 182). QseE is downstream of QseC in this signaling cascade, given that transcription of *qseE* is activated through QseC (183). In addition to sensing these host hormones, QseC also senses the bacterial signal AI-3 (36). QseE, however, does not sense AI-3, thereby discriminating between host and bacterial-derived signals (182). Upon sensing their respective signals, QseC and QseE autophosphorylate and activate the virulence gene expression and pathogenesis *in vitro* and *in vivo* in EHEC (36, 180, 182). QseC transfers its phosphate to three response regulators (RR): QseB, QseF and KdpE, which upon phosphorylation are activated and function as transcription factors (90). QseE only transfers its phosphate to QseF (261). The concerted action of these RRs activates the EHEC virulence repertoire, including the LEE and *espFu* genes (Figure 4.1A). The QseF RR is necessary for the expression of EspFu (183), and it is known to regulate the sRNA GlmY, encoded right upstream from the *qseEGFglnB* operon (Figure 4.1B) (184). This sRNA is known to act as a molecular mimic (71), stabilizing another sRNA (GlmZ), which directly binds to the mRNA of the gene encoding for glucosamine synthetase (*glmS*), and activates its translation by breaking a hairpin loop and revealing the RBS (238).

Here we show that both the QseB and QseF RRs directly activate expression of *glmY*. GlmY and GlmZ coordinate LEE and EspFu expression post-transcriptionally through two

different mechanisms. We used a combination of genetic, biochemical and cell biology approaches to show that GlmY and GlmZ post-transcriptional regulation of the LEE and EspFu ensures the correct timing and dynamics of AE lesion formation by EHEC on epithelial cells. We propose that sRNA-mediated post-transcriptional regulation is responsible for the plasticity and dynamic rewiring of expression of different components of bacterial complex machineries that allow successful interactions with mammalian cells.

RESULTS

Transcriptional Regulation of *glmY* and *glmZ*

The QseC/QseE signaling system controls a plethora of virulence genes in EHEC that have to be coordinately expressed to ensure optimal AE lesion formation on epithelial cells, leading to host infection (37, 90, 154, 180). AE lesion formation is a dynamic process that requires plasticity and rapid adaptation of bacterial gene expression. Coupling transcriptional and post-transcriptional regulation within a signaling transduction cascade in the bacterial cell is key to ensuring fine tuning, and rapid adaptation of gene expression towards regulation of complex processes, such as AE lesion formation. Upstream of the *qseEGFglnB* operon is *glmY* (Figure 4.1B). The *glmY* gene is known to have two overlapping promoters, one that is driven by a σ^{70}-RNA polymerase (the homeostatic form of this enzyme), and another that is driven by a σ^{54}-RNA polymerase (Figures 4.1B and C). Transcription of g*lmY* is known to be regulated by the σ^{54}-dependent transcriptional activator QseF (184). Additionally, a sequence matching the known consensus sequence of QseB (38), another RR

involved in inter-kingdom signaling was identified *in silico* in this promoter region (Figure 4.1C).

Transcriptional β-galactosidase reporters of the promoters of both *glmY* and *glmZ* were constructed. As previously reported, in the *qseF* mutant *glmY* expression is starkly decreased and almost ablated, and the *qseB* mutant, while still expressing *glmY*, expressed significantly less than wild-type (wt) (Figure 4.1D). This result was confirmed by northern blot against the GlmY RNA (Figure 4.1E). Neither RR had any effect on *glmZ* expression (Figure 4.1F). The almost complete ablation of expression of *glmY* in Δ*qseF* is due to the σ^{54}-RNA polymerase acting as a repressor in the absence of QseF (184). The σ^{54}-RNA polymerase cannot promote the formation of the DNA open complex to initiate transcription by itself; it requires a σ^{54}RR, such as QseF, for this process (78). Because the σ^{54} promoter overlaps with the σ^{70}, in the absence of QseF the σ^{54}-RNA polymerase occupies the σ^{54} binding site and prevents access to the σ^{70}-promoter by the σ^{70} -RNA polymerase (184).

Electrophoretic mobility shift assays (EMSAs) demonstrated that both QseB and QseF directly bind to the *glmY* regulatory region (Figure 4.1G). Interestingly, while the QseB consensus sequence within the *glmY* regulatory region differs slightly between the EHEC strain 86-24, and the K12 strain MG1655 and the enteropathogenic *E. coli* (EPEC) E2348/69 strains, all are capable of binding to QseB (Figure 4.2).

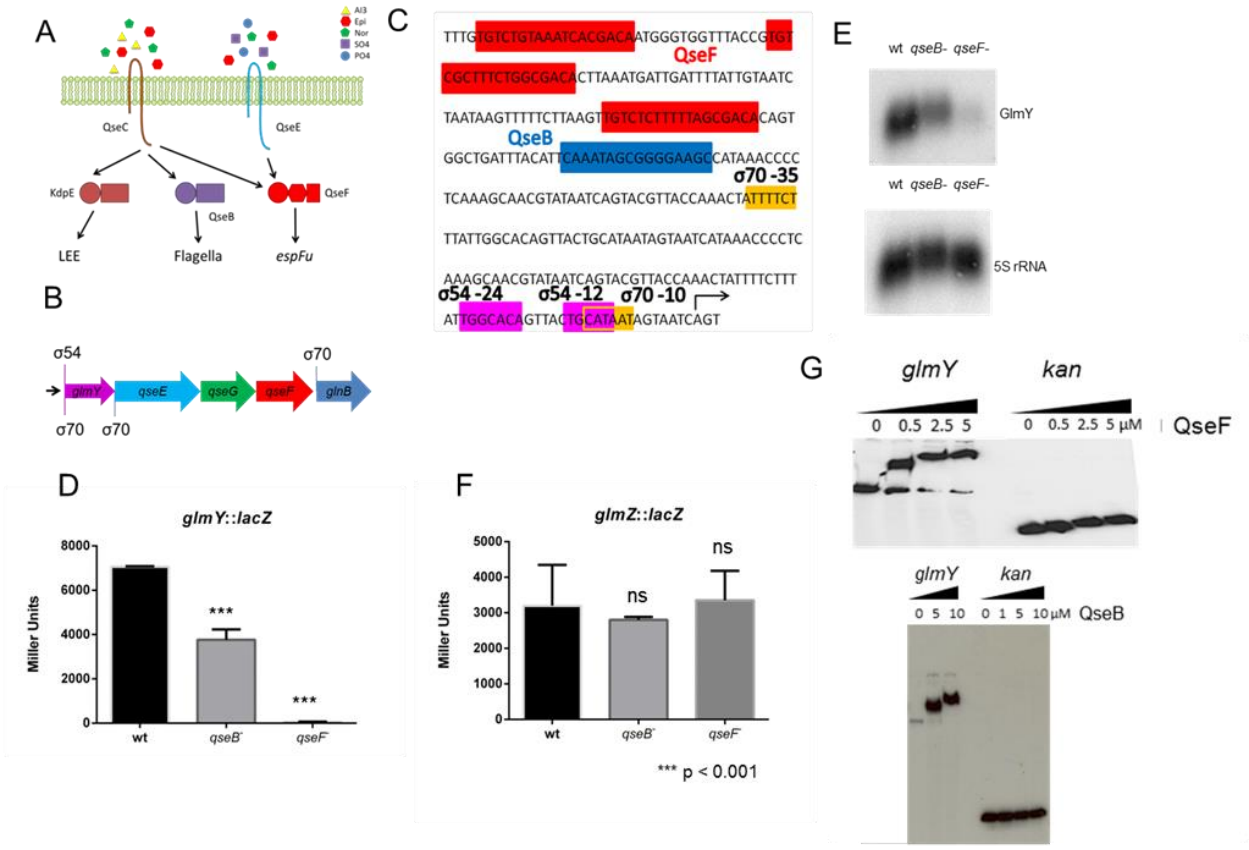

Figure 4.1: Figure 1. QseF and QseB regulation of *glmY*. (A) Schematic representation of the QseC and QseE transduction signaling systems showing the signals the kinases respond to and the response regulators they are capable of phosphorylating. (B) Schematic representation of *glmY* and *qseEGFglnB* operon depicting the location of σ^{54} and σ^{70} promoters. (C) Diagram showing the layout of the *glmY* regulatory region. QseF binding sites are depicted in red, QseB binding sites are depicted in blue, σ^{54} and σ^{70} promoters are depicted in magenta and yellow, respectively. (D) β-galactosidase assay of the *glmY::lacZ* transcriptional fusion in wt, Δ*qseB* and Δ*qseF* strains. (E) β-galactosidase assay of the *glmZ::lacZ* transcriptional fusion in wt, Δ*qseB* and Δ*qseF* strains. (F) Northern blot with a *glmY* probe of RNAs from wt, ΔqseB and Δ*qseF*. Northern blot of a 5S rRNA probe of the same RNAs, loading control. (G) EMSAs of the *glmY* promoter with increasing amounts of QseF or QseB protein in the presence of acetyl phosphate. EMSAs of the *kan* promoter with QseF and QseB, negative control.

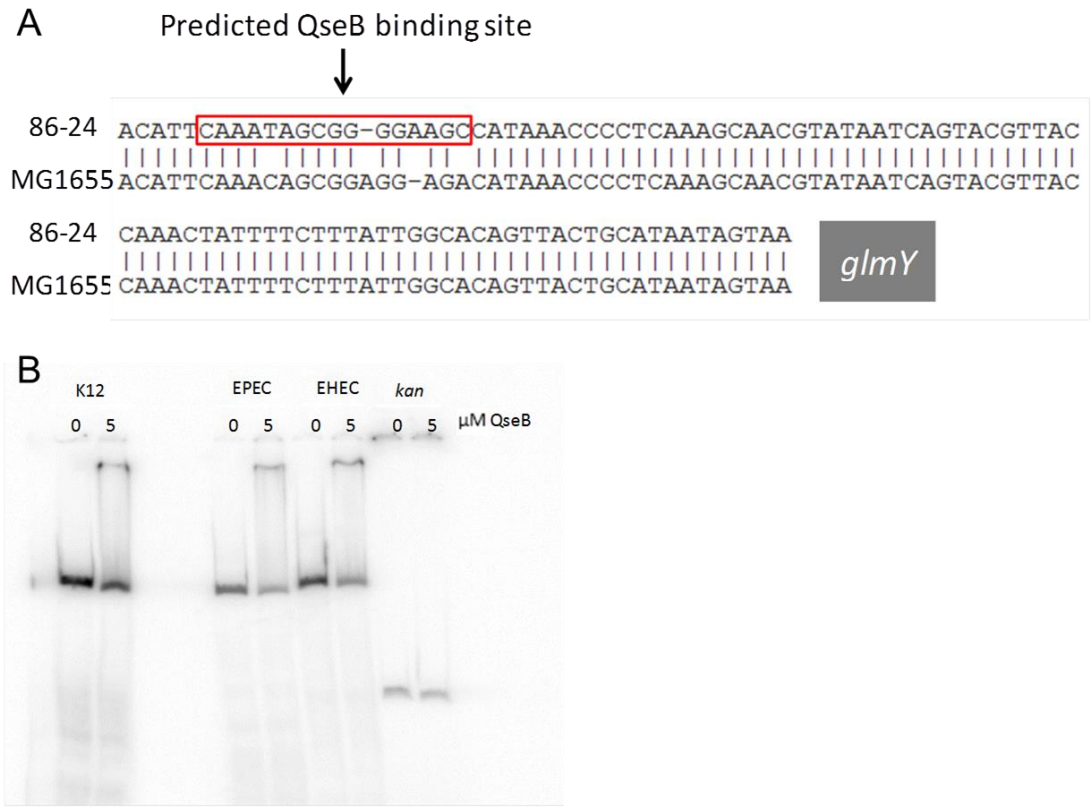

Figure 4.2: QseB binding sites in EHEC, EPEC and K-12. (A) Alignment of the *glmY* regulatory region region between depicting the QseB binding site of EHEC 86-24 and K-12 MG1655. (B) EMSAs of the *glmY* regulatory region from EHEC 86-24, K-12 MG155, and EPEC E2348/69 with the QseB protein.

Regulation of EspFu by QseF

Because the QseF RR controls AE lesion formation by indirectly promoting expression of the EspFu T3SS effector (183), next we investigated whether GlmY and/or GlmZ also played a role in EspFu expression. EspFu interacts with another effector, Tir, through IRTKS and acts as an Nck mimic to recruit N-WASP and Arp2/3 to the site of bacterial attachment causing the formation of the characteristic actin-rich pedestal (Figure 4.3A) (28, 66, 239). The expression of this effector is dependent on QseF, with the *qseF*

mutant having the same phenotype as Δ*espFu* , which is the almost complete lack of AE lesion formation on HeLa cells (Figure 4.3A and 4.3B) (28, 66, 183).

Since the regulation of *espFu* by QseF is known to be indirect (183), we tested the ability of its known target, GlmY, as well as its downstream target GlmZ, to complement a *qseF* mutant. The *glmY* and *glmZ* genes were cloned under the control of an inducible promoter and transformed into the Δ*qseF* strain. These strains were then used to infect HeLa cells to perform the fluorescein actin staining (FAS) test to visualize AE lesions. In the FAS assay the HeLa cytoskeleton was stained in green with fluorescein isothiocyanate (FITC)-labeled phalloidin, and the bacteria and nuclei were stained in red with propidium iodide, pedestals were visualized as brilliant patches of green underneath a red bacterium. Both sRNAs were able to rescue AE lesion formation in the *qseF* mutant, indicating that these sRNAs are the intermediaries between QseF and *espFu*. (Figure 4.3B).

Since sRNAs act post-transcriptionally, we investigated the *espFu* mRNA levels using northern blots. EspFu is encoded outside of the LEE within a prophage. Upstream of the *espFu* gene is the *espJ* gene that encodes another T3SS effector (Fig. 4.3E) (130). In the WT, there is a major band the size of the predicted *espFu* transcript (1100bp), as well as a much fainter upper band of 2100 nucleotides in length. In the *qseF* mutant, the lower band is still present, however the upper band is much more pronounced. This upper band corresponds to the expected size of an *espJ-espFu* transcript (Figures 4.3C and 4.3E). There are 320bp between these two genes, and previous work suggested they were not co-transcribed. However, since only RNA from wt bacteria was used in those experiments, it

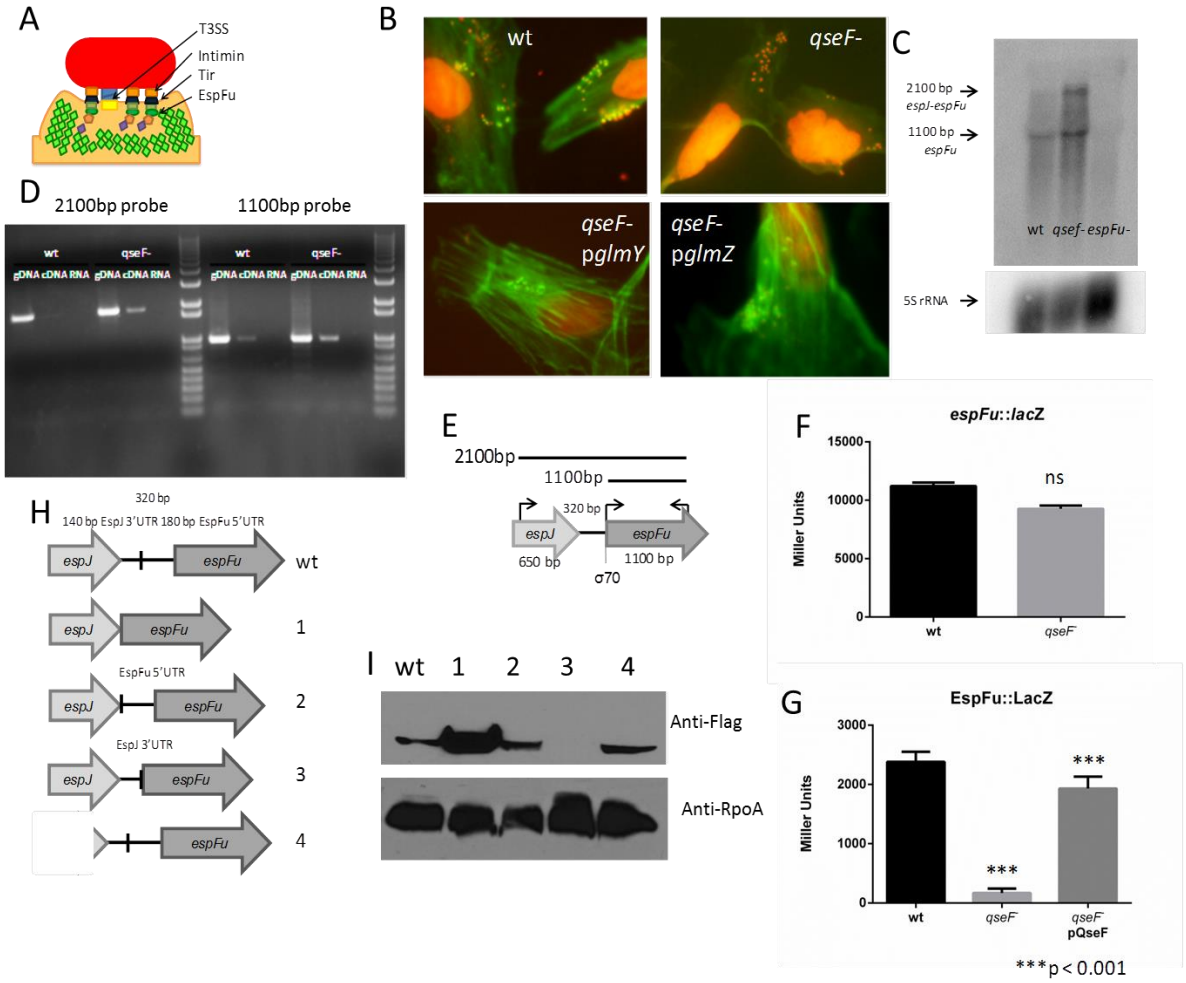

Figure 4.3: . Post-transcriptional regulation of EspFu. (A) Schematic representation of AE lesion formation. (B) FAS of HeLa cells infected by wt, Δ*qseF*, and Δ*qseF* complemented with pGlmY or pGlmZ. (C) Northern blot using an *espFu* probe on RNA from wt, Δ*qseF*, and Δ*espFu*, Northern with a 5S rRNA probe with the same RNAs, loading control. (D) RT-PCR of cDNA from wt and Δ*qseF* using primer sets to the entire *espJ-espFu* region or just *espFu*. (E) Schematic representation of the *espJ-espFu* region and the 2100 *espJ-espFu* and the 1100 *espFu* transcripts. (F) β-galactosidase assay of the *espFu*::*lacZ* transcriptional fusion in wt and Δ*qseF*. (G) β-galactosidase assay of a EspFu::LacZ translation reporter plasmid in wt, Δ*qseF*, and Δ*qseF* complemented. (H) Diagram of the espJ-espFu WT and deletion constructs. (I) Western blots of wt EHEC expressing *espJ-espFu* WT and deletion Flag-tagged constructs probed with an anti-flag antibody.

is possible that the less abundant larger transcript was not detected (183). To confirm whether this larger transcript is *espJ-espFu*, RT-PCR was performed using primers spanning this region. While a very faint band corresponding to *espJ-espFu* (2100bp) transcript is observed in WT EHEC, it is much more pronounced in the *qseF* mutant (Figure 4.3D). Therefore the lack of EspFu expression in a *qseF* mutant is not due to the absence of its transcript, but the lack of a processing event of the *espJ-espFu* transcript necessary for EspFu expression.

Transcriptional and translational reporters of *espFu* were constructed. As expected, the Δ*qseF* had no defect in *espFu* transcription (Figure 4.3F); however it is required for the translation of EspFu (Figure 4.3G). To determine the regions of the *espJ-espFu* required for this regulation, various deletions in the intergenic region between these genes were constructed. These deletions were cloned into a vector with a FLAG-tag on the C-terminus of *espFu*. Four deletion mutants were constructed using the previously identified 5' untranslated region (UTR) of the *espFu* transcript as a reference point (183): p1 that lacks the entire intergenic region, p2 that lacks the *espJ* 3' UTR, p3 that lacks the *espFu* 5' UTR, and p4 that does not have the *espJ* gene but still has the intergenic region (Figure 4.3H). Western blots using anti-flag antiserum were performed on whole cell lysates of EHEC expressing the WT *espJ-espFu*-Flag plasmid and each of these four deletions constructs. The p2 and p4 constructs expressed similar levels of EspFu::Flag to the WT plasmid, while p1 expressed more protein, and p3 did not express EspFu (Figure 4.3I). These data indicate that the 3' UTR of *espJ* acts negatively on the translation of *espFu*, and this QseF/GlmY/GlmZ-

mediated processing event is required for the translation of *espFu* since the resulting transcript lacks the 3' UTR of *espJ*. Additionally, the presence of EspFu::FLAG from the p4 plasmid indicates that in addition to being co-transcribed with *espJ*, *espFu* also has its own promoter (Figure 4.3I).

The role of GlmY and GlmZ in pedestal formation

Given that EspFu is involved in pedestal formation, we further investigated the role of GlmY and GlmZ in AE lesion formation by constructing Δ*glmY* and Δ*glmZ* strains, and performing FAS assays with these mutants. Since they were both capable of rescuing the *qseF* phenotype, we expected that both mutants would present a decreased ability to form pedestals similarly to the Δ*qseF* and Δ*espFu* strains. Surprisingly, both sRNAs mutants attached to and formed pedestals on HeLa cells at levels far higher than the wt strain. Both the *glmY* and *glmZ* plasmids were capable of complementing the *glmY* mutant, which is an expected result (Figures 4.4A and 4.4B). The GlmY sRNA is known to stabilize the GlmZ sRNA (238), and GlmZ in a Hfq-dependent manner exposes the RBS of the *glmS* mRNA to promote its translation (238). Thus far, the only known target for GlmY and Z regulation in *E. coli* was *glmS*. The "effector" sRNA that base-pairs with the *glmS* mRNA is GlmZ, and thus the effect of GlmY in *glm*S is indirect and solely attributed to its stabilization of GlmZ (238). However, the *glmY* plasmid was also capable of complementing the *ΔglmZ*, which suggests that GlmY may have additional functions besides preventing the degradation of GlmZ (Figures 4.4A and 4.4B). Because in addition to *espFu*, AE lesion formation also

requires the expression of the LEE genes, we assessed LEE regulation by these sRNAs. Expression of the *stx2a* gene encoding Shiga toxin and of *ler* (*LEE1* operon), the master regulator of the LEE, was unchanged, but expression of the *LEE4 (espA)* and *LEE5* (eae) operons was decreased in the wt strain expressing GlmY or GlmZ on a plasmid (Figures 4.4C and 4.4D), suggesting that these sRNAs decrease *LEE4* and 5 expression post-

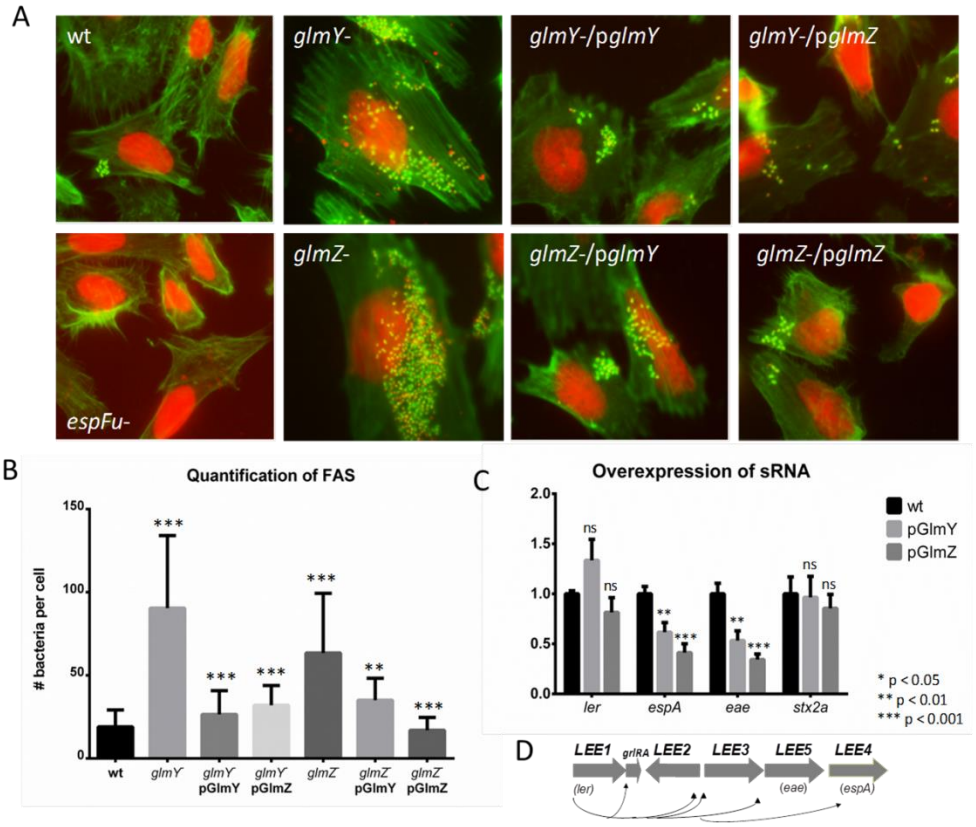

Figure 4.4: GlmY and GlmZ regulation of AE lesion formation and the LEE genes. (A) FAS of HeLa cells infected with wt, Δ*espFu*, Δ*glmY*, Δ*glmZ* complemented with pGlmY or pGlmZ, Δ*glmZ*, and Δ*glmZ* complemented with pGlmY or pGlmZ. (B) Quantification of the FAS experiment measuring the average number of bacteria attached per cell. (C) qPCR of *ler*, *espA*, *eae*, and *stx2a* using RNA extracted from wt, wt overexpressing pGlmY and wt over-expressing pGlmZ. (D) Schematic representation of the LEE region depicting the *LEE1-5* and *grlRA* operons. Ler is encoded by the first gene in the *LEE1* operon and is the transcriptional activator of all the LEE genes.

transcriptionally. These data offer an explanation for why deletion of the genes encoding these sRNAs increases AE lesion formation. In the absence of these sRNAs, the transcripts of *LEE4* and *LEE5* operons (containing many genes essential for pedestal formation) would be stabilized, increasing pedestal formation.

The role of GlmY and GlmZ in regulation of AE lesion formation by promoting EspFu translation and destabilizing LEE transcripts (Figures 4.3 and 4.4), seems to be initially confounding. However, AE lesion formation is a dynamic process, where pedestals are constantly being formed and unformed during infection, and the precise modulation of the levels of LEE and EspFu expression are important for the efficiency of this phenotype. To better understand the dynamics of pedestal formation responsible for the phenotype of the *glmY* and *glmZ* EHEC knockouts, we wanted to visualize the cells in real time. The F-actin binding peptide Lifeact (187) proved to be the most effective at visualizing the pedestals formed by EHEC. For ease of experimentation, a cell line stably expressing Lifeact::GFP was created. Bacteria were visualized by expression of mCherry (Figure 4.5A-C). The Δ*glmY* and Δ*glmZ* strains attached to and formed pedestals much more efficiently and faster than the WT strain, suggesting that these sRNAs regulate the proper timing and amount of AE lesion formation on epithelial cells.

GlmY and Z are known to promote translation of *glmS* that encodes the glucosamine synthase enzyme in the *E. coli* K-12 strain MC4100. GlmS is necessary for the synthesis of N-acetylglucosamine-6-P, which is used for cell wall biosynthesis (Figure 4.6A) (238). A

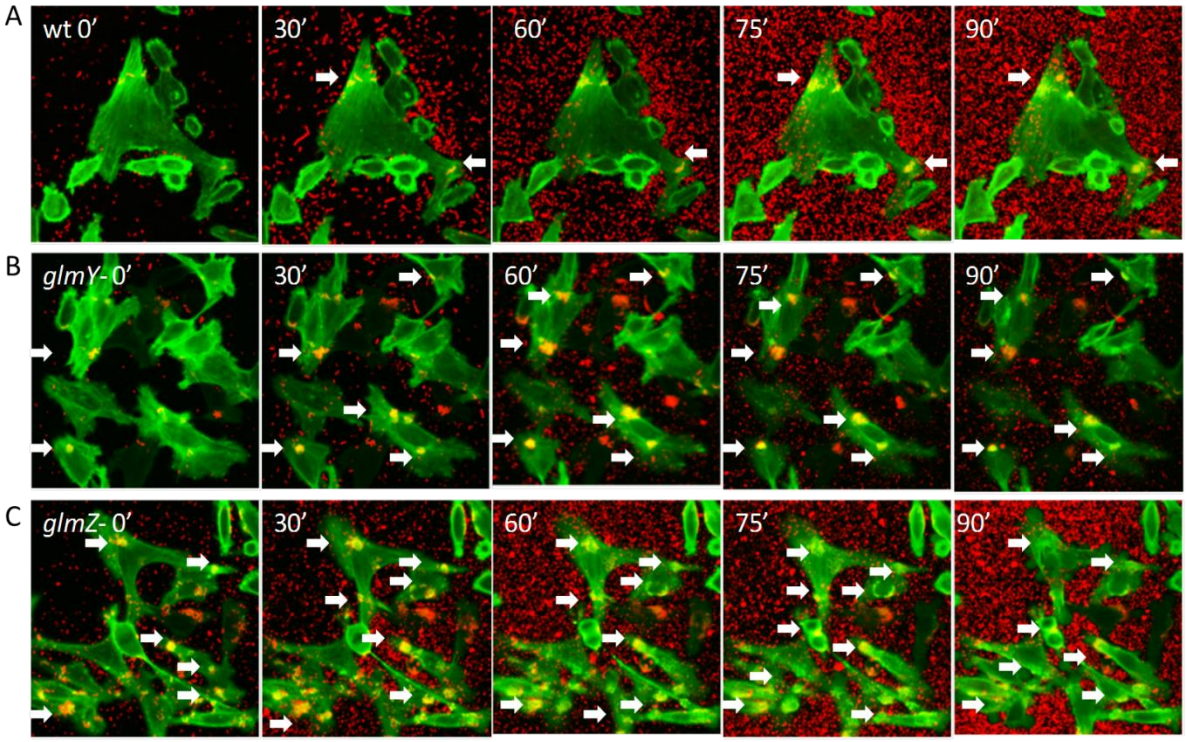

Figure 4.5: GlmY and GlmZ regulation of AE lesion timing and dynamics. (A) Time lapse microscopy of Lifeact::GFP expressing HeLa cells being infected with mCherry expressing wt EHEC. White arrows show clusters of EHEC AE lesions. (B) Time lapse microscopy of Lifeact::GFP expressing HeLa cells being infected with mCherry expressing Δ*glmY* EHEC. (C) Time lapse microscopy of Lifeact::GFP expressing HeLa cells being infected with mCherry expressing Δ*glmZ* EHEC.

glmS mutant is lethal because it is defective for cell wall biosynthesis. However, addition of 1% N-acetyl-glucosamine (GlcNAC) to the media allows the survival of a *glmS* mutant, because the GlcNAC sugar needed for cell wall synthesis is being provided exogenously (242). To rule out the possibility that the AE lesion phenotype governed by GlmY and GlmZ is due to defects in cell wall synthesis in these sRNAs mutants because of the decreased expression of GlmS, FAS assays were repeated in media containing 1% GlcNAC. The AE lesion phenotypes of the *glmY* and *glmZ* mutants, which form pedestals at

a much higher rate than the wild type, was the same both in the absence or presence of GlcNAC (Figure 4.6B and C). Additionally, point mutations in *glmZ* that abolish

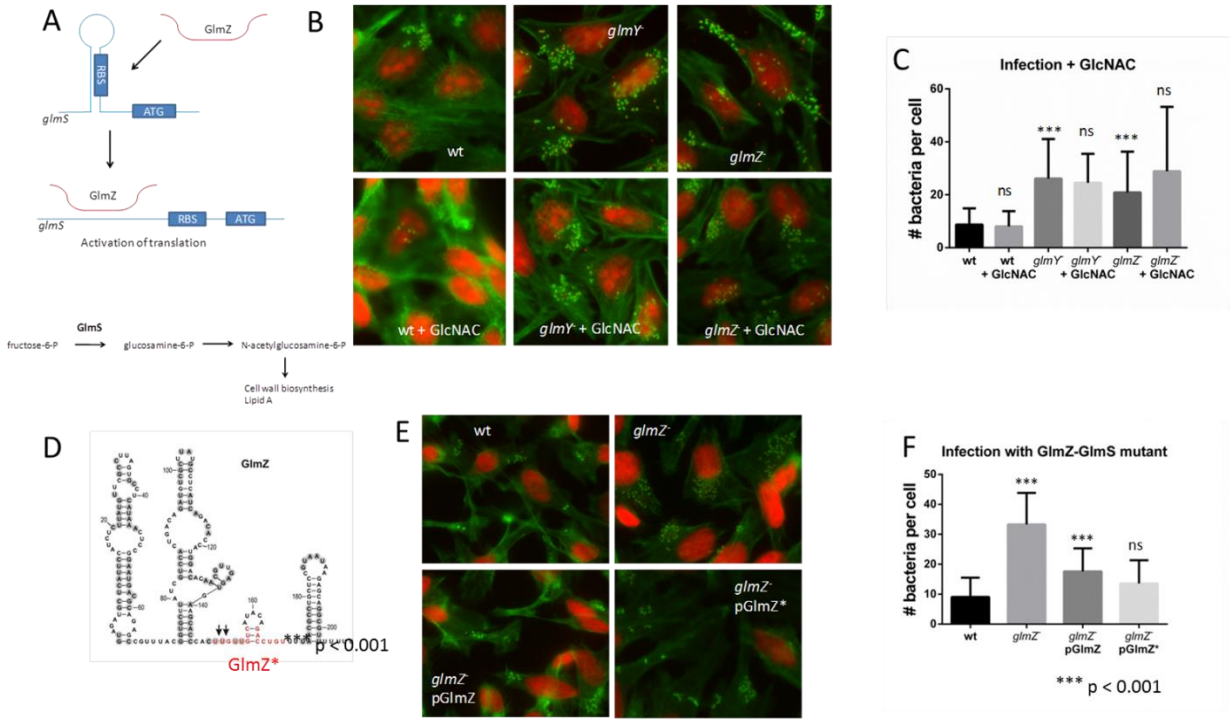

Figure 4.6: GlcNac and GlmS are not involved in the GlmY/GlmZ-dependent AE lesion phenotype. (A) Scheme depicting the mechanism of GlmZ regulation of *glmS* translation. (B) FAS of HeLa cells infected with wt, Δ*glmY*, Δ*glmZ* in the absence and presents of 1% GlcNAC. (C) Quantification of the FAS of HeLa cells infected with wt, Δ*glmY*, Δ*glmZ* in the absence and presence of 1% GlcNAC depicting the average number of bacteria attached per cell. (D) Sequence and structure of the GlmZ sRNA . Arrows depict the site-directed mutations created to disrupt GlmZ/*glmS* pairing. This mutant is named GlmZ*. (E) FAS of HeLa cells infected with wt, Δ*glmZ*, Δ*glmZ* complemented with pGlmZ and Δ*glmZ* pGlmZ*, a mutant of GlmZ that is incapable of binding to *glmS*. (F) Quantification of the FAS of HeLa cells infected with wt, Δ*glmZ*, Δ*glmZ* complemented with pGlmZ and Δ*glmZ* pGlmZ*, a mutant of GlmZ that is incapable of binding to *glmS* depicting the average number of bacteria attached per cell.

the regulation of *glmS* by GlmZ were created as previously reported, and the mutant GlmZ was named GlmZ* (Figure 4.6D) (238). This GlmZ* was still capable of complementing

pedestal formation in the *glmZ* mutant, indicating that the pedestal formation phenotype is not mediated through downstream effects of diminished GlmS expression or issues with cell wall biosynthesis (Figure 4.6E and F). To further assess the levels of GlmS expression promoted by GlmY and Z in EHEC, northern blots of *glmS* were performed on RNA from wt EHEC, and wt EHEC expressing GlmY, GlmZ and GlmZ* on a plasmid. It has been previously reported that in *E. coli* K-12 strain MC4100, expression of the *glmZ* mRNA is increased by expression of these sRNAs on plasmids due to the more efficient translation of *glmS* (238). In EHEC, however, expression of both of these sRNAs on plasmids, did not affect the levels of the *glmS* transcript under the conditions we assayed (Figure 4.7A). We also created a translational reporter of GlmS. Previous studies indicate that overexpression

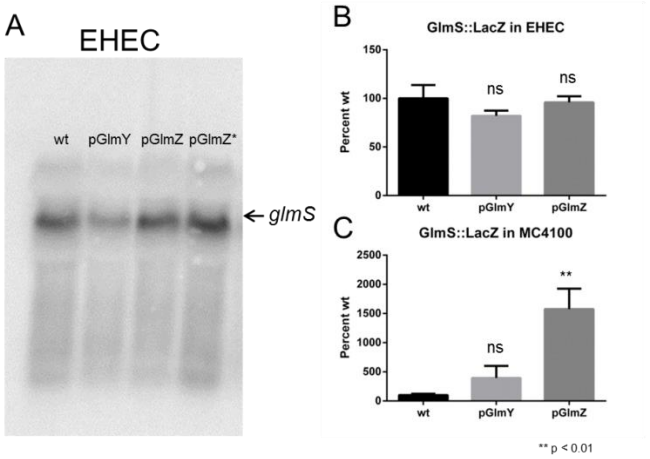

Figure 4.7: GlmY and glmZ regulation of *glmS* in EHEC and K-12 MC4100. (A) Northern blot against *glmS* using RNA from with wt, Δ*glmZ*, Δ*glmZ* pGlmZ and Δ*glmZ* pGlmZ*. (B) β-galactosidase assay of the pGlmS::LacZ translation reporter in wt EHEC, wt overexpressing *glmY*, and wt overexpressing *glmZ*. (C) β-galactosidase assay of the pGlmS::Lac translation reporter in MC4100, MC4100 overexpressing *glmY*, and MC4100 overexpressing *glmZ*.

of either *glmY* or *glmZ* should lead to an increase in β-galactosidase activity with this reporter construct. However, in EHEC, overexpressing *glmY* or *glmZ* did not change GlmS::LacZ expression (Figure 4.7B). This same reporter plasmid was then assayed in MC4100, the *E. coli* K12 strain used in previous study on *glmS* (184, 238), and it behaved as previously reported (Figure 4.7C). Since the sequences of both *glmS* and *glmZ* that interact are invariant between these two strains, it is likely there is another level of regulation that is masking the regulation of g*lmS* by GlmZ in EHEC 86-24 that is not present in MC4100.

Post transcriptional regulation of *LEE5* and *LEE4* by GlmY and GlmZ

The *LEE5* operon in EHEC consists of three genes that encode the translocated intimin receptor (*tir),* its chaperone (*cesT),* and the bacterial adhesin intimin that Tir interacts with (*eae*) (Figures 4.5A and 4.2A) (55, 58). While this operon is transcribed by a single promoter upstream of *tir* (1, 54, 216), there is a processing event that results in the separation of *cesT-eae* from *tir* (Figures 4.8A-D). Inasmuch as GlmY and GlmZ over-expression decreased the levels of the *eae* transcript (Figure 4.8A), we investigated the mRNA levels of each gene in this operon by northern blot (Figure 4.8B). Over-expression of both sRNAs decreased the levels of the *cesT-eae* transcript (3300bp) (Figures 4.8C and D), while the *tir* transcript was largely unaffected (1600bp) (Figure 5B). The levels of transcription of the entire *LEE5* operon (4900bp) were also decreased by these sRNAs (Figures 4.8B-D). Since the first gene of this operon is unaffected, GlmY and GlmZ must be acting post-transcriptionally. One of the primary ways sRNAs affect gene stability is by blocking

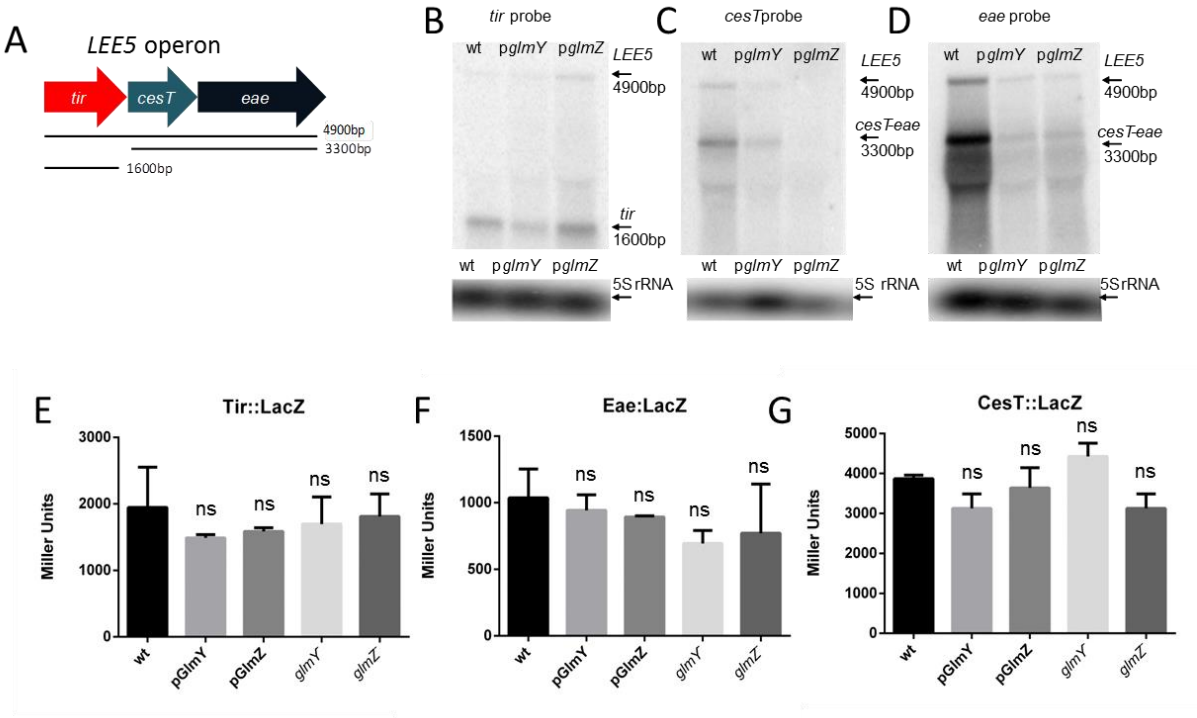

Figure 4.8: GlmY and GlmZ regulation of the *LEE5* operon. (A) Schematic depiction of the *LEE5* operon and its transcripts. (B) Northern blots using a probe against *tir* using RNA from wt, bacteria overexpressing *glmY*, and bacteria overexpressing *glmZ* with the 5S rRNA serving as a loading control. (C) Northern blots using a probe against *cesT* using RNA from wt, bacteria overexpressing *glmY*, and bacteria overexpressing *glmZ* with the 5S rRNA serving as a loading control. (D) Northern blots using a probe against *eae* using RNA from wt, bacteria overexpressing *glmY*, and bacteria overexpressing *glmZ* with the 5S rRNA serving as a loading control. (E) β-galactosidase assays of Tir::LacZ translational fusion in wt, wt/pGlmY, wt/pGlmZ, Δ*glmY*, and Δ*glmZ*. (F) β-galactosidase assays of Eae::LacZ translational fusion in wt, wt/pGlmY, wt/pGlmZ, Δ*glmY*, and Δ*glmZ*. (G) β-galactosidase assays of CesT::LacZ translational fusion in wt, wt/pGlmY, wt/pGlmZ, Δ*glmY*, and Δ*glmZ*.

translation by binding to the RBS (251). An mRNA being translated is largely protected from nuclease by the ribosomes, so blocking translation can lead to the degradation of the transcript. To test this possibility, translational LacZ reporters were constructed of all three genes of *LEE*, and β-galactosidase assays were performed (Figure 4.8E-G). Neither the knockouts of *glmY* and *glmZ* nor did their overexpression have any effect on the translation

of any of the three reporter proteins, suggesting that they are not acting through this mechanism.

The *LEE4* operon encodes the SepL regulator of effector translocation into host cells, the EspA protein that forms a filament that creates a sheath around the T3SS needle , the EspBD proteins that create a pore through the eukaryotic cell membrane, a chaperone CesD2, the EscF structural protein of the needle, an uncharacterized protein Orf29, and the effector EspF (41, 49, 58, 116, 136, 202). The first gene of this operon *(sepL*) is processed from this operon in a RNase E dependent manner (123), and there is a terminator in the *cesD2* gene that leads to lower expression of the last three genes (136) (Figure 4.9A). Similarly to our studies concerning the post-transcriptional regulation of *LEE5* (Figure 4.8), northern blots were performed for *LEE4* genes in wt, wt overexpressing *glmY* or *glmZ,* and the *glmY* and *glmY* mutants (Figure 4.9B). Similarly to *LEE5*, over-expression of both sRNAs leads to lower levels of the *espA-cesD2* transcript, while the *sepL* transcript was mostly unaffected. The level of the *espA-cesD2* transcript was noticeably higher in the sRNAs mutants (Figure 4.9B). The transcript of the last three genes of the *LEE4* operon could not be detected by northern blot, because of their much lower expression due to the transcription terminator in *cesD2*, however qPCR demonstrated that they were also down regulated by overexpression of *glmY* and *glmZ* (Figure 4.9C). Translational fusions of SepL, EspA, EspD, and EspB were constructed and assayed, and again expression of these reporters were unaffected in the Δ*glmY* or Δ*glmZ* strains, and by over-expressing the two sRNAs (Figure 4.9D-G). In a

similar scenario to the post-transcriptional regulation of *LEE5* (Figure 4.8), GlmY and GlmZ are not destabilizing the *LEE4* transcripts by blocking translation (Figure 4.9).

Some sRNAs bind directly to the coding region of their mRNA target transcript, and cause its degradation through the recruitment of a ribonuclease (251). The IntaRNA software (25) was used to predict potential binding sites of GlmY and GlmZ to the coding regions of *LEE5* and *LEE4*, then point mutants that would affect binding were created for each prediction. These mutants were then assayed by their ability to complement the *glmY* and *glmZ* mutants in FAS test and qRT-PCR of target genes. While we have been unable to find any direct binding site (either *in silico* or empirically) of GlmZ or GlmY to the *cesT-eae* transcript, one prediction of GlmZ binding to the *orf29* region of the *LEE4* operon was promising (Figures 4.9H and I). We generated a GlmZ mutant changing two C residues to G residues to prevent base pairing with the *LEE4* operon (Figure 4.9I). This GlmZ mutant, named GlmZ-CG, was unable to complement the pedestal formation of the *glmZ* knockout (Figure 4.9K), nor did it complement expression of the *LEE4* gene *espA* (Figure 4.9J). To confirm this result, the corresponding compensatory mutations were made in a plasmid containing the entire *LEE4* operon, so that in the presence of both GlmZ-CG and *LEE4*-GC the interaction would be restored (Figure 4.9J). This was tested using qPCR against *espA*. The *glmZ*-GC plasmid is unable to complement the increased levels of *espA* in Δ*glmZ*; however *LEE4*-GC restores this regulation (Figure 4.9J). This indicates that GlmZ is interacting with *LEE4* at the predicted site, and that this interaction is responsible for the regulation of *LEE4* by GlmZ.

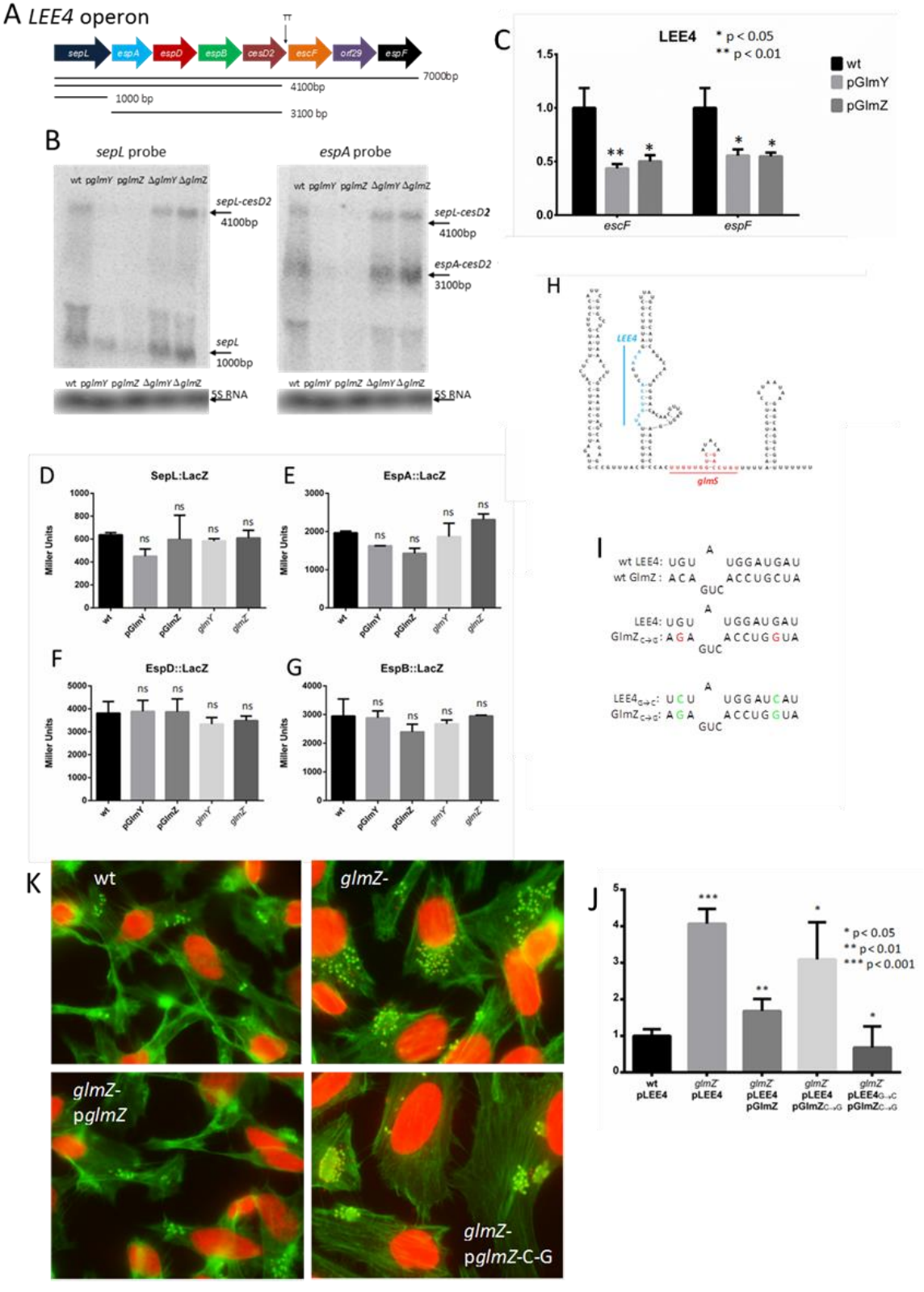
A LEE4 operon
sepL
espA
espD
espB
cesD2
escF
orf29
espF
7000bp
4100bp
1000 bp
3100 bp
B
sepL probe
espA probe
wt pglmY pglmZ ΔglmY ΔglmZ
sepL-cesD2
4100bp
espA-cesD2
3100bp
sepL
1000bp
5S RNA
C
LEE4
* p < 0.05
** p < 0.01
wt
pGlmY
pGlmZ
escF
espF
D
SepL::LacZ
E
EspA::LacZ
F
EspD::LacZ
G
EspB::LacZ
Miller Units
ns
H
LEE4
glmS
I
wt LEE4: UGU A UGGAUGAU
wt GlmZ: ACA ACCUGCUA
GUC
LEE4: UGU A UGGAUGAU
GlmZC→G: AGA ACCUGGUA
GUC
LEE4C→C: UCU A UGGAUCAU
GlmZC→G: AGA ACCUGGUA
GUC
J
* p< 0.05
** p< 0.01
*** p< 0.001
wt pLEE4
glmZ pLEE4
glmZ pLEE4 pGlmZ
glmZ pLEE4 pGlmZC→G
glmZ pLEE4G→C pGlmZC→G
K
wt
glmZ-
glmZ- pglmZ
glmZ- pglmZ-C-G

Figure 4.9: GlmY and GlmZ regulation of the *LEE4* operon. (A) Schematic depiction of the *LEE4* operon and its transcripts. (B) Northern blots using probes against *sepL* and *espA* using RNA from wt, wt/pGlmY, wt/pGlmZ, Δ*glmY*, and Δ*glmZ*, with the 5S rRNA serving as a loading control. (C) qPCR of *escF* and *espF* using cDNA from wt, wt over-expressing *glmY* or *glmZ*. (D) β-galactosidase assays of SepL::LacZ translational fusion in wt, wt/pGlmY, wt/pGlmZ, Δ*glmY*, and Δ*glmZ*. (E) β-galactosidase assays of EspA::LacZ translational fusion in wt, wt/pGlmY, wt/pGlmZ, Δ*glmY*, and Δ*glmZ*. (F) β-galactosidase assays of EspD::LacZ translational fusion in wt, wt/pGlmY, wt/pGlmZ, Δ*glmY*, and Δ*glmZ*. (G) β-galactosidase assays of EspB::LacZ translational fusion in wt, wt/pGlmY, wt/pGlmZ, Δ*glmY*, and Δ*glmZ*. (H) Sequence and secondary structure of the GlmZ sRNA depicting the regions that interact with the *glmS* and *LEE4* mRNAs. (I) Schematics showing the predicted interaction between GlmZ and *LEE4*, the point mutants made in GlmZ to abolish binding and the compensatory mutations made in *LEE4*. (J) qRT-PCR of *espA* with cDNA from wt p*LEE4*, Δ*glmZ* pLEE4, Δ*glmZ* pGlmZ p*LEE4*, Δ*glmZ* pGlmZ-CG p*LEE4*, and Δ*glmZ* pGlmZ-CG p*LEE4*-GC. (K) FAS of HeLa cells infected with wt, Δ*glmZ*, Δ*glmZ* pGlmZ, and Δ*glmZ* pGlmZ-CG.

Since GlmY and GlmZ mediated regulation of *LEE4* and *LEE5* does not act through repression of translation, it is likely that they recruit a nuclease that then degrades the transcripts. The major *E. coli* ribonuclease RNAse E is known to be recruited to many sRNA-mRNA complexes, and it has been previously shown to mediate processing of the *LEE4* transcript (123). While *rne* is an essential gene, there is a well characterized *E. coli* K-12 temperature sensitive mutant (8) that we used to test whether RNAse E is involved in the GlmZ-dependent post-transcriptional regulation of *LEE4* and *LEE5*. Since this strain is a K12 strain, plasmids containing the entire *LEE4* or *LEE5* operons along with the *glmZ* over-expression plasmid were transformed into the rne^{wt} (wild-type) and rne^{ts} (temperature sensitive mutant) strains. The bacteria were heat-shocked for 15 minutes before RNA was extracted. This was sufficient to stop the RNAse E mediated processing of *sepL* from *espA*

as previously reported (123), however, it had no effect on the GlmZ downregulation of the *espA-cesD2* transcript. (Figure 4.10). The processing of *tir* from *cesT-eae* is also RNase E dependent, but GlmZ is able to function without the presence of RNase E (Figure 4.10). These data show that in both cases, *LEE4* and *LEE5* are processed by RNase E, however GlmZ does not act by recruiting this nuclease. Another potential nuclease known to be recruited by sRNAs is RNaseIII. While the *rnc* gene is not essential, the knockout in 86-24 proved to be extremely sick and has a severe defect in growth, so we were unable to assay this mutant.

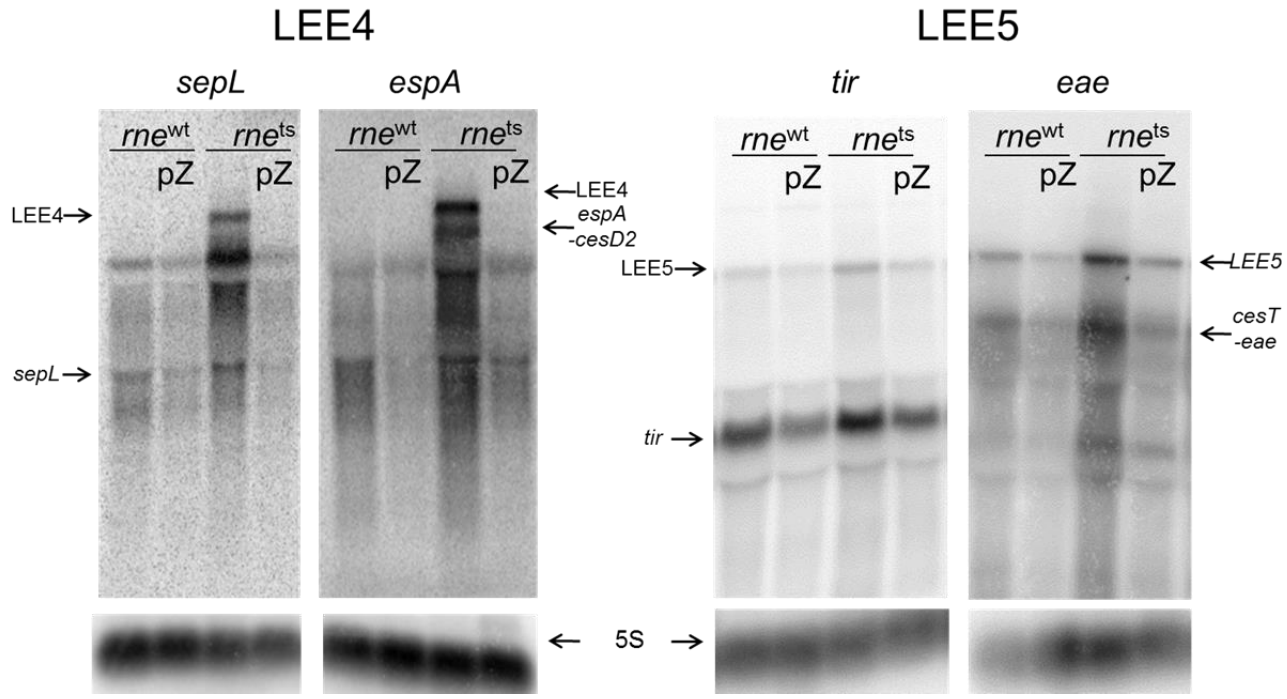

Figure 4.10: GlmY and GlmZ regulation of the *LEE4* operon. Northern blots against *sepL*, *espA*, *tir*, and *eae* with the 5S rRNA serving as a loading control on RNA from heat shocked rne^{wt}, rne^{wt} overexpressing *glmZ*, rne^{ts}, and rne^{ts} overexpressing *glmZ*

DISCUSSION

By asking the question of how an extracellular bacterial pathogen rapidly and precisely coordinates expression of its molecular circuitry to engage expression of an array of genes necessary to encode the molecular structures and effectors that rearrange host actin dynamics, we uncovered three new targets and two different molecular mechanisms of action of the GlmY and GlmZ sRNAs (Figure 4.11). Post-transcriptional regulation allows for more plasticity and rapid responses in gene expression within cells. While research into post-transcriptional regulation of genes in bacteria is a growing field, very little is known about the details of post-transcriptional regulation of virulence genes in pathogens at the bacterial/host interface.

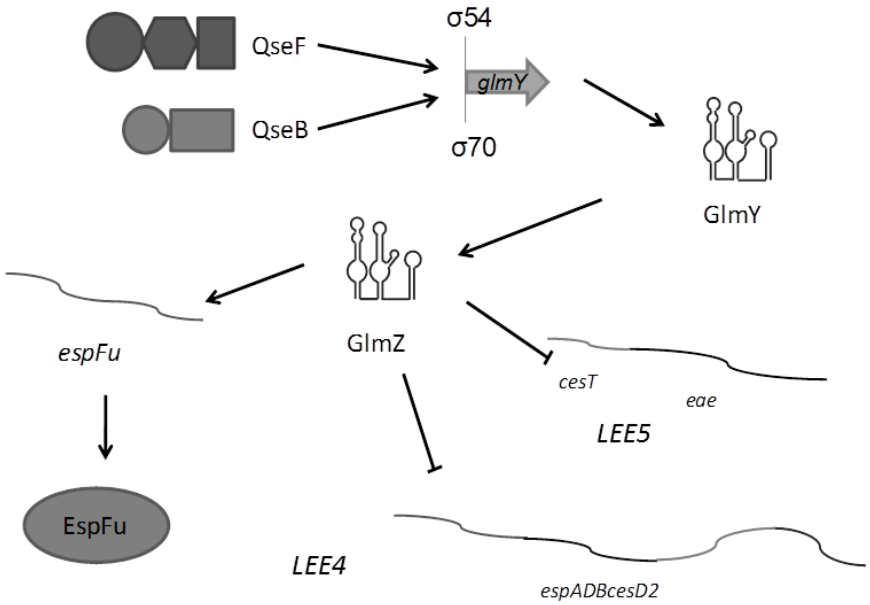

Figure 4.11: Cartoon diagram of the proposed model of GlmY and GlmZ regulation of *LEE4*, *LEE5*, and EspFu. QseB and QseF activate the transcription of GlmY which stabilizes GlmZ. GlmZ activates the translation of *espFu* and destabilizes parts of the *LEE4* and *LEE5* transcripts.

Post transcriptional regulation of bacterial virulence gene expression

Our data establish that both the LEE and *espFu* genes necessary for pedestal formation on epithelial cells are post-transcriptionally regulated by the GlmY/GlmZ sRNAs through two different mechanisms. Several previous reports recognized that the LEE region is highly post-transcriptionally regulated (19, 20, 29, 122, 123, 205). However, no sRNA has yet been shown to be responsible for this regulation. One system known to be involved is the RNA binding protein and global regulator CsrA, which directly binds to the *LEE4* operon, and indirectly regulates a wide array of virulence factors (19). Additionally, the RNA chaperone Hfq is required for the proper expression of many virulence genes (81, 107, 205), suggesting that *trans*-acting sRNAs are involved at some level of regulation. Here we described the first known sRNAs that regulate the LEE, GlmY and GlmZ. Previous to this work, GlmY and GlmZ had only one target, the *glmS* mRNA, and one known molecular mechanism of action. GlmY was described as a molecular mimic of GlmZ, protecting GlmZ from degradation. In the case of *glmS*, GlmZ is the "effector sRNA" base pairing with the *glmS* mRNA to break a hairpin loop to expose the RBS and promote translation of this gene (71, 238). Our data unraveled two new mechanisms of action for these sRNAs. GlmY and Z promote cleavage of the intergenic region between *espJ* and *espFu* to allow translation of EspFu (Figure 4.2). Moreover, through destabilization of the *LEE4* and *LEE5* transcripts, these sRNAs fine tune LEE expression (Figures 4.4, 4.5 and 4.7).

Co-option of a metabolic regulatory pathway

GlmZ is well characterized as the activator of *glmS* translation. This core metabolism regulating sRNA was co-opted to regulate the LEE and *espFu* , both horizontally acquired islands, at some point in evolutionary history. Horizontal acquisition of pathogenicity islands (PAI) contributes to virulence of an organism, allowing exploitation of other niches and hosts for colonization (159). Our results suggest that the interplay between ancient and recent evolutionary acquisitions has shaped EHEC pathogenicity. An inverse example of this phenomenon comes from the InvR sRNA from *Salmonella enterica* (240), where a co-opted sRNA that is adjacent to the SPI-1 pathogenicity island regulates many core chromosomal genes.

While we did not directly observe regulation of *glmS* by GlmZ in any conditions we assayed in EHEC, we have evidence from the Δ*qseF* transcriptome that suggests amino sugar metabolism, and cell wall synthesis are still affected at some level in this mutant (181). The tying of glucosamine synthesis to inter-kingdom chemical signaling and pathogenesis may reflect the need to integrate both host and bacterial physiological cues to ensure a successful association between these organisms. It is also noteworthy that the LEE encoded T3SS must span the perisplasm and pass through the peptidoglycan layer. Since EHEC creates dozens of these injectisomes, there is likely significant remodeling of the cell wall during host infection.

Timing and dynamics of infection

One of the key advantages of post-transcriptional regulation for an organism is it is faster than regulation at the level of transcription, allowing for much finer tuning of gene expression. In the case of GlmY and GlmZ, it also allows for the differential regulation of genes within the *LEE4* and *LEE5* operons. This decoupling of the regulation of the genes of a polycistronic mRNA from each other enables a much more varied pattern of gene expression. EHEC infection of an epithelial cell, is a prolonged and dynamic process with the bacteria growing and replicating while adhering to the pedestal. GlmZ specifically down regulates the downstream portion of the *LEE4* operon, including the filament EspA, the pore EspDB and the needle EscF, while leaving SepL unaffected (Figure 4.9). SepL is an important regulator of the translocation of effectors (48) and is likely required for effector delivery to host cells even when many of the structural proteins of the T3SS translocon are not. The post-transcriptional regulation of *LEE4* mediate by GlmZ enables EHEC to tightly regulate the process of AE lesion formation (Figures 4.2-4.9).

CHAPTER FIVE

Post-transcriptional regulation by GlmY and GlmZ in enterohemorrhagic *E. coli*

INTRODUCTION

Enterohemorrhagic *Escherichia coli* (EHEC) must precisely time the expression of its virulence genes to successfully infect its host and attach to intestinal epithelial cells. From the initial infection event the bacteria must pass through the acidic stomach, traverse the small intestine and then infect the colonic epithelium. This requires the assembly of two complex proteinaceous machines, first the flagella that it uses to cross the mucosal layer and then the LEE T3SS that is required to form the AE lesion. These systems are regulated by the Qse cascade, which also controls the expression of the sRNAs GlmY which stabilizes another sRNA GlmZ. Post-transcriptional control is faster than transcriptional control and is often used to fine tune gene expression. My previous work has shown that GlmY and GlmZ are involved in the post-transcriptional control of selective parts of the LEE with GlmZ directly binding and destabilizing part of *LEE4*. GlmZ also activates the translation of GlmS, glucosamine synthetase. Multiple targets are common in bacteria, and it is possible that GlmZ has other targets. Additionally, there is some evidence that indicates that GlmY has another function besides stabilizing GlmZ.

Trans-encoded sRNAs such as GlmY and GlmZ can act as global regulators in bacteria (236). Identifying these trans-encoded sRNAs has been facilitated due to advances in next generation sequencing technology (194). However, identifying their targets has proven to be more challenging. In some cases, such as GlmY and GlmZ, simple

overexpression of the sRNA was sufficient in revealing a target, as overexpression of either in the strain MC4100 resulted in GlmS being visible on an Coomassie stained protein gel (238). Bioinformatics have proven useful in many cases, but there are limitations. Many of the more effective approaches use evolutionary conservation of sequences to find targets (242). While this works well for core metabolism and other highly conserved genes, for horizontally acquired genes such as pathogenicity islands, there usually are too few species to make this method effective. In cases such as this, global analysis of the transcriptome is often more effective to determine the targets of sRNAs. The aim of this work is to use RNA sequencing and microarray technology to identify other GlmY and GlmZ targets in EHEC.

RESULTS

Global targets of GlmY and GlmZ

In order to determine other GlmY and GlmZ targets, several transcriptome analyses were performed. Transcripts from wt, Δ*glmY*, and Δ*glmZ* were analyzed by RNA sequencing. The Nugene Ovation RNA-Seq System was used in conjunction with the Nugene RNA Encore Complete RNA-Seq system to create a library enriched in mRNA transcripts that was sequenced on an Ilumina machine. This resulted in ~40 million paired reads per sample that were then analyzed using Rockhopper (131).

Many genes of the LEE pathogenicity island were upregulated in Δ*glmY* and Δ*glmZ*, with *ler* being an interesting exception in Δ*glmZ* (Figure 5.1A). A few of the *mur* cell wall synthesis genes were affected in the RNAseq data, but failed to be confirmed by qPCR

(Figure 5.1B). It was expected that some of these would be regulated due to GlmZ's interaction with GlmS. Many stress-related genes such as the gene encoding the osmotic stress protein OsmY and the acid resistance *gad* genes were down regulated in both mutants. The fucose utilization genes, which have been associated with virulence regulation in EHEC (165), were all upregulated. The *tnaLAB* tryptophanase operon was also very strongly regulated (Figure 5.1A).

Two of the most strongly regulated genes were the outer membrane protein *nlpD* and the alternative sigma factor *rpoS* (Figure 5.1B). The *rpoS* promoter overlaps with the open reading frame of *nlpD*, and the alignment of reads to the genome showed that this difference is due to this promoter and not regulation of *nlpD* itself (Figure 5.1C).

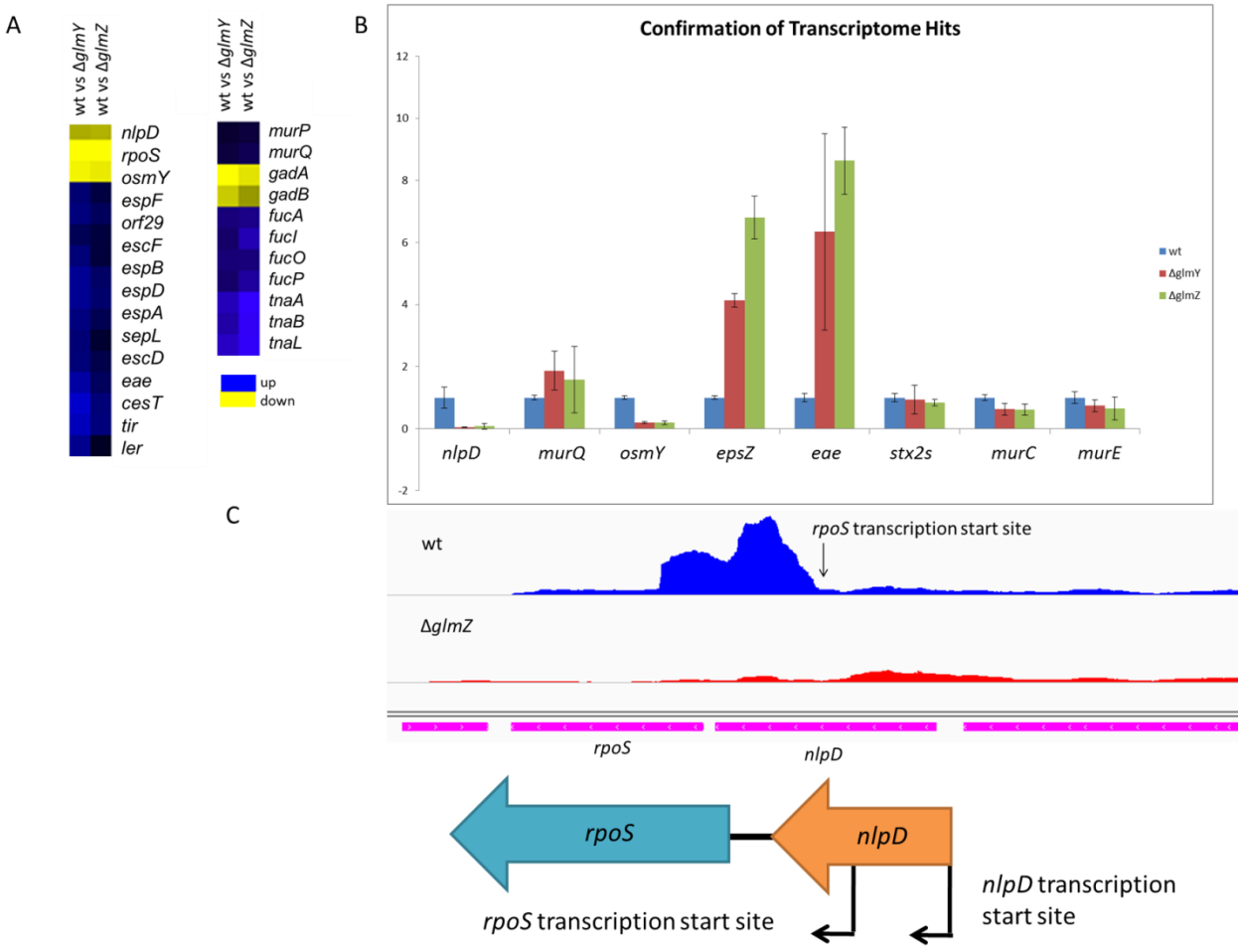

Figure 5.1: RNA seq for Δ*glmY* and Δ*glmZ*. (A) Heat maps of stress proteins, virulence genes fucose utilization genes, cell wall metabolism. (B) qPCR of various hits from the RNA seq data. The stress protein *osmY* was confirmed, however none of the cell wall metabolism genes were affected. (C) Alignment of RNA seq reads to the genome showing that it is *rpoS*, not *nlpD* that is differentially regulated in the Δ*glmZ* strain.

RpoS

The resultant pattern of expression closely matched the transcriptome pattern previously reported for an *rpoS* knockout in the EHEC strain EDL933, suggesting that many, if not most, of the difference in gene expression seen in the RNA seq data may be due to regulation of *rpoS* (50). RpoS is regulated post-transcriptionally by three separate trans-encoded sRNAs, OxyS, RprA, and DsrA (12). It is regulated transcriptionally by the ArcA-ArcB and BarA-UvrY two component systems and Crp. All of these were unaffected in the Δ*glmY* and Δ*glmz* microarrays, as were their known transcriptional regulators.

To further investigate this, RpoS transcriptional and translation fusions were constructed. The transcriptional fusion proved to be nonviable in EHEC, however the

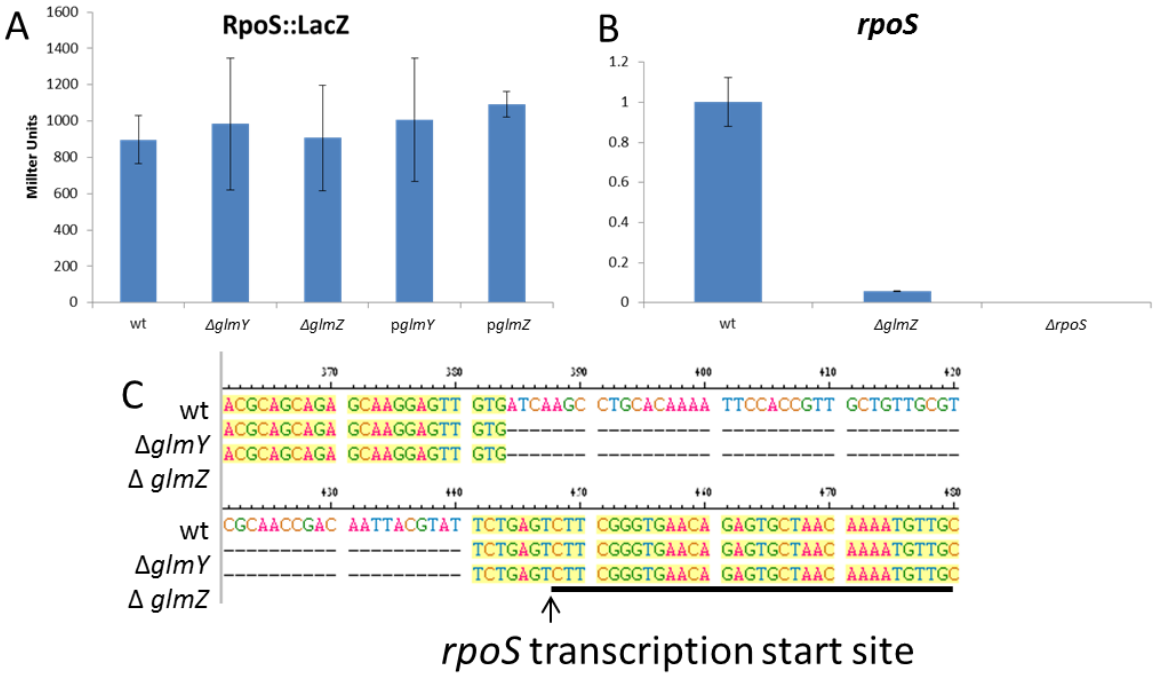

Figure 5.2: Regulation of *rpoS* in ΔglmYZ. (A) Translational reporter fusion of RpoS::LacZ in wt, Δ*glmY*, Δ*glmZ*, p*glmY*, and p*glmZ* showing no difference in expression levels (B) qPCR of *rpoS* levels in wt, Δ*glmZ*, and Δ*rpoS*. RpoS levels in the *glmZ* mutant are down 20-fold and for comparison are down 10,000-fold in the *rpoS* mutant. (C) Sequence alignment of the *rpoS* promoter region from wt 86-24, Δ*glmY*, and Δ*glmZ* shows the same 57bp deletion in both mutants.

expression of a RpoS:LacZ translation reporter was unaffected in both the *glmY* and *glmZ* knockouts and overexpression of the two sRNAs (Figure 5.2A). The *rpoS* gene and promoter often spontaneously undergo mutagenesis. To test for this possibility, the entire region was sequenced from wt 86-24 and the Δ*glmY* and Δ*glmZ* strains (Figure 5.2C). Both sRNA knockout strains possess the same 57bp deletion. This deletion covers the -35 and -10 sequences, effectively destroying the *rpoS* promoter. However, *rpoS* is also transcribed from

the *nlpD* promoter, resulting in a knockdown phenotype in the Δ*glmYZ* strains with approximately 5% of wildtype levels (Figure 5.2B).

Since the Δ*glmY* and *Z* are also *rpoS* knockdowns, it is necessary to determine which of the phenotypes observed are due to a deficit of *rpoS* and which are due to the lack of the sRNAs. FAS of the *rpoS* mutant was performed and it over attaches to HeLa cells in a phenotype similar to the strain Δ*glmZ*. (Figure 5.3A). qPCR comparing the expression of virulence genes between Δ*glmZ* and Δ*rpoS* was also performed. The expression levels of *espA*, *tir*, and *eae* were higher in both strains; however, the levels in Δ*rpoS* were far higher than in the *glmZ* mutant, while *stx2* levels were unaffected in both (Figure 5.3B). Interestingly, expression of the gene encoding the LEE master regulator differs between the Δ*glmZ* and Δ*rpoS*. Expression of *ler* was elevated in the Δ*rpoS* strain, but there was no statistically significant increase in Δ*glmZ* (Figure 5.3B). These data, coupled with the observation that the *glmZ* mutant can be complemented by providing GlmZ *in trans* (Figure 5.3A), suggests that the Δ*glmZ* and Δ*rpoS* phenotypes are partially overlapping, and that post-transcriptional regulation of *LEE4* and *LEE5* by GlmZ does not involve RpoS.

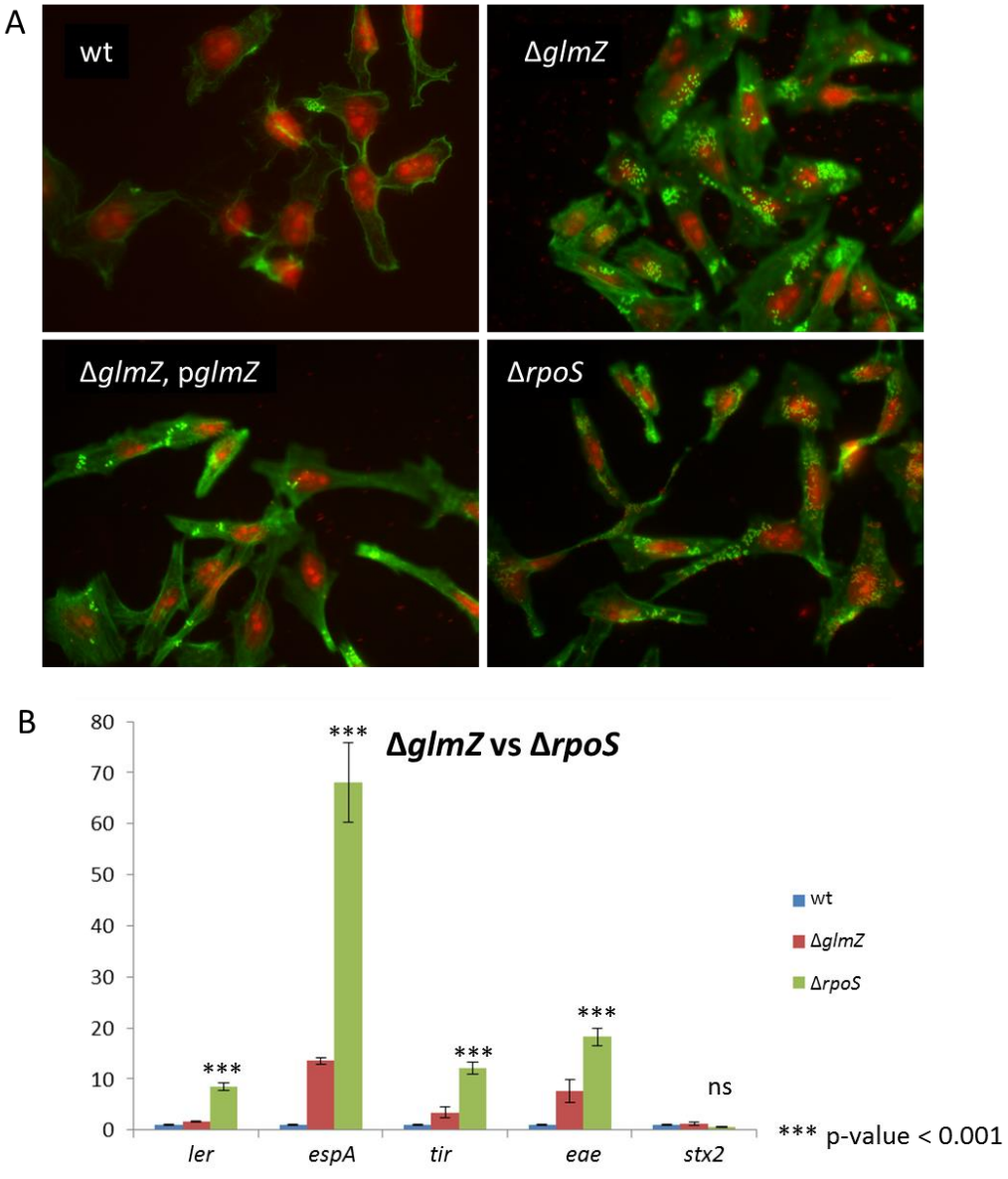

Figure 5.3: Comparison of ΔglmZ and ΔrpoS. (A) FAS of wt EHEC, of Δ*glmZ*, Δ*glmZ* p*glmZ* and Δ*rpoS*. The Δ*glmZ* and Δ*rpoS* strains both overattach to HeLa cells, however ΔglmZ is fully complementable (B) q-rtPCR of virulence gene expression in Δ*glmZ* and Δ*rpoS*. l*er* is stastically unchanged in Δ*glmZ* and up in Δ*rpoS*. *espA*, *tir*, and *eae* are up in both mutants, although significantly more in the Δ*rpoS* strain. *Stx2* expression is unchanged

In order to determine which of the targets identified from the RNA seq transcriptome are actually due to Δ*glmY* and Δ*glmZ* or the *rpoS* knockdown, we used Affymetrix GeneChip *E. coli* Genome 2.0 arrays to compare the transcripts of Δ*glmY* and Δ*glmZ* to Δ*rpoS*, and wt to Δ*rpoS*. The global effects are summarized in Table 5.1. There are significant differences between both ΔglmY and ΔglmZ and ΔrpoS suggesting that many of these GlmY and GlmZ regulated genes are not due to the knockdown of *rpoS* in the *glmY* or *glmZ* mutants. Hence,

Comparison	Δ*glmY* vs Δ*rpoS*	Δ*glmZ* vs Δ*rpoS*	Δ*glmY* vs Δ*glmZ*	wt vs Δ*rpoS*
Increase	781	669	676	1121
Marginally Increase	61	42	415	62
No Change	8281	7848	8399	7779
Marginally Decrease	72	93	58	61
Decrease	1013	1556	1034	1195
Differential Expression	1927	2360	1809	2429

Table 5.1: Table of changes in gene expression in Δ*glmY* and Δ*glmZ* vs Δ*rpoS*

certain genes seem to be regulated by GlmY and GlmZ and not *rpoS* (Figure 5.4A). The annotation file format used by Rockhopper excludes sRNAs, whereas the *E. coli* 2.0 array does not. The Δ*glmY* vs Δ*glmZ* microarray data revealed that many ncRNAs are enriched in Δ*glmY* (Figure 5.4B). GlmY is already known to stabilize GlmZ due to its interaction with RapZ. It's possible that GlmY regulates other sRNAs as well. Additionally, the maltose ABC transporter seems to be differentially regulated by GlmY and GlmZ, with mRNA levels being much higher in Δ*glmY* (Figure 5.4C).

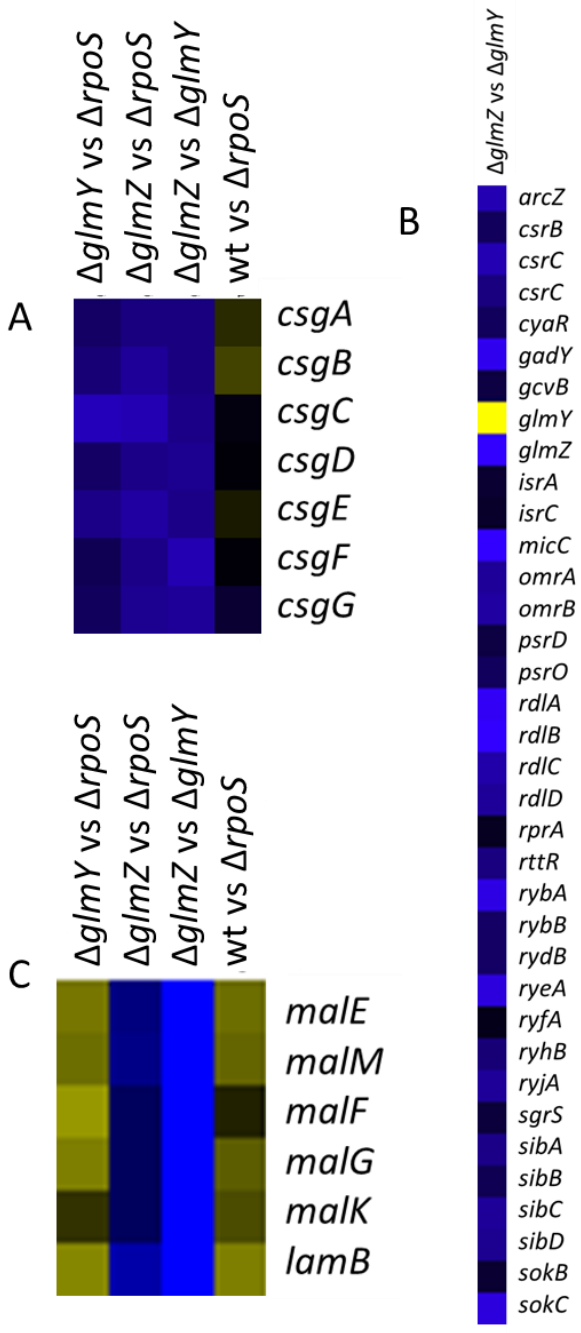

Figure 5.4: Heatmaps of ΔglmY and Δ*glmZ* vs Δ*rpoS*. (A) Curli genes are down in Δ*glmY* and Δ*glmZ* but not Δ*rpoS*. (B) Various sRNAs are up regulated in Δ*glmY*. (C) The maltose ABC transported is differentially regulated by GlmY and GlmZ

Regulation of Curli by GlmZ

One of the major pathways that the microarray data indicated was regulated in a *rpoS* independent manner by GlmY and GlmZ was curli, a major class of adhesion molecules that are involved in bacterial attachment to abiotic surfaces, biofilm formation, and attachment to host cells (12, 170). The curli genes are regulated by the transcription factor CsgD that activates the transcription of the other *csg* operons. The other components are exported to the perisplasm through the sec secretion system. CsgE and CsgF stabilize the other subunits and transport them to CsgG which exports them out of the cell. Then the major subunit, CsgA, is assembled by the homologous protein CsgB (Figure 5.5A) (12).

Expression of *csgE* and *csgF* was measured by qPCR (Figure 5.5B). While there was no difference the Δ*rpoS* strain in the transcript, levels of both genes were decreased in the *glmZ* mutant congruent with the microarray data. Curli are capable of binding to the Congo red dye. Cells that express curli at high levels turn bright red on Congo red plates while those that cannot express curli remain white. This is a convenient assay for the detection of functional curli on bacteria cells. Wt EHEC, Δ*glmY*, Δ*glmZ* and Δ*rpoS* were plated on Congo red plates (Figure 5.5C). Contrary to the qPCR data, the Δ*glmZ* strain appears to make more curli than wt EHEC. Interestingly, the Δ*rpoS* mutant is deficient in curli production, as previously published in K12 (79), which also conflicts with the qPCR data. In *E. coli* K12, the promoters for both operons are σ^S promoters and the sequence identity is 99%. The discrepancy between the two readouts may be due to post-transcriptional regulation.

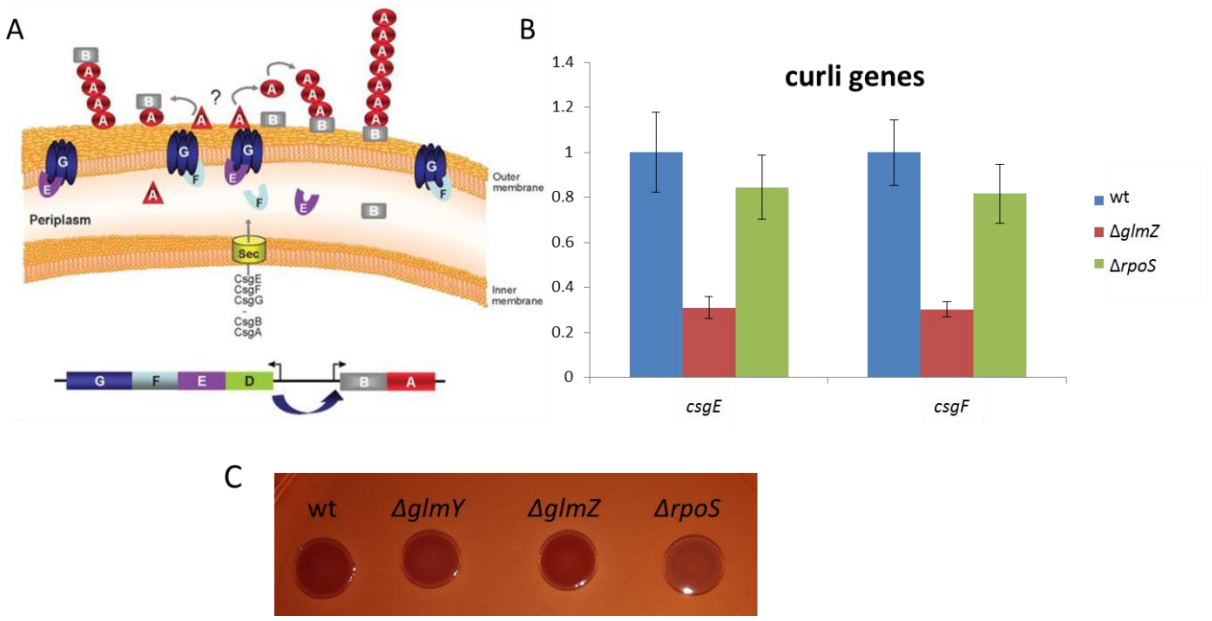

Figure 5.5: Regulation of curli by GlmYZ. (A) Diagram of curli in *E. coli* (12). (B) qPCR of curli gene expression in wt, Δ*glmZ*, and Δ*rpoS*. Expression of *csgEF* is decreased in Δ*glmZ* but not in Δ*rpoS*. (C) Congo red plate of wt, Δ*glmY*, Δ*glmZ*, and Δ*rpoS*. Curli production is visible and unchanged in Δ*glmY,* increased in Δ*glmZ*, and decreased in Δ*rpoS*.

Regulation of NleA

NleA is a non-LEE encoded type three secreted effector protein that disrupts the tight junctions of epithelial cells, which has been shown to be important for virulence in the *Citrobacter* animal model (75). While *nleA* is *rpoS* regulated according to microarray data, it was not significantly regulated our transcriptome data did not suggest it was regulated by GlmY or GlmZ independently of RpoS. In order to determine if the *nleA* regulation was solely due to the effects of *rpoS*, qPCR of the gene was performed in EHEC overexpressing the *glmY* or *glmZ* sRNA (Figure 5.6A). Expression of the *nleA* gene was decreased when either sRNA was overexpressed.

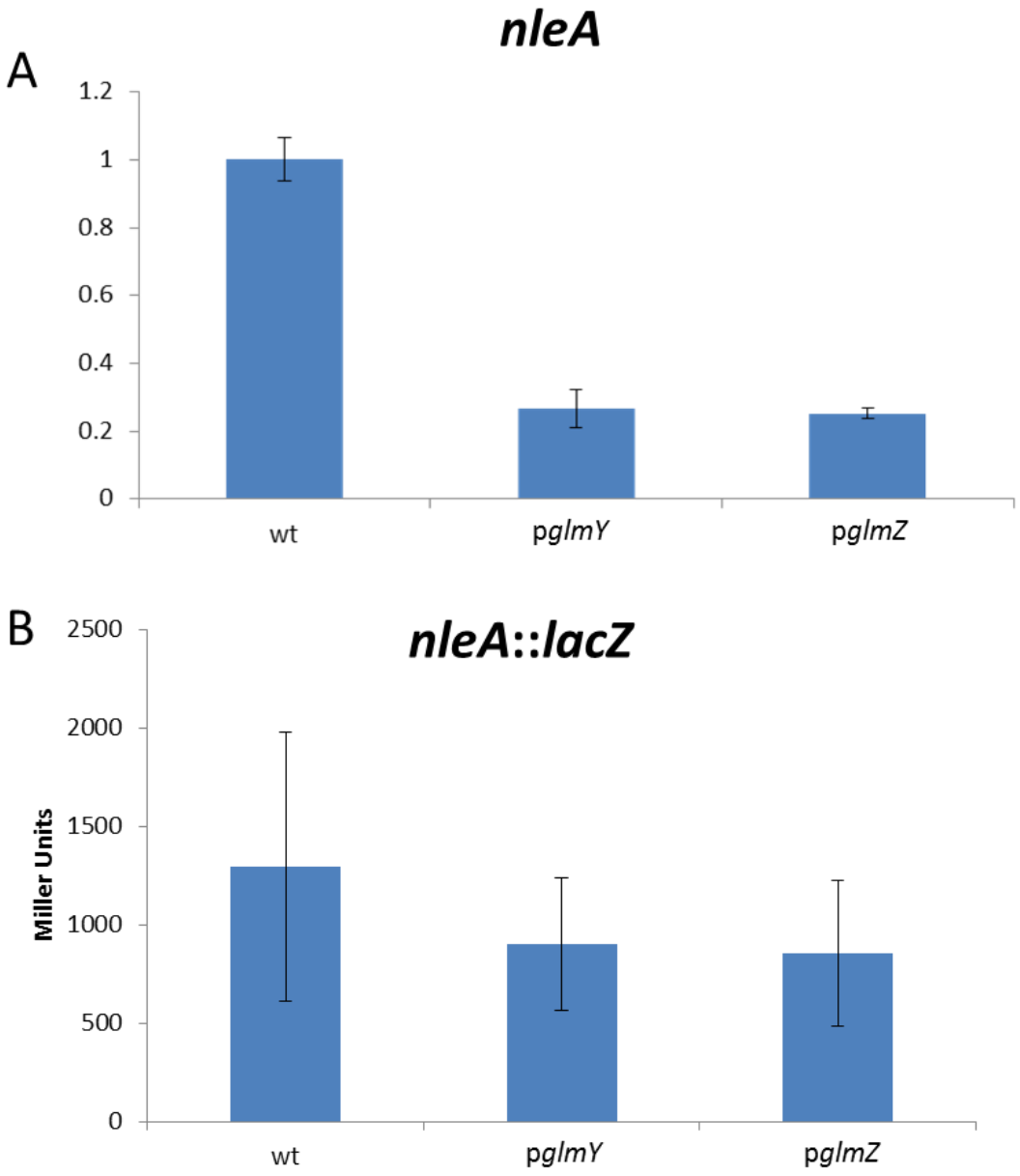

Figure 5.5: Regulation of NleA by GlmYZ. (A) qPCR of *nleA* expression in wt EHEC and strains over expressing *glmY* or *glmZ*. Overexpression of either sRNA results in decreased levels of *nleA* (B) Transcriptional *nleA* fusion in cells overexpressing *glmY* or *glmZ*. LacZ expression is unaffected by overexpression.

An *nleA*::*lacZ* transcriptional fusion was constructed and used to determine if the difference in the expression levels was due to effects at the level of transcription, and overexpression of *glmY* and *glmZ* had no effect. GlmY and GlmZ are likely working at the post-transcriptional level, although it may be an indirect effect.

DISCUSSION

E. coli is estimated to encode 200 small RNAs, with 55 being experimentally confirmed (86, 241). For comparison, two-component systems are considered one of the major regulator systems in bacteria and *E. coli* has only 36 (163). These sRNAs represent a major source of regulation that is largely unexplored. Most studies into *E. coli* sRNAs have used a K12 strain, so regulation of virulence genes present in pathogenic strains such as EHEC has been neglected. GlmY and GlmZ have been shown to directly regulate *glmS* (238) and *LEE4* and also *LEE5* and *espFu*. In this study we investigated the global role of GlmY and GlmZ in EHEC gene regulation.

GlmYZ and RpoS

The discovery that both Δ*glmY* and Δ*glmZ* have a deletion in the *rpoS* promoter is intriguing. While mutations in the *rpoS* gene are not uncommon, the fact that both mutants have the exact same deletion that is absent in the parent strain suggests that there is significant selection against overt *rpoS* expression in these mutants.

RpoS mutants are much more likely to become persister cells, bacterial cells that are extremely resistant to bacteriostatic antibiotics, such as those that affect the cell wall synthesis (89). Given the known role of GlmY and GlmZ in amino sugar metabolism and thus cell wall formation, it is possible that without these sRNAs the bacterial cell is under a similar form of stress at some point during their *in vitro* growth. RpoS is also known to be involved in the regulation of the LEE and other virulence genes (50). It is possible that it works in concert with GlmY and GlmZ. Both of these sRNAs are reported to be more abundant during stationary phase, suggesting that there may be a complex interplay between these regulators of virulence.

GlmY without GlmZ

To date, GlmY has only been reported to act by binding to RapZ and preventing the degradation of GlmZ (71). We previously found evidence that GlmY is capable of acting independently, as it was capable of partially complementing a Δ*glmZ* strain. However, GlmY does not bind Hfq, so it is unknown how it would directly act on any target.

Our RNAseq and microarray data show that the Δ*glmY* transcriptome has several major differences from that of Δ*glmZ*. Interestingly, several sRNAs are much more abundant in the Δ*glmY*. GlmY is known to stabilize GlmZ. These data suggest it is involved in the regulation of other sRNAs through a still unknown mechanism. The regulation of the maltose ABC transporter system is vastly different between Δ*glmY* and Δ*glmZ*, its expression is increased in Δ*glmY* and decreased in Δ*glmZ*. The glucose polymers that would result in

free maltose are a favored energy source for *E. coli* and are present in the host. Additionally, the maltose porin LamB is the lambda phage receptor. It is unclear why this system would be differentially regulated by two sRNAs in the same pathway.

Regulation of Other Virulence Genes by GlmYZ

The curli genes in EHEC have been implicated in the attachment of the bacteria to abiotic surfaces, plants, and host cells (113, 170). These genes have been reported to be regulated by RpoS in K12 (176). However, we did not observe *rpoS*-dependent regulation of *csgEF* during our assays. Instead we observed that transcript levels of *csgEF* were decreased in Δ*glmY* and Δ*glmZ*, but unchanged in Δ*rpoS*. Conversely, we observed increased curli formation on Congo red plates in Δ*glmZ*, and the loss of culi in the Δ*rpoS*. CsgEF are required for the formation of curli (152), suggesting that post-transcriptional control of curli transcripts results in differences in protein and mRNA levels.

EHEC has an estimated 51 effector proteins, only 6 of which are encoded within the LEE itself (231). While the LEE is the largest and most important pathogenicity island, EHEC also has several O-islands that encode for other virulence proteins such as the non-LEE encoded effectors. We have shown that GlmY and GlmZ regulate at least one of these effectors, NleA, and that this regulation is post-transcriptional. This raises the possibility that GlmYZ are global regulators of virulence in EHEC. Other O-island encoded genes will need to be investigated.

CHAPTER SIX

Conclusions and Future Directions

CONCLUSIONS

This work was focused on increasing our understanding of the downstream targets of the QseC and QseE signaling pathway, specifically the response regulator QseF. QseF was reported to activate the transcription of the gene encoding the sRNA GlmY, and we determined that the other Qse response regulator QseB also activates its transcription. GlmY is known to bind to RapZ and displace another sRNA, GlmZ preventing its degradation. GlmZ subsequently activates the translation of GlmS, glucosamine synthetase (71, 100, 184, 238). We showed that GlmY and GlmZ also regulate the translation of the effector *espFu* and post-transcriptionally regulated the *LEE4* and *LEE5* mRNA, with GlmZ directly targeting *LEE4*. While investigating the global regulation of GlmYZ using RNA sequencing, we determined that both knockouts had a mutation in *rpoS* promoter region, creating a knockdown effect, suggesting a connection between *rpoS* and *glmYZ*. We then showed that *glmY* and *glmZ* regulate expression of curli and the effector *nleA* post-transcriptionally. We also identified differences between Δ*glmY* and Δ*glmZ*, specifically regarding the expression of various sRNAs and the maltose ABC transporter. Our data suggest that GlmYZ may be a global regulator of virulence in EHEC.

The role of RpoS in GlmYZ regulation

The alternative sigma factor RpoS controls a vast array of genes involved in cell survival in stationary phase, biofilm formation, metabolism, and virulence (14, 50). Mutants

for *rpoS* are common in both environmental and laboratory populations of *E. coli* and they often have a selective advantage under conditions of nutrient limitation. However *rpoS* mutants perform poorly during other stress conditions such as acid stress (157). Since both the *glmY* and *glmZ* mutants have a conserved deletion in the *rpoS* promoter, it is likely that the *glmY* and *glmZ* mutants nonviable when *rpoS* is expressed at normal levels. This may be due to the enhanced ability of *rpoS* mutants to enter a persister state (89). Persister cells are in a largely dormant state, and are capable of surviving antibiotic treatment (204). As *glmYZ* are known to regulate *glmS* and thus amino sugar metabolism and cell wall biogenesis, it is possible that the lack of these sRNAs causes a similar stress to the cell. While we did not observe *glmS* regulation by GlmZ in the growth conditions we tested in EHEC, the sequence of GlmZ and *glmS* that interact are identical and the *qseF* array (181) suggests it still occurs at some point during the growth phase (181). An alternative possibility that is equally supported by our data is that *glmYZ* and *rpoS* have overlapping regulation, and that in the absence of *glmY* or *glmZ*, *rpoS* is toxic due to other factors. The *glmZ* mutant, which contains the *rpoS* promoter deletion, was complemented by *glmZ* on a plasmid, which suggests that GlmZ has RpoS-independent functions.

Post transcriptional regulation of bacterial virulence genes

We have shown that the required genes for pedestal formation are post-transcriptionally regulated by GlmYZ. The *espJ-espFu* mRNA undergoes a processing event in a manner somewhat reminiscent of the *gadWX* operon (161, 234). This processing event

requires GlmYZ and is needed for efficient expression of *espFu*. Additionally, the latter fragments of both *LEE4* and *LEE5* operons are downregulated by GlmYZ, which in the case of *LEE4* is due to direct binding of GlmZ to the *orf29* region of *LEE4*. GlmYZ are also able to downregulate the expression of the effector *nleA* in a post-transcriptional manner. The curli genes also seem to be regulated by GlmY and GlmZ. The transcript levels of curli genes are decreased in the *glmYZ* mutants in both the microarray data and according to qPCR; however, according to Congo red plates, curli production is increased. There are multiple possibilities for this discrepancy; there could be post-transcriptional regulation or differences in gene expression due to different growth conditions, static cultures vs. plates. It is also possible that these sRNAs are involved in the timing of curli expression, decreasing translation once enough curli have been expressed, and perhaps promoting stabilization of curli proteins through regulation of chaperones. These data all suggest that GlmYZ play an important role in virulence gene regulation (Figure 6.1).

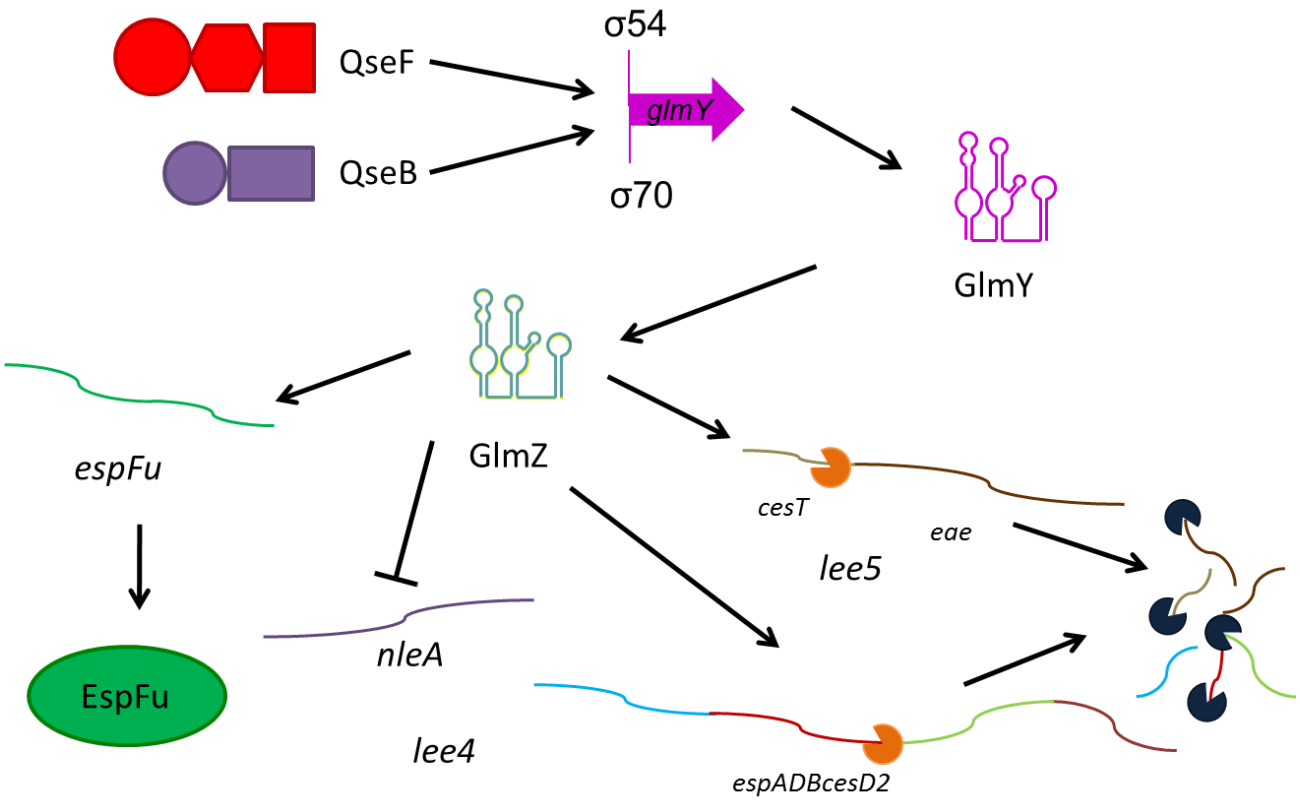

Figure 6.1: Virulence gene regulation by GlmYZ. Cartoon diagram of the proposed model of GlmY and GlmZ regulation of LEE4, LEE5, NleA and EspFu. QseB and QseF activate transcription of GlmY which stabilizes GlmZ. GlmZ activates the translation of *espFu* and destabilizes *LEE4* and *LEE5*. GlmY and GlmZ also downregulate *nleA* post-transcriptionally and are involved in the regulation of curli expression.

GlmY vs. GlmZ

Currently, the only published role for GlmY is to act as an anti-adapter RNA to prevent GlmZ degradation (71). GlmY and GlmZ are both conserved in other enterobacteria, suggesting that this pathway is also conserved (70). This system may exist to allow for multiple levels of control to regulate GlmZ function. Alternatively GlmY may have a function apart from GlmZ. Our transcriptome data show that there are many differences in gene expression between Δ*glmY* and Δ*glmZ*. The most strongly differentially regulated

genes are those of the maltose ABC transporter, which are slightly increased in Δ*glmY* and strongly decreased in Δ*glmZ*. This system is responsible for the uptake of α(1→4) glucose polymers of 7-8 monomers in length, which are degradation products of many common polysaccharides including amylose and glycogen. The maltose porin, LamB, is also the receptor for the lambda phage (21). It is currently unclear why or how *glmY* would regulate these genes. The known target of the GlmY/Z pathway, *glmS*, is involved in sugar metabolism, so this may be the link with maltodextrins. Trans-acting sRNAs such as GlmZ lack perfect sequence complementarity and require the RNA chaperone Hfq to act. GlmY is incapable of binding Hfq (47), so its mechanism of action is likely distinct from other reported sRNAs.

Our microarray data also indicate that many sRNAs are differentially regulated between Δ*glmY* and Δ*glmZ*. The only known function of GlmY is to stabilize GlmZ, however our data show that other sRNAs are destabilized in the absence of GlmY. The RNase E binding protein RapZ is responsible for the degradation of GlmZ has only been found to bind to GlmY and GlmZ (71). This suggests that the mechanism that GlmY uses to affect the stability of other sRNAs is potentially through a different mechanism. All of these data, along with our finding that GlmY can partially complement a *glmZ* KO (Figure 4.4B) show that *glmY* can act through a *glmZ* independent mechanism.

Tying metabolism to virulence

The activation of translation of GlmS by GlmZ is well studied. The regulation of this essentially metabolic pathway was co-opted to regulate horizontally acquired virulence genes. To be useful to the cell, these newly acquired genes must be integrated into the bacteria's genetic machinery. There are many known examples of core metabolism transcription factors that have been coopted to regulate virulence genes (10), but GlmY and GlmZ are the first reported sRNAs to do so.

While we did not find any direct evidence of *glmS* regulation in EHEC under the growth conditions we tested, microarray data from Δ*qseF* suggests that it does occur. Additionally, our results also tie this system to maltose uptake. Metabolism and virulence are intimately intertwined in EHEC and other pathogens (155, 156, 165), and GlmYZ are another example of this. In this case the bacteria tied both glucosamine and pathogenesis to interkingdom signaling. This may be advantageous for EHEC since the LEE T3SS must cross the perisplasm and the peptidoglycan cell wall, which would require significant remodeling of the cell wall.

Timing is everything

Post-transcriptional regulation has many advantages. It is faster than transcriptional control and it allows for differential regulation of different genes within an operon. This uncoupling of transcription from gene expression allows for greater control of polycistronic mRNAs. We showed that GlmY and GlmZ specifically down regulate parts of the *LEE4* and *LEE5* operons. GlmZ destabilize the portions of *LEE4* that encode the EspA filament, the

EspDB, and the EscF needle while leaving SepL unaffected. In the case of *LEE5*, GlmY and GlmZ downregulate the chaperone CesT and the adhesin intimin while leaving the translocated intimin receptor Tir alone. In both cases, the genes that are downregulated are likely genes that are needed earlier in the infection. For *LEE5*, CesT and intimin both stay associated with the bacteria cell while Tir enters the host cell. In the case of *LEE4*, EspADB and EscF are required for the assembly of the needle and the translocon while SepL is required for the regulation of effector secretion. The process of infecting an epithelial cell and forming an AE lesion requires tight control of several layers of gene expression and the post-transcriptional regulation of these two operons is likely responsible for the fine tuning of this process.

GlmY is regulated by the quorum sensing QseEC kinase systems, and previous work has shown that this system is central to the regulation of virulence genes in EHEC. (37, 38, 90, 154, 156, 182, 219). My work has added the GlmYZ sRNA cascade to this regulon (Figure 6.2).

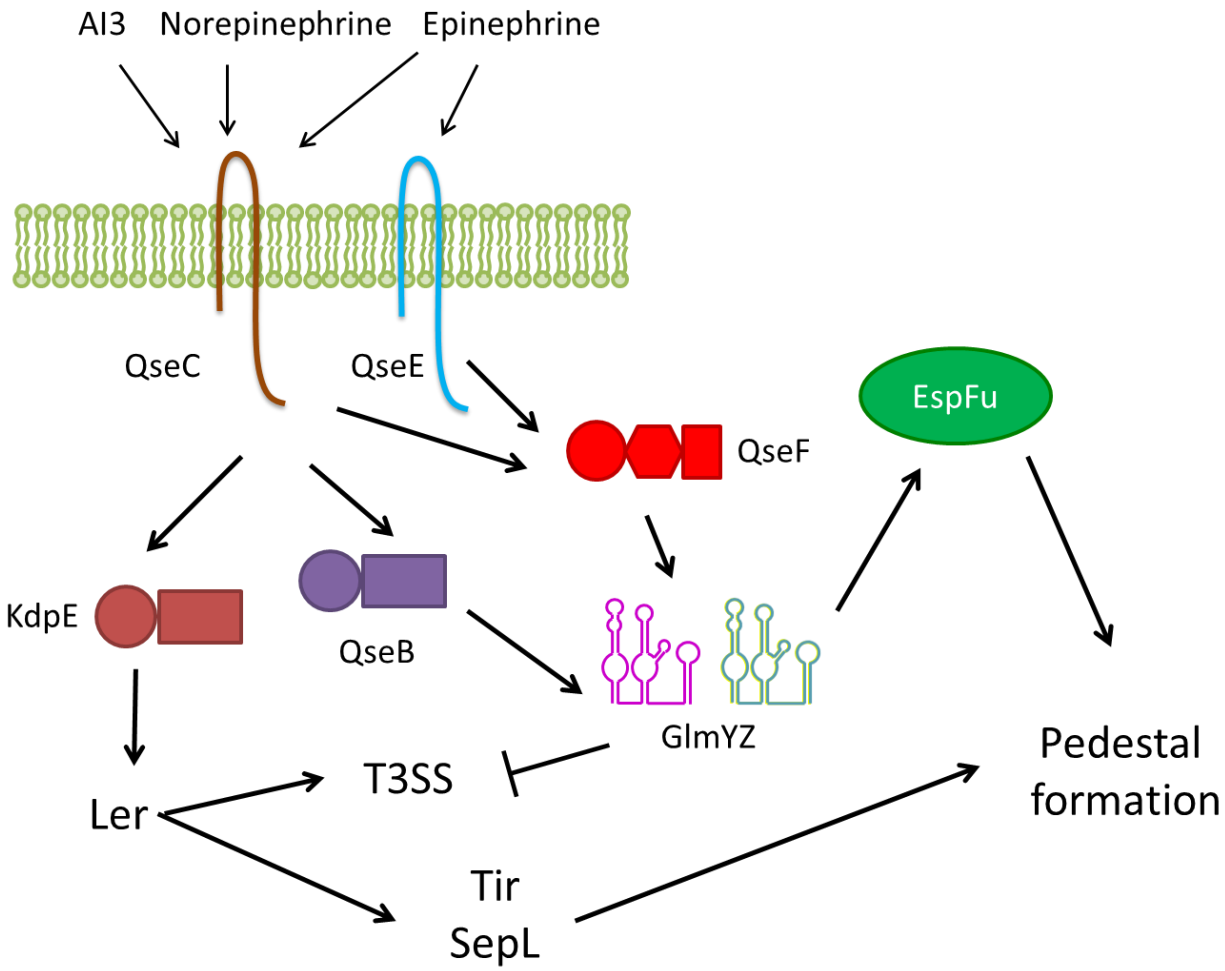

Figure 6.2: QseEC cascade and GlmYZ. Cartoon model of cell to cell signaling regulation of virulence genes through GlmYZ. QseC and QseE sense bacterial and host derived signaling molecules and phosphorylate three different response regulators, KdpE, QseB, and QseF. KdpE activates expression of *ler*, which then activates the rest of the LEE, forming the T3SS. QseB and QseF both activate the transcription of GlmY and thus increase the levels of GlmZ. GlmZ activates the translation of *espFu*, which with the rest of the T3SS is required for pedestal formation. GlmZ also destabilizes the mRNAs for the structural genes of the LEE, selectively turning off gene expression.

FUTURE DIRECTIONS

While my studies have shown that GlmY and GlmZ are important regulators of virulence in EHEC, there is much work that still needs to be done. While QseB and QseF both regulate *glmY*, there is nothing known about the transcriptional regulation of *glmZ*, except that QseF and QseB are not involved. Additionally, the factors that lead to the accumulation of *glmY* in glucosamine limiting conditions are also unknown, as are regulators of *rapZ*. To fully understand the context of GlmY and GlmZ regulation, more information on the regulation of this pathway is needed.

We discovered that the Δ*glmY* and Δ*glmZ* both contained the same deletion in the *rpoS* promoter region. This is extremely unlikely to have happened by chance, so there must be some very strong selection for this. To test this, we will need to restore *rpoS* to wildtype levels in the Δ*glmY* and Δ*glmZ* strains, and determine if they have a growth defect or are viable. If expression of *rpoS* does not kill these bacteria, it would also be interesting to attempt to recreate these mutants and isolate clones that do not have this mutation. The Δ*rpoS* phenotype has some similarities to that of Δ*glmYZ*, but there are also differences between the two that need to be further investigated. Since *glmZ* on a plasmid complemented all of the *glmZ* mutant phenotypes assessed, this suggests that there several GlmZ targets are independent of RpoS. Additionally, since the *glmY* and *glmZ* mutants do express some *rpoS* from the *nlpD* promoter, a construct that mimics this level of expression should be constructed and used to complement the *rpoS* mutant. These data suggest the intriguing possibility that these sRNAs are important for the proper function of the *rpoS* regulon, and that in the absence of theses sRNAs, overt RpoS expression is deleterious to the bacteria cell.

Our data have raised the possibility that GlmY has a role independent of GlmZ. The FAS data of the *glmY* and *glmZ* mutants first suggested this as overexpressing *glmY* partially complemented the Δ*glmZ* phenotype. Overexpression of Hfq-binding sRNAs often have pleiotropic effects due to the sequestration of Hfq. However GlmY cannot bind Hfq, making this scenario unlikely. The regulation of the maltose operon provides another example of GlmY being involved in sugar metabolism in a GlmZ independent manner. More work is needed to determine if this is a direct interaction, and if it is at the transcriptional or translational level. The effect of *glmY* on many other sRNAs also suggests that *glmY* may act as a global regulator of other sRNAs. The only known way to affect so many sRNAs at once is to affect Hfq (47), because in its absence most trans-acting sRNAs are destabilized. Hence the effect of GlmY on the transcription and translation of Hfq needs to be assessed.

We showed that GlmY and GlmZ regulate virulence genes in EHEC. The *espJ-espFu* mRNA is processed in a QseF dependent manner, and its expression can be restored through overexpression of *glmY* or *glmZ*. Investigations into the regulation of *espFu* are complicated by the extremely low expression of this effector. The only way this protein can be detected by western blot is in an artificial system where the pUC plasmid is used to have several hundred copies of the gene per cell. An enzymatic reporter system such as LacZ may be better suited to study *espFu*. We determined that GlmZ directly binds to *LEE4*; however we have not identified the ribonuclease responsible. RNase E, the primary RNase in *E. coli* had no effect. The secondary RNase, RNase III, has also been known to act on the targets of various sRNAs. We successfully constructed an *rnc* (RNase III) mutant, however while this

gene is not essential, the mutant is extremely ill and has severe growth defects. There is no temperature sensitive allele, so an inducible promoter construct needs to be made so expression of *rnc* can be shut off at will. While we showed that GlmZ regulates *LEE5*, we were unable to find a direct interaction site. Bioinformatics have not worked, so a more brute force approach may be necessary. For example, fragments of the *LEE5* operon can be cloned into a reporter until the region is found. As *glmY* and *glmZ* overexpression downregulate NleA post-transcriptionally, a translation fusion of the gene will need to be made to determine if it is regulated akin to *LEE4* or GlmS. Expression of other non-LEE encoded effectors should also be investigated since the microarray and RNA seq data did not reliably identify NleA as being regulated by GlmYZ. The *rpoS*/*glmYZ* regulation of curli is contradictory. The expression of *csgA*, the major structural protein and *csgD*, the master regulator will need to be investigated. It is possible that this discrepancy is simply due to the differences in gene expression on an agar plates vs. statically at 37ºC and 5% CO_2, or that there is differential regulation of transcript and protein levels by these sRNAs.

To study the role of *glmY* and *glmZ* in pathogenesis in *vivo*, the *Citrobacter rodentium* model could be used. The *Citrobacter* genomic sequence reveals that GlmY and GlmZ are conserved and have the same promoter elements, in contrast to other species such as *Salmonella* where *glmZ* has a σ^{54} promoter (70). The main difficulty would be constructing the knockouts of the genes, preferably avoiding the *rpoS* promoter deletion. This system may function similarly with the *Citrobacter* LEE, making it a useful infection

model. Our data indicate that GlmY and GlmZ are key regulators of virulence in EHEC, and this study has laid the groundwork for future investigations.

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