THE FUNCTIONAL CHARACTERIZATION OF QSEC A BACTERIAL ADRENERGIC RECEPTOR AND THE LUXR HOMOLOGUE SDIA IN EHEC

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DEDICATION

To my wife Amy for her poetry, her ceaseless love, and unwavering support

To my parents David and Sophia Hughes ,
my grandparents JoAnn (MeMe) and Paul Schmidt, Margaret Hughes and Jerry and Dottie Mannarino,
my sisters Alisha and Lauren Hughes and Jenny Caterinacci,
my brother Mark Caterinacci
my Uncle Mike Mannarino
and my second family Cindy, Joe, David, and Erin Meyer.
For all the love, support, and encouragement you have graciously given to me

In memory of my grandfather, Thomas Hughes

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by

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Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 is a human pathogen responsible for outbreaks of hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS). The histidine sensor kinase QseC is an inner membrane adrenergic receptor which responds to the bacterial signal autoinducer-3 (AI-3) and the host signals epinephrine and norepinephrine. EHEC senses these signals in the gut in order to coordinate expression of multiple virulence factors. These factors include the locus of enterocyte effacement (LEE) genes which facilitate attachment and effacement (AE) of the gut epithelium, Shiga toxin (Stx) which causes HUS, and secreted effectors like NleA. We had previously reported that QseC is autoregulatory and regulates the flagellar genes through its cognate response regulator QseB. Here, we examined the global role of QseC in EHEC gene regulation. Microarray analysis of Δ*qseC* along with real time RT-PCR (qPCR) revealed QseC's regulation of Stx, NleA, and Ler, the master regulator of the LEE. Additionally, phosphotransfer studies between QseC and thirty two *E. coli*

response regulators, revealed two new QseC phosphotransfer partners: QseF and KdpE. qPCR confirmed a role for QseC in QseF and KdpE genetic regulation. Additionally, QseC appears to regulate the LEE genes through KdpE and regulates Stx through QseF. Finally, Δ*qseC* and Δ*qseB* do not have the same phenotype. We examined this phenomenon by monitoring the flagellar response in Δ*qseC* and Δ*qseB*. It appears that, QseB plays a dual role in gene regulation based on its phosphorylation state.

We also studied the role of EHEC cell-cell signaling in cattle, the asymptomatic natural reservoirs of EHEC. We have shown that mutation of the LuxR homologue SdiA, decreases EHEC's ability to colonize the bovine intestine. The LuxR proteins are transcription factors that are activated or repressed by the quorum sensing molecules, autoinducer-1 (AI-1) which are N-acyl homoserine lactones (AHL). Generally, in these systems, LuxI synthesizes the AHL that LuxR senses. EHEC does not encode a LuxI homologue, indicating that it can respond to AHLs through SdiA, but cannot produce them. EHEC uses SdiA to sense its environment through other AHL-producing bacteria.

Microarray analysis and qPCR confirmed that, in response to AHL, SdiA represses the transcription of the LEE genes, which encode bovine colonization and human virulence factors. Additionally, electrophoretic mobility shift assays have indicated that SdiA binds the promoter of *ler*.

Previous reports have indicated that glutamate-dependent acid resistance (AR2) is required for EHEC to survive in cattle. qPCR comparing WT EHEC to Δ*sdiA* showed a decreased expression of AR2 genes. When AHL was added to WT EHEC an increase was seen in AR2 gene expression. This effect was absent in Δ*sdiA*. Functional acid

resistance tests have confirmed that SdiA is essential in facilitating acid resistance specifically through the AR2.

Finally, previous reports have indicated that *sdiA* is required for EHEC to survive in cattle. To this end, we have confirmed the presence of AHLs in the bovine rumen and have shown that hydrophobic rumen extracts containing AHLs can decrease LEE gene expression and increase AR2 gene expression. This effect is enhanced in the presence of SdiA.

These findings have led us to compose a more complete picture of adrenergic signaling in EHEC and given us a greater understanding of the role of cell-cell signaling in cattle, the natural reservoir of EHEC.

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LIST OF ABBREVIATIONS

86-24	Enterohemorrhagic *E. coli* wild-type strain 86-24
ACP	Acyl carrier protein
AE	Attaching and effacing
AHL	N-acyl homoserine lactone
AI	Autoinducer
Ala	Alanine
AR1	Acid resistance system 1
AR2	Acid resistance system 2
AR3	Acid resistance system 3
Asp	Aspartate
bp	Base pair
BSA	Bovine serum albumin
C6-HSL	N-Hexanoyl-L-homoserine lactone
C6-oxo-HSL	N-(β-Ketocaproyl)-L-homoserine lactone
C8-HSL	N-Octanoyl-L-homoserine lactone
C8-oxo-HSL	3-Oxooctanoyl-L-homoserine lactone
C8-oxo-HTL	N-(3-Oxooctanyl)-DL-homocysteine thiolactone
CDC	Centers for Disease Control and Prevention
CFU	Colony forming unit
CNS	Central nervous sustem
DAEC	Diffusely adherent *E. coli*
DMEM	Dulbeco's modified Eagle's medium

DMHF	4-hydroxy-2,5-dimethyl-furanone
DNA	Deoxyribonucleic acid
dNTP	dideoxy-nucleotide triphosphate
DPD	4,5-dihydrody-2,3-pentanedione
DTT	Dithiothreitol
EAEC	Enteroaggregative *E. coli*
E. coli	*Escherichia coli*
EDTA	Ethylenediamine-tetraacetic acid
EHEC	Enterohemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EMSA	Electrophoretic mobility shift assay (gel shift)
ENS	Enteric nervous system
EPEC	Enteropathogenic *E. coli*
Epi	Epinephrine
Epi/NE	Epinephrine / norepinephrine
ETEC	Enterotoxigenic *E. Coli*
GABA	γ-amino butyric acid
GI	Gastrointestinal Tract
Glu	Glutamate
GPCR	G-protein coupled receptor
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
His	Histidine
HK	Histidine kinase

HSL	Homoserine lactone
HUS	Hemolytic Uremic Syndrome
Gad	Glutamate dependent acid resistance
IL	Interleukin
IPTG	β-D-thiogalactopyranoside
IR-1	Interleukin-1
IR-10	Interleukin-10
kDa	kiloDalton
LB	Luria-Bertani broth
LEE	Locus of Enterocyte Effacement
Ler	Lee-encoded regulator
LPS	Lipopolysaccharide
Map	Mitochondria associated protein
MFS	Major Facilitator Superfamily
MHF	4-hydroxy-5-methyl-furanone
NCBI	National Center for Biotechnology Information
NE	Norepinephrine
Nle	Non-LEE encoded regulator
NMR	Nuclear magnetic resonance
O.D.	Optical density
PAGE	Polyacrylamide gel electrophoresis
PAI	Pathogenicity Island
PBS	Phosphate-buffered saline

PCR	Polymerase chain reaction
PE	Phentolamine
PNK	Polynucleotide kinase
PO	Propranolol
qPCR	Quantative PCR
QS	Quorum Sensing
Qse	Quorum Sensing *E. coli* Regulator
QseB	Quorum Sensing *E. coli* Regulator B (the response regulator)
QseBC	Quorum Sensing *E. coli* Regulators B and C (the two-component system)
QseC	Quorum Sensing *E. coli* Regulator C (the sensor kinase)
RAJ	Rectal-anal junction
RDEC	Rabbit diarrheagenic *E. coli*
REPEC	Rabbit enteropathogenic *E. coli*
RNA	Ribonucleic acid
RpoA	RNA polymerase subunit A
RR	Response regulator
R-THMF	(2R,2S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran
RT-PCR	Reverse transcriptase PCR
SAM	S-adenosyl-methionine
SdiA	Supressor of cell division inhibition
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel
SMAC	sorbitol MacConkey agar

Stx	Shiga toxin
Ti	Tumor inducing
Tir	Translocated intimin receptor
TLC	Thin layer chromatography
TNFα	Tumor necrosis factor alpha
TSS	Transcriptional start site
TTSS	Type III secretion system
Tyr	Tyrosine
UPEC	Uropathogenic *E. Coli*
WT	Wild type
X-gal	5-bromo-4-chloro-3-indolyl β-galactopyranoside

CHAPTER ONE

LITERATURE REVIEW

TAXONOMY AND HISTORICAL PERSPECTIVE OF ENTEROHEMORRHAGIC *E. COLI*

Escherichia coli was first isolated in 1885 by the German pediatrician and bacteriologist Theodor Escherich, whom the genus was eventually named after by Castellani and Chalmers in 1919 (86). *E. coli* is classified as a member of the Enterobacteriaceae family of gamma-proteobacteria. *E. coli* is a Gram-negative, facultative, anaerobic bacillus that is a major constituent of the endothermic lower gastrointestinal (GI) tract. Enterohemorrhagic *Escherichia coli* or EHEC was first described in 1983 by Ripley *et al*. as a rare serotype, O157:H7, isolated from beef patties, which caused severe hemorrhagic colitis. The "O" in O157 refers to the unique surface antigen on this strain while the "H" in H7 refers to the flagellin antigen (302).

Commensal *E. coli* strains are the most abundant facultative anaerobes of the human GI tract and humans are colonized with *E. coli* within a few hours of birth. Pathogenic *E. coli* strains are highly adapted version of these commensal strains that have acquired multiple virulence factors through incorporation of genetically-encoded mobile (or previously mobile) elements like plasmids, pathogenicity islands, and phages. Six categories of *E. coli* exist that cause gastrointestinal clinical syndromes in healthy individuals. They include: enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC) (148) (31).

EHEC is a human pathogen that causes major outbreaks of hemorrhagic colitis and hemolytic uremic syndrome (HUS). In the U.S.A., EHEC causes approximately 73,000 illnesses, 2,000 hospitalizations, and 69 deaths annually (210). Following the initial finding by Ripley *et al.,* several subsequent reports characterized the EHEC infection as being associated with bloody diarrhea, hemorrhagic colitis, hemolytic uremic syndrome (HUS) and thrombotic-thrombocytopenic purpura (302) (154) (264) (312). These and other studies have lead to the general paradigm of EHEC infection: EHEC colonizes the large intestine where it forms attaching and effacing (AE) lesions and releases Shiga toxin which is responsible for HUS (194) (252). HUS is the major contributor to the mortality and morbidity seen in infected patients (31).

Although, the first outbreaks occurred in 1983 at the McDonald's restaurant chain, EHEC did not become nationally recognized until the 1993 Jack-in-the-Box outbreak. This EHEC outbreak occurred in four states and included 73 restaurants, where over 700 people were sickened and four died. During this outbreak, it became clear that EHEC is an effective pathogen because it has a low infectious dose. The contaminated Jack in the Box hamburgers had an average of 67.5 organisms per patty (370). Since 1993, in addition to processed meat, major outbreaks have been attributed to produce (1), unpasteurized milk (156) and juice (3), petting zoos (4) and fresh water sources (2) .

THE NATURAL RESERVOIRS OF ENTEROHEMORRHAGIC *E. COLI*

The first EHEC infections were associated with the consumption of ground beef, leading investigators to suspect cattle as the natural reservoir of EHEC (302). The correlation of EHEC infection with cattle was strengthened by two investigations in the late 1980s. In 1986, Martin *et al*. isolated EHEC from dairy cattle associated with two HUS cases

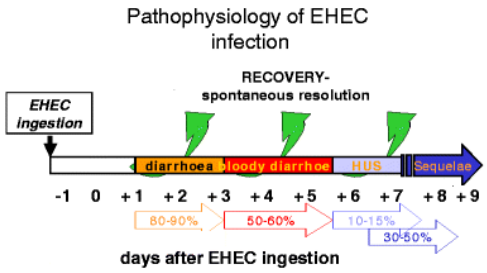
(227). Similar conclusions were made the following year when several kindergarten children developed HUS after visiting a dairy farm and drinking unpasteurized milk. This farm contained six head of cattle that tested positive for EHEC (30). Several subsequent studies have confirmed cattle as the primary reservoir of EHEC (120) (417). Prevalence rates vary from country to country. The prevalence rates of EHEC cattle colonization in the U.S.A. indicates a high degree of variability based on the farm, feed, season, and numerous other conditions. In the U.S.A, over 70% of cattle herds have at least one EHEC positive cow. The number of EHEC positive cows on a given farm ranges from 0-28% depending on the study (121) (166) (190) (76). In humans, Shiga toxin (Stx), the causative agent of hemolytic uremic syndrome, acts through the globotriaosylceramide (Gb3) receptor. In 2000, it was discovered that cattle lack this receptor, explaining the asymptomatic nature of the cow EHEC relationship (287). Additionally, in cattle, EHEC principally colonizes the rectal-anal junction (RAJ) while in humans, EHEC colonizes the large intestine (282). Differences in the site of colonization may also lead to differences in diarrheal disease.

In addition to cattle, other small ruminants (mainly sheep and goats) are asymptomatic natural reservoirs of EHEC, explaining the frequent EHEC outbreaks associated with petting zoos (181). EHEC outbreaks have been associated with numerous other food sources including: apple cider, lettuce, radishes, sprouts, and spinach all of which have had an association with ruminant feces (27) (374) (237) (32).

CLINICAL PRESENTATION, DIAGNOSIS, AND TREATMENT

EHEC illness initially presents with severe abdominal cramps and non-bloody diarrhea. On the second to third day of illness the diarrhea can become bloody. Bloody diarrhea

occurs in 35-90% of infected patients with severity ranging from streaks of blood in the stool to all blood and no stool (24). Fifty percent of patients also have nausea and vomiting. EHEC is unique among infectious agents that present with similar symptoms, in that little or no fever is associated with illness (114). These initial symptoms usually subside within one week. However, 2-14 days after the initial symptoms, HUS develops in 6% of infected patients (152). HUS is most likely to occur in children and the elderly and is characterized by microangiopathic hemolytic anemia, thrombocytopenia, renal failure, and neurologic complications, including seizures, coma and hemiparesis (115) (280) (47). The mortality rate is 3-5%, with 5% of survivors experiencing severe symptoms such as end-stage renal disease and permanent neurologic injury (335) (Figure 1.1).



(319)

Figure 1.1: EHEC course of infection. About two days after EHEC consumption patients present with watery diarrhea, nausea, and vomiting. Two to three days later patients develop bloody diarrhea. Eighty to ninety percent of patients recover from EHEC infection after one week. The remaining infected

The easiest, cheapest, and most routine way to detect and diagnose EHEC is by the use of sorbitol-MacConkey agar. EHEC, unlike most *E. coli* of the GI tract, can not ferment sorbitol. When feces from a suspected infection are streaked on the sorbitol-MacConkey agar plate, sorbitol fermenters will turn red while non-fermenters (EHEC) will be white or opaque (222). This test is usually followed by use of a commercially available ELISA for Shiga toxin (267).

Prior to the onset of HUS, the only effective treatment is intravenous rehydration (360). Use of antibiotics to treat EHEC is not advised because several studies indicate that antibiotics can exacerbate the production of Shiga toxin which would lead to an increased chance for HUS onset (266). If the patient experiences an increase in platelet count, no diarrheal symptoms, a negative sorbitol-MacConkey test, and has no rise in creatinine concentration, then the HUS risk period has passed and the patient can be discharged (360). If a patient does progress to HUS, treatment is mostly supportive. The goal of the physician is management of renal failure. Treatment options include fluid and electrolyte balance, treatment of anemia, control of hypertension, nutritional support, and dialysis (334).

PATHOLOGY

The intestinal pathology of EHEC occurs mainly in the ascending colon. Colonoscopic studies revealed that during infection the ascending colon appears shaggy and has thickened folds (271). The most in depth human histological study examined nineteen colonoscopic biopsies of EHEC infected individuals. This study revealed that all patients had hemorrhage and edema in the lamina propria. Based on histology, patients were separated into three groups. Group one consisted of nine individuals whose symptoms matched individuals experiencing acute ischemic colitis. These symptoms included necrosis, hemorrhage, and inflammation of the superficial mucosa while the deep crypts were unaffected. Patients from group two showed symptoms similar to group one, but with neutrophils infiltrating the lamina propria and the crypts, a symptom indicative of infectious colitis. The third group had all of the above symptoms with the addition of inflammatory pseudomembranes, a symptom usually seen in patients infected with

Clostridium difficile (113). A second study examining surgically excised tissue and tissue from two autopsies of EHEC infected individuals had similar findings. All individuals in the second study showed ulceration, hemorrhage, neutrophil infiltration, and microvascular thrombi as well as the inflammatory pseudomembranes seen in the previous study (158).

The hallmark pathological characteristic of EHEC infection is the attaching and effacing (AE) lesion. These lesions are characterized by modulation of the host actin cytoskeleton into a pedestal-like structure and effacement of the brush-border microvilli (148).

VIRULENCE FACTORS OF ENTEROHEMORRHAGIC *E. COLI*

The Locus of Enterocyte Effacement (LEE)

A hallmark of EHEC infection is the effacement of the brush-border microvilli and the rearrangement of the host actin cytoskeleton into a pedestal-like structure (Figure 1.2).

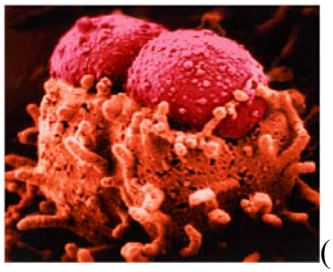
 (148)

Figure 1.2: Pedestal formation. A classic pathology of EHEC infection is pedestal formation. EHEC uses the genes encoded within the LEE pathogenicity island to modify the host actin cytoskeleton and form pedestals.

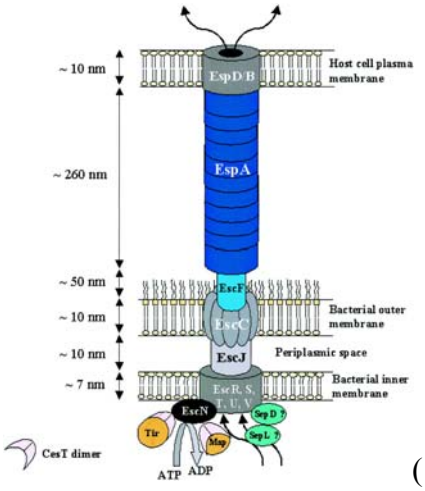
These morphological changes are referred to as AE lesions (172) (243) (373). Most of the genes required for the formation of AE lesions are encoded within a chromosomal pathogenicity island termed the locus of enterocyte effacement or LEE

(228). The LEE is composed of 41 genes most of which are separated into five operons termed *LEE1*, *LEE2*, *LEE3*, *LEE5*, and *LEE4* (82) (36) (233). Encoded within these operons are the transcriptional regulators Ler, GrlR, and GrlA, (233) (64), a type III secretion system (142), the adhesin intimin (*eae*) (144), the adhesin receptor Tir (162), and several secreted effectors (79) (146) (164) (229) (368).

Genetic regulation of the LEE is complex. *LEE1* encodes *ler* the master regulator of the LEE genes (81) (233) (345). The protein encoded by *ler* directly activates transcription of the LEE genes by antagonizing H-NS mediated silencing (36). A multitude of additional proteins and genes have been implicated as transcriptional regulators of the LEE genes, including: H-NS (36), GadX (332), Per (233), EtrA, EivF (415), QseA (326), SdiA (147), CpxR (219), LexA (234), Pch (138), and Hha (325). Posttranscriptional regulation has also been proposed as a mechanism of LEE gene regulation (306) (205).

The type III secretion apparatus is used to transfer bacterial effectors into the host cell to manipulate host processes and facilitate pathogenesis (130). *LEE1, LEE2, LEE3* and *LEE4* encode the EHEC basal type III secretion apparatus and the translocation machinery (82). Initially, construction of the type III secretion apparatus occurs sequentially using the *sec* secretion machinery. EscRSTUV compose the inner membrane base, EscC forms the outer membrane pore, and inner and outer membrane complexes are connected in the periplasm by EscJ (Figure 1.3) (106) (56) . EscF constitutes the needle. It connects the outer membrane component, EscC, to the EspA filament which forms the hollow conduit around between the bacterial cell and host cell

(405) (57) (173). EspA connects to EspB and EspD which form the translocation pore in the host plasma membrane (Figure 1.3) (135) (176).



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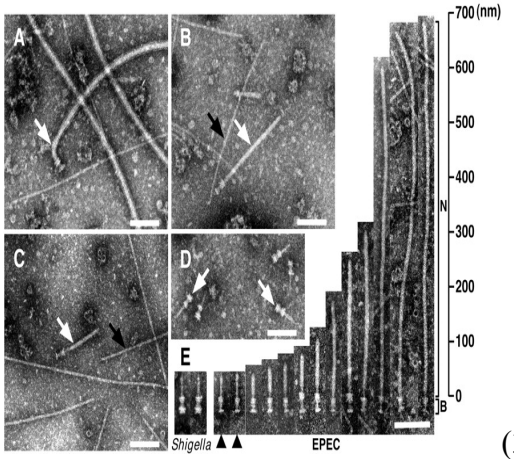

(324)

Figure 1.3: The EHEC type III secretion system EHEC encodes a TTSS within the LEE pathogenicity island. EscRSTUV compose the inner membrane base, EscC forms the outer membrane pore, and inner and outer membrane complexes are connected in the periplasm by EscJ (106) (56) . EscF constitutes the needle. It connects the outer membrane component, EscC, to the EspA filament which forms the hollow conduit around between the bacterial cell and host cell (405) (57) (173). EspA connects to EspB and EspD which form the translocation pore in the host plasma membrane ((135) (176). (B). An electromicrograph of the EHEC TTSS

Several other LEE encoded proteins are involved in secretion but are not structural components of the machinery. EscN is the ATPase that provides the energy for protein transport (13). SepD and SepL are also involved in type III secretion, but their mechanism of action remains undefined. SepD and SepL are known to interact with each other (54). Interestingly, mutating *sepD* abolishes secretion of the translocator and effector proteins and eliminates AE lesion formation. *sepL* mutants secrete effectors normally, but do not secrete translocator proteins and are deficient in AE lesion formation (259) (64).

Many of the effector proteins also have chaperones encoded within the LEE. Currently, five chaperones have been identified in the LEE. CesF is the chaperone for EspF (80). CesT is required for translocation of Tir and Map and it interacts with the ATPase EscN (5) (53) (78) (105). CesD, CesD2, and CesAB are all involved in EspD and EspB translocation (384) (256). Finally, CesAB is required for EspA filament biogenesis (55).

The genes encoding Intimin and Tir are both located within *LEE5* (82). The interaction of these two proteins is responsible, at least in part, for the intimate attachment between the bacterium and the host plasma membrane. Intimin is a 94 kDa protein that is secreted to the outer membrane by the general secretory pathway. *eae*, the gene encoding intimin, was the first gene product found to be involved in AE lesion formation (144). Multiple animal and *ex vivo* models have proven the necessity of intimin for intestinal colonization, AE lesion formation, and pathogenesis (70) (69) (125). Initially, the intimin receptor was thought to be a mammalian protein termed Hp90 (308). However, it was later discovered that Hp90 was actually the bacterial protein Tir and that Tir was secreted into the mammalian cell through the type III secretion apparatus (162). Most of the early work on Tir focused on the protein encoded in EPEC. Interestingly, the mode of action of EHEC and EPEC Tir is not the same. In fact, EHEC *tir* mutants expressing EPEC *tir* can form pedestals, but EPEC *tir* mutants expressing EHEC *tir* cannot (65) (161). It was discovered that EHEC Tir containing a 12-amino-acid sequence of EPEC Tir, encompassing Tyr474 (a residue that is phosphorylated in the mammalian cell), was sufficient for EPEC *tir* mutant to form pedestals (38). For EPEC, Tir is the only translocated protein required for pedestal formation. EPEC uses the

interaction of phosphorylated Tir and the mammalian adaptor protein Nck to promote actin assembly (38) (116). EHEC, however, does not utilize Nck and requires the secreted effector EspFu for pedestal formation (39) (104). In the mammalian cell, EHEC Tir interacts with the mammalian protein IRTKS which interacts with EspFu which recruits N-WASP. N-WASP activates actin assembly by stimulating the actin nucleating complex Arp2/3 (381).

In addition to Tir, there are five other secreted effectors in the LEE region. They include: Map, EspF, EspG, EspZ (previously SepZ), and EspH. All of the effectors are required for pathogenicity in an animal models (304). These effectors have multiple and highly variable functions. Most of the effectors show some level interdependence, cooperativity, and redundancy (61). Currently, Map (mitochondrion-associated protein) has three known functions. Map inhibits mitochondrial function (164), it is involved in actin rearrangement (163), and it disrupts intestinal tight junctions (62). Additionally, Map has been shown to interact with EBP50/NHERF1, a host protein involved in ion channel regulation, suggesting it may play a role in EHEC diarrheal disease (337). EspF disrupts intestinal barrier function, possibly through redistribution of the tight junction proteins (230) (275). Like Map, EspF is localized to the mitochondria where it is involved in mitochondrial membrane permeabilization. This effect, as well as an induction of cytochrome c release into the cytosol and cleavage of caspases 9 and 3 indicates that EspF plays a role in the mitochondrial programmed cell death pathway (248) (258). Additionally, EspF may be involved in EHEC diarrheal disease through its ability to decrease the expression of NHE3, a protein involved in Na^{2+} exchange in the intestine (127). EspG has been shown to modify the actin cytoskeleton and compromise

microtubule networks under the intestinally adhered bacterium (328). The exact role of EspH is not currently known, although it has been shown to localize to the host plasma membrane and modulate the actin cytoskeleton (368). EspZ is known to be secreted into the host cell, but has no known function.

Other EHEC Effectors

In addition to the LEE-encoded secreted effectors, EHEC contains at least 39 proteins encoded throughout the chromosome, that are confirmed as secreted effectors, although only a small fraction have been studied in detail. The majority of these effectors are encoded within λ phages indicating recent acquisition through horizontal gene transfer (364). Currently, EspFu, NleA, EspL2, EspJ, and NleH have been characterized to some extent. EspFu has been well characterized as the bacterial intermediate required for EHEC Tir to interact with N-WASP and mediate actin assembly (104) (39). NleA disrupts protein secretion at the ER through a direct interaction with Sec24, the cargo determining factor of COPII coated protein transport vesicles (169). EspL2 has been shown to modify the actin cytoskeleton through a direct interaction with annexin 2, a host protein known to modulate actin organization (241). EspJ is involved in inhibition of receptor-mediated phagocytosis. Expression of EspJ in phagocytes can inhibit their ability to internalize opsonized red blood cells (223). Finally, mice infected with a *C. rodentium nleH* mutant had decreased levels of NF-κB activity in the colonic mucosa.

pO157

Most of the EHEC O157:H7 strains carry a 92 kb F-like plasmid containing 100 putative open reading frames. Approximately, 20 of these genes have been classified as potential virulence factors (34). This plasmid was first discovered, in 1990, through its ability

modulate the adherence of pO157$^-$ strains to HEp-2 epithelial cells (365). EHEC strains bearing the plasmid are associated with an increased incidence of hemorrhagic colitis, HUS, and bovine colonization (359) (195) (198). One of the most well characterized plasmid encoded virulence factors is StcE, a metalloprotease that cleaves the protein backbone of mucin-type glycoproteins (356). StcE, has been shown to alter the functions of neutrophils and contributes to EHEC adherence to host cells (356) (118). StcE is secreted by a pO157 encoded type II secretion system. This type II secretion system plays a role in adherence and intestinal colonization in an infant rabbit model of EHEC infection (183) (126). The pO157 plasmid also contains the genes required for the EHEC hemolysin activity, called enterohemolysis (21) (28). Regulation of the hemolysin genes is linked to the LEE genes through the LEE encoded regulator GrlA (314). The role of pO157 in bovine colonization has also been partially elucidated since the plasmid encoded autotransporter EspP, a serine protease, has been implicated as a bovine colonization factor (71).

Stx

Stx is the causative agent of HUS. Direct injection of Stx into a rabbit model resulted in non-bloody diarrhea and death. Histological features included, edema and hemorrhage in the large intestine, edema, hemorrhage, and necrosis in the brain and spinal cord, and renal lesions (301). HUS is the leading cause of mortality in patients infected with EHEC, and the leading cause of acute renal failure in children (151). The association between HUS and EHEC was first made in 1985, when Karmali *et al*., discovered that 75% of pediatric patients experiencing HUS were infected with some form of Shiga toxin-producing *E. coli* (152). *stx* genes from all EHEC subspecies are prophage encoded

and can be divided into two unique antigenic groups (353). Stx1, which is absent in 86-24 (the strain used in this body of work), is antigenically identical to Stx from *Shigella dysenteriae* type 1 (352). Stx2, which is present in 86-24, is structurally and antigenically unique and cannot be neutralized with an Stx1 antibody (88) (353). Stx2 was shown to be 1000x more cytotoxic, when applied to human renal microvascular cells, that Stx1 (208) . Additionally, patients infected with EHEC strains encoding *stx2* alone were 7 times more likely to develop HUS than patients infected with EHEC strains bearing *stx1* or both *stx1* and *2* (263).

Stx is a classic AB-cytotoxin. It contains a single A polypeptide and 5 B polpeptides. Stx is internalized through clathrin-dependent endocytosis, mediated by a direct interaction of the B subunit with the glycolipid receptor globotriaosylceramide (Gb3) (168) (201) (139). Once inside the host cell, the A subunit, an N-glycosidase, removes a single adenine from the 28S host rRNA, inhibiting translation (316) (83).

Regulation of *stx* in EHEC is linked to the SOS response. The EHEC *stx* gene is located within a prophage of the λ family (255) (281). λ prophages are maintained in a quiescent state through prophage encoded repressors, called cI repressors (290). During the SOS response, the presence of ssDNA (indicative of DNA damage) activates RecA. Normally, RecA is used to activate autocleavage of LexA, a repressor of DNA repair proteins (202). In the case of prophages, RecA is used to cleave cI, a process called induction (305). An induced prophage will enter the lytic cycle and transcription of prophage encoded genes, in this case *stx*, will occur (371). Multiple antibiotics, including ciprofloxacin, an antibiotic commonly prescribed for diarrheal *E.coli* infections, have been shown to induce the SOS response (157). This phenomenon may explain the

increased likelihood of HUS development in EHEC patients treated with antibiotics (313).

IMMUNE INVOLVEMENT

The immune response to EHEC has not been well characterized. Early studies indicated that humans can develop an immune response to EHEC LPS and that this response may affect EHEC colonization (43) (29). Pediatric patients examined for antibody production after EHEC infection were found to have high antibody titers to Tir and modest titers to EspA, EspB, and intimin (196). HUS affected patients have lower levels of IL-2 production indicating that EHEC infection could inhibit immunologic memory (399). Several groups have focused on the proinflammatory response, because multiple studies have indicated that tumor necrosis factor alpha (TNFα) and interleukin-1 (IL-1) could induce expression of the Stx receptor on human endothelial cells (375) (207) (155). Additionally, higher ratios of proinflammatory cytokines (TNFα, IL-1/inteleukin-10 (IL-10)) have been found in EHEC infected/HUS patients (399) (286). These studies imply that the immune response to EHEC could exacerbate HUS and could potentially explain the variability in the association of EHEC infection and the development of HUS. Although the proinflammatory-Stx uptake relationship has been proven *in vitro*, attempts to correlate EHEC inflammation with HUS in infected patients have been unsuccessful (206).

Stx is also thought to be a potent immune activator. Current theories speculate that since EHEC is a non-invasive pathogen it must damage the epithelial barrier to facilitate the transport of Stx. Stx has been shown to upregulate the production of the cytokines and chemokines IL-8, MGSA, MIP-2α and β, and ENA-78 (362). These

factors would lead to PMN recruitment which could lead to damage of the intestinal barrier and increase Stx absorption. Other groups have argued that these effects may be attributed to the host's response to flagellin as opposed to Stx (307).

VACCINOLOGY

EHEC vaccination falls into three general categories. The first category relates to strategies used in preventing cows from carrying EHEC, pre-harvest interventions. The second category focuses on preventing HUS in infected individuals by neutralizing Stx. The final strategy uses EHEC antigens to elicit an adaptive immune response towards EHEC in animal models of infection.

Vaccine strategies have been attempted to reduce or eliminate EHEC from the cow population. Several antigens have been used with varying results. Three resent studies using an EHEC culture supernatant enriched with type III secreted proteins have shown strong efficacy in reducing EHEC colonization. In the most recent in this series of stidies, 718 cattle were tested in the trial. With a two-dose vaccination strategy, cows were 92% less likely to be colonized with EHEC (342) (278) (283). Other antigens (siderophore receptors, porins, H7-flagellin, EspA, intimin, and Efa-1) have been tested as cow vaccines against EHEC with limited success (361) (231) (73) (376).

Most therapeutic EHEC vaccination attempts have focused on neutralizing Stx before it can cause HUS. No Stx vaccines have been tested in humans. The majority of vaccines have been tested in mice, with the exception of one study in non-human primates (355). Multiple antigens and delivery mechanisms have been explored. Mice immunized with a Stx2 toxoid are completely protected from Stx2 challenge (397). In a follow up study, the same toxoid was cloned into a plant. Mice were fed the plant and

were completely protected against Stx2 challenge (398). Similar studies using various Stx antigens have shown complete or partial protection in mice (367), (221), (37).

Intimin and the O157 antigen have also been used as antigens. Suckling piglets, an animal model of EHEC, can be protected against EHEC infection if their mothers are vaccinated against EHEC with intimin (60). Despite being tested for safety in phase I and II clinical trials, the O157 antigen as a vaccine, has shown no effect in preventing EHEC colonization of the mouse intestine (174) (9) (52).

Attenuated EHEC strains have also been tested as vaccines in animal models. A truncated intimin mutant in RDEC-1, a rabbit enteropathogenic *E. coli*, was highly effective at preventing hemorrhagic colitis following challenge (8). Vaccination with a *ler* mutant has also resulted in complete protection of rabbits following WT challenge (419).

ANIMAL MODELS

Animal models for EHEC can be separated into two groups. The first models are used to study the pathogenesis or colonization of EHEC, or related species. The second models are used to study the effects of Stx. Mice, rabbits, chickens, dogs, pigs, cows, ferrets, macaques, and baboons have all been utilized to study aspects of EHEC infection (235).

Rabbits are most often utilized to study EHEC-like infections. The rabbit specific strains RDEC (O15:H-) and REPEC (O103:H2) have been successfully used to study specific EHEC-like virulence mechanisms. RDEC was discovered as a weanling rabbit diarrheal pathogen in 1977 (40). This model was developed throughout the 1980s with a paramount discovery in 1990 showing that EPEC, EHEC and RDEC all contain the same loci required for AE lesion formation (144). The LEE region from RDEC was

subsequently cloned and sequenced and was nearly identical to the LEE region from EPEC and EHEC (150) (418). Furthermore, the addition of an EPEC adherence plasmid to RDEC facilitated formation of AE lesions on human tissue culture cells (143). This adherence to human epithelial cells activated human signaling cascades resulting in tyrosine phosphorylation of human proteins and increases in intracellular calcium (279). REPEC has been used similarly to RDEC. Specifically, REPEC has been used to help characterize the LEE genes, Tir, intimin, EspA and EspB, as virulence factors (6), (224). One of the challenges in using these rabbit systems is the necessity to perform all genetic manipulations on at least two strains: the human strain (EHEC) and the rabbit strain. Additionally, neither strain naturally encodes genes for Stx2, although a RDEC strain has been engineered to express Stx1 (339).

The development of the infant rabbit model for EHEC helped to overcome these obstacles. When inoculated with EHEC, 3-day old rabbits experience diarrhea, colonic inflammation, and death. These results are absent in 11-day old rabbits (265) (284). This observation was first utilized to study the virulence genes *stx2*, *eae*, and *tir*. Interestingly, mutations in *stx2* increased severity and duration of EHEC-induced diarrhea, while mutations in *eae* and *tir* eliminated EHEC intestinal colonization (303). Additionally, the LEE encoded secreted effectors, EspH, Map, EspF, and EspG have all been shown, using this model, to be involved in EHEC pathogenicity (304).

Like infant rabbits, gnotobiotic piglets can be used to directly study EHEC, but these studies are more challenging because they require a complex animal facility (303). Although this animal model is technically challenging, it has been used to study the effects of EHEC pathogenesis related to the LEE genes. Mutations in EHEC *eaeA*

(intimin) were incapable of intimate attachment to the pig colonic epithelium, colonized the colon poorly, and induced little or no diarrhea (70) (372).

Weaned 3- to 4-month-old calves have been used to study EHEC pathogenesis and colonization. This model, however, suffers from several drawbacks. It requires a complex and expensive animal facility. Additionally, in order to develop diarrhea and AE lesions, weaned calves require intragastric inoculation of 10^{10} cfu of EHEC (59). This model is useful for studying EHEC colonization factors. It has been used to study the role of probiotics in limiting EHEC colonization and has been used to identify genes that may be required for EHEC colonization (363) (209).

For EHEC, the current mouse models of infection are only useful for studying colonization and the effects of Stx. These models are not used to study AE lesion formation or hemorrhagic colitis. Colonization studies are generally conducted with oral inoculation in either conventional mice, germ-free mice, or mice treated with streptomycin or mitomycin C (382) (95) (74). Streptomycin and mitomycin C treatments allow for an increased susceptibility to EHEC colonization, while mitomycin C treatment also facilitates an increase in Stx production (331). The effects of Stx have been studied through oral inoculation and through injection of EHEC or Stx itself into various sites (74). Stx symptoms in mice are similar to humans experiencing HUS including renal tubular necrosis and multiple neurological sequelae (382) (383) (74), (170).

Citrobacter rodentium is a natural mouse enteric pathogen that shares LEE gene homology with EHEC, and is a widely used model to study *in vivo* AE lesion formation. Typically, mice are orally challenged and after 2-3 days organisms can be detected in the distal colon. Colonic hyperplasia, the most common symptom during infection, occurs 5-

14 days post infection. Most mouse strains clear the infection within 21-28 days and show little or no mortality. C3H/HeJ mice are the exception with high mortality 10 days post infection (404). The mortality phenotype of C3H/HeJ is routinely used to assess the virulence of *Citrobacter rodentium* genetic mutations (245). The *C. rodentium* LEE region is highly similar to the EHEC LEE containing the same 41 open reading frames. However, the *C. rodentium* LEE contains multiple insertion sequences. Additionally, the EHEC LEE region is located in the *selC* tRNA gene, while the *C. rodentium* LEE region is flanked by an operon containing an ABC transport system and sequences homologus to EHEC and Shigella plasmids (63).

The *C. rodentium* system has been highly effective at establishing the necessity for virulence of multiple factors. In fact, all 41 open reading frames in the LEE region, several of the newly discovered putative non-LEE encoded effectors, and multiple other previously suspected virulence factors have been mutated and tested for their role in virulence using this model (64) (246) (117) (318) (171).

The ease of use of a murine model has also facilitated signature tagged mutagenesis as a tool to discover new virulence and colonization factors. This strategy was used to discover the virulence factor NleA, a non-LEE encoded secreted effector and NleB and a novel type IV pilus gene cluster required for gastrointestinal colonization (246) (247) (159).

The *C. rodentium* model also benefits from the ease with which we can genetically manipulate mice. Mouse mutations in TLR4, TLR2, IL-1R and MyD88 have all been tested in the *C. rodentium* model (108) (107) (167) (186). These studies have

helped to understand the role of the innate immune response to luminal pathogens like EHEC.

GENETIC CONTENT

Three EHEC genome sequences have been completed and 17 more sequences are listed on the National Center for Biotechnology Information (NCBI) website as being in progress. The genome is approximately 5.5 megabases. The divergence of EHEC from K-12 occurred approximately 4.5 million years ago (300). The two strains share a common co-linear backbone sequence of approximately 4.1 megabases. Divergent regions in EHEC are referred to as O-islands and they encompass 1.34 megabases. K-12 contains 0.34 megabases not found in EHEC. About 26% of the EHEC genes are found in these O-islands. These regions encode multiple virulence factors including Stx and the LEE genes (276) (123).

EHEC is believed to have diverged from its most common ancestor, the EPEC strain O55:H7, within the last millennia (192). These strains share 81% of the EHEC O-islands and 85% of the variation among the two strains is a result of prophages (403). Two subgroups have emerged from the O55:H7 ancestor and are characterized by the presence or absence of the H7 flagellar antigen. The H7 positive group, to which EHEC belongs, is further divided into three clusters which are categorized by their inability to ferment sorbitol. The strain used in all studies presented in this body of work, 86-24, is divergent from the other two O157:H7 clusters based on the presence of the *stx2* gene and the absence of the *stx1* gene (Figure 1.4) (192).

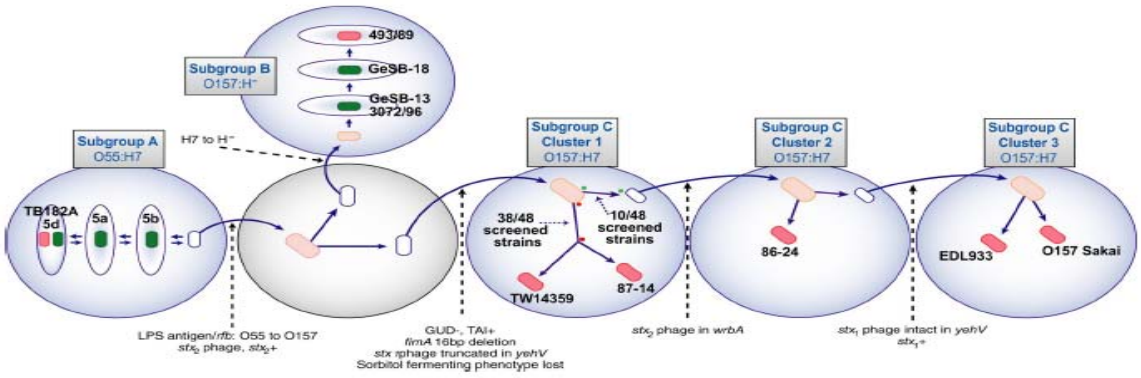

Figure 1.4: The evolution of EHEC presumably occurring within the last millennia, (192) EHEC, 8624, the strain used in this study, falls into subgroup C, cluster 2. This cluster is characterized by the presence of *stx2* and the absence of *stx1*.

CELL-TO-CELL SIGNALING IN BACTERIA

Cell-cell signaling mechanisms in bacteria can be broken down into four general categories. These include the AI-1 and AI-3 systems which are used solely by Gram-negative bacteria, the autoinducing peptide system which is used by Gram-positive bacteria, and the AI-2 system which is used by both.

THE AI-1-LUXI and LUXR SYSTEM

AI is the abbreviation for autoinducer, the molecule that regulates autoinduction. This term was given to the phenomenon by which the marine bacterium *Vibrio fischeri* produced light based on its population density (254) (253). This observation formed the basis of the quorum sensing field. Two proteins control quorum sensing in the AI-1 system, LuxI and LuxR. LuxI is the autoinducer synthase, which produces the acyl-homoserine lactone (AHL) autoinducer. LuxR is the transcription factor that binds to the autoinducer and directly regulates (in the case of *Vibrio fischeri*) the luciferase operon (84) (75). As AHLs are produced they diffuse freely, or in some cases are pumped, in and out of the cell (149). The signal increases as the bacterial cell density rises.

Eventually the signal concentration reaches a critical threshold and begins diffusing back into the cell at levels high enough to bind to and activate LuxR. LuxR activates expression of the *lux* genes which constitute the luciferase operon. Additionally, LuxR activates expression of *luxI* leading to a positive feedback loop which floods the environment with AHLs (350). Many Gram-negative bacteria use this system for signaling and they have adopted high level of specificity when coordinating their cognate AHL to their LuxR protein. This specificity is based on the mechanism by which AHLs are synthesized. LuxI proteins catalyze lactonization of S-adenosylmethionine (SAM) and lactone acylation with fatty acyl chains carried on acyl-acyl carrier proteins (270) (244). Different bacterial species incorporate unique acyl chains onto the homoserine lactone in order to garner an AHL that is specific for their version of LuxR. The specificity of the acyl chain is governed by the binding pocket of the LuxI protein of the given bacterial species (393). The final product can have various chain lengths (C4 to C18), multiple degrees of saturation, and modifications at the third carbon of the acyl chain (401) (226). Two other classes of AHL synthetases have also been described. The LuxM family comprises the second class and includes three proteins all found in *Vibrio* species (18). This group is unique because it can use either acyl-ACP or acyl-CoA as a substrate for synthesis. The third class is comprised of a single protein, HdtS, a member of the lysophosphatidic acid acyltransferase family, whose AHL synthesis mechanism has not been completely elucidated (185).

The LuxIR system has also been extensively characterized in the human opportunistic pathogen *Pseudomonas aeruginosa*. Quorum sensing is essential for *P. aeruginosa* virulence because it controls adhesion, biofilm formation, and virulence

factor expression (343). Mutations in the *P. aeruginosa* quorum sensing regulators have resulted in decreased virulence in multiple animal models of infection (309) (310) (358) (272). *P. aeruginosa* encodes two LuxIR systems termed LasIR and RhlIR which respond to two unique AHLs (101) (260) (273) (274). LasR and RhlR have been shown to control hundreds of genes in *P. aeruginosa,* making it an ideal organism to study quorum sensing genetic regulatory mechanisms (321). Promoter specificity studies indicate that promoters respond to either LasR alone or to LasR and RhlR, although the elements of specificity are not well understood (402). LasR and RhlR, have been shown to bind to conserved palindromic sequences called *las–rhl* boxes. These regions share some homology to the well characterized *lux* box, the promoter element bound by LuxR in *V. fischeri* (311) (277). DNA binding requires dimerization and association with the cognate AHL molecule (322). *P. aeruginosa* also contains an orphan (no cognate LuxI homologue) LuxR homologue, termed QscR that is believed to bind to the same AHL molecule as LasR (193). Mutations in the gene encoding QscR lead to hypervirulence in an animal model of infection, indicating that QscR may act as a transcriptional repressor (46). QscR's mode of action remains unclear. It is known to form heterodimers with LasR and RhlR which may inhibit their ability to act as transcriptional activators (187). Additionally, QscR may act as a sink for AHLs and would delay or prevent transcriptional activation by LasR (193). At least 5 other LuxR homologues have been described to have repressor functions. These include: EsaR from *Pantoea stewartii*, YpsR from *Yersinia pseudotuberculosis*, SpnR from *Serratia marcescens*, and VirR from *Erwinia* species (240) (15) (129) (35). It is believed that these LuxR homologues act as transcriptional repressors in the AHL unbound state and addition of the cognate AHL

leads to derepression (240) (129) (41) (35). Among these proteins, EsaR has the best characterized mode of action. In the absence of AHL, EsaR forms a homodimer that directly represses transcription of its own gene, *esaR*, through an interaction with the *esaR* promoter. AHL addition, in some experiments, inhibited DNA binding and lead to derepression of the *esaR* gene (240).

Transcriptional regulation of LuxR homologues is also well characterized in the plant pathogen *Agrobacterium tumefaciens*, a bacterium that can directly transform plant cells and cause crown gall tumor formation. The LuxR homologue in *A. tumefaciens* is termed TraR. Interestingly, TraR, the synthetase TraI, and all of the genes regulated by TraR are located on and control conjugation of the Ti plasmid, which encodes the genes for transformation and tumor formation (97) (133) (96). The promoter binding sites and the mechanism of TraR have been thoroughly studied. Four TraR binding sites have been discovered. They are upstream of the five operons regulated by TraR and they share an 18 bp consensus sequence called the *tra* box. TraR binds to these promoters as a dimer complexed with C8-oxo-HSL (its cognate AHL) and recruits RNA polymerase by at least two unique mechanisms (400).

Structural studies of TraR have also help to understand the role AHL binding and activator function. These studies have shown that AHL binding results in large conformational changes that modify the transcriptional activity of the protein. In fact AHLs are absolutely required for TraR to fold properly, indicating that the protein is unstructured in the absence of ligand (421). Additionally, the crystal structure of TraR complexed with AHL and a *tra* box DNA element indicates that the AHL molecule is completely engulfed in the core of the protein and protected from solvent. This finding

suggests that the ligand was present during protein folding or that extensive conformational changes occur during protein-ligand interaction (379) (416).

SDIA, A LUXR HOMOLOGUE

SdiA (suppressor of cell division inhibition) is a LuxR homologue that was originally discovered in a screen looking for genes that could modify cell division in *E. coli*. Overexpression of *sdiA* activated expression of the *ftsQAZ* genes which are known to activate cell division. *ftsQAZ* is regulated by at least six promoters four internal and two upstream of the operon (12). SdiA was shown to activate *ftsQAZ* by interacting with the P2 promoter (390) (338) (410). Overexpression studies with SdiA have also indicated that it plays a role in multidrug resistance. In these studies, *sdiA* overexpression lead to increased mitomycin C, quinolone, kanamycin, tetracycline, chloramphenicol, and erythromycin resistance (395) (293). Array analysis of an *E. coli* strain overexpressing *sdiA* implicated the multidrug efflux pump AcrAB as the factor mediating SdiA based drug resistance (394). This was confirmed when overexpression of *sdiA* lead to an increase in the AcrA and AcrB protein levels in *E.coli*. Additionally, mutations in *acrA* or *acrB* led to a loss of SdiA mediated drug resistance (293). SdiA is also involved in biofilm formation. Mutating *sdiA* in *E.coli* leads to a 50-fold increase in the production of biofilms. Indole is thought to play a role in SdiA mediated biofilm repression but the exact mechanism of action has not been determined (188).

In typical AHL quorum sensing systems, the bacterium encodes two proteins that are essential for interpreting its population density LuxRI. Interestingly, *E. coli* (and several other species) encodes a LuxR homologue (SdiA) but lacks a LuxI homologue and therefore lacks the ability to use the AI-1 system to determine its own population

density. In 2001, Michael *et al*. proposed that SdiA is used to sense the presence of AHL producing bacteria (236). In a previous study they discovered a series of gene promoters in *S. typhimurium* that are regulated by SdiA (11). They then used the promoter of *rck* (one of the genes discovered in their screen, that had no homology to *E.coli* genes) fused to the luciferase operon to show that the addition of purified AHL to *S. typhimurium* could activate light expression. This reporter strain was then used to show that *S. typhimurium* could also detect AHL produced by bacteria in a mixed culture and that this effect was dependent on the presence of SdiA (236). If the purpose of SdiA is to detect the presence of the bacteria surrounding it, then it would make sense for SdiA to detect numerous versions of AHLs to facilitate surveillance of a broad range of bacteria. SdiA has been shown to respond to a multitude of AHLs with various chain lengths and modifications. In every instance this response was stronger than the LuxR response to the same AHL. Additionally, SdiA has the ability to respond to AHLs with thiol modifications on the lactone ring. This modification inhibited the molecule from activating LuxR (141). NMR studies of SdiA indicate that the interaction of AHL with the binding pocket is heterogeneous and that multiple AHL molecules can bind to the AHL binding pocket. The NMR study also indicated that the mechanism of SdiA is that of a "folding switch". In the absence of ligand the protein is unstructured, however when the protein encounters AHL it assumes a folded confirmation. It is believed that folding of the protein activates it and allows it to act as a transcription factor (412).

Three other studies have also been used in an attempt to understand the role of SdiA, two in EHEC and one in K-12. In EHEC, overexpression of SdiA led to reduced adherence in a tissue culture model. This reduced adherence was attributed to a decrease

in two of the LEE genes. Northern blot analysis indicated that overexpression of SdiA reduced the expression of *espD* from *LEE4* and *eae* (intimin) from *LEE5*. Western blot analysis indicated that the proteins encoded by these genes showed the same pattern (147). In the second study, signature tagged mutagenesis was performed on EHEC looking for genes required for bovine colonization. *sdiA* was one of the genes required for EHEC to colonize the cow (72). The final study utilized a promoter trap assay to find promoters that were activated by AHL, only in the presence of *sdiA*. One of the promoters identified in this study was that of *gadA*, which encodes one of the proteins required for the glutamate-dependent acid resistance response (AR2) in *E.coli*. The authors then attempted to quantify acid resistance in the K-12 WT and *sdiA* mutant strains in the presence and absence of AHL. They found that the addition of AHL, after 44 hours of growth, could increase the acid resistance of K-12 by 0.2 log (CFU/mL) (377). Unfortunately, this study was performed in LB media. When grown in LB, *E.coli* does not use the AR2 for acid resistance (42).

THE AI-2 SYSTEMS

The AI-2 quorum sensing system has been extensively characterized in multiple bacteria and is remarkably complex. This system was originally discovered in the common marine bacterium *Vibrio harveyi* as an alternative to the classic LuxIR system in the regulation of luminescence (19). Depending on the bacterial species the AI-2 signal is either (2R,4S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (R-THMF) (*E.coli*, *Salmonella*, and others) or S-THMF-borate (for *Vibrio* spp.). AI-2 is a byproduct of the activated methyl cycle which generates S-adenosyl-L-methionine a molecule used in the methylation of proteins, RNA, DNA and certain metabolites. Durring the activated methyl cycle S-adenosyl-L-

methionine is converted to S-adenosyl-L-homocysteine which is enzymatically cleaved by the enzyme Pfs into adenine and S-ribosylhomocysteine the substrate of LuxS. LuxS converts S-ribosylhomocysteine into homocystine and 4,5-dihydroxy-2,3-pentanedione (DPD) the precursor or AI-2. DPD then spontaneously cyclizes into the aforementioned AI-2 furanones (380).

There are two AI-2 signaling mechanisms. The first system is used by various *Vibrio* species and has been characterized extensively. In this system, AI-2 diffuses into the periplasm where it interacts with the periplasmic binding protein LuxP, which then interacts with the inner membrane histidine kinase LuxQ activating its phosphatase activity (19). In the absence of AI-2, LuxQ autophosphorylates and transfers its phosphate to LuxU, which then transfers its phosphate to the response regulator LuxO (90) (91) (92) (20). Phopho-LuxO interacts with σ^{54} to activate transcription of small regulatory RNAs. Through Hfq, these small regulatory RNAs destabilize the mRNA encoding LuxR (197) (191). LuxR is the transcriptional activator of the genes required for light production (19). The presence of AI-2 inhibits the phosphrelay by activating the phosphatase activity of LuxQ which removes the phosphate from LuxU and subsequently allows for the production of LuxR.

A second AI-2 signaling mechanism has been described in *Salmonella typhimurium* and *E. coli*. In these organisms, AI-2 controls the production of the *lsr* operon. This operon encodes the genes required for the uptake and processing of AI-2. The *lsr* operon encodes an ABC transporter, that is required for active uptake of AI-2 (357). Once inside the cell AI-2 is phosphorylated by LsrK (407) (408). Genetic studies indicate that phospho-AI-2 derepresses the *lsr* operon thereby rapidly increasing the

internalization of AI-2. Phospho-AI-2 binds to and is believed to antagonize, LsrR, a repressor of the *lsr* operon. LsrG another gene product of the *lsr* operon cleaves AI-2 and therefore deactivates the uptake cycle (408). Other than the *lsr* operon, no other genes have been found to be directly regulated by LsrR. This observation led to the proposal that the purpose of this second AI-2 system is to intercept and destroy the AI-2 signals produced by other bacteria (406). Several groups have used mutations in *luxS,* in at least 24 bacterial species, as a means to understand the role of AI-2 mediated cell-cell signaling (369). The findings of these studies should be reexamined based on the critical role of LuxS in metabolism (386).

The AI-3 SYSTEM

The AI-3 system is the least understood of all the QS systems and is best characterized in EHEC. Work in this field was based on the discovery that a small molecule signal could regulate the LEE genes and the flagellar regulon in EHEC (348). The chemical nature of the AI-3 signal remains unknown, but may be a group of signals similar to how AI-1 constitutes a group of signals (269). AI-3 is produced by many Gram-negative bacteria, including human commensal species like non-pathogenic *E.coli* and *Enterobacter cloacae* as well as by the human pathogens of the *Shigella*, *Salmonella* and *Klebsiella* species (386). The receptor for AI-3 is the sensor kinase QseC (48). Regulation by QseC occurs through its cognate response regulator QseB which is autoregulatory and regulates the flagellar regulon through a direct interaction with the *flhDC* promoter (49) (50). Two-component systems are the major signal transduction systems in bacteria. Typically the system is composed of an inner membrane sensor kinase (QseC) and a cytoplasmic response regulator (QseB). Usually, the sensor kinase receives some kind of signal (AI-

3) and autophosphorylates on a conserved histidine residue. It then transfers the phosphate to a response regulator on a conserved aspartate. The response regulator is a transcription factor that then can activate genes through a direct interaction with their promoters (136). Interestingly, QseC also contains a putative EAL domain (48). Proteins with EAL domains hydrolyze the second messenger cyclic dimeric GMP (320). The presence of an EAL domain indicates that QseC may be involved in extracellular and intracellular bacterial signaling mechanisms.

The means by which AI-3 regulates the LEE genes is not yet clear, but it likely occurs through the LysR-like regulator QseA (348) (344) (326). It is believed that EHEC uses AI-3 to determine its location and the bacterial population density in the human GI tract. Using this knowledge the bacterium will activate its virulence factors in a manner that will allow for their most efficient use.

BACTERIA-HOST CATECHOLAMINE SIGNALING

In mammals, the stress response catecholamine hormones, epinephrine and norepinephrine, regulate multiple processes; most notably, the stress related "fight or flight" response. In the gut, which is rich in adrenergic neurons, these hormones are responsible for the intestinal smooth-muscle contraction, submucosal blood flow and chloride and potassium secretion (128) (77). Norepinephrine is produced by the adrenergic neurons of the enteric nervous system and at homeostasis it is likely the predominant of the two hormones present in the gut with levels reaching the micromolar range (77) (99). During the stress response, epinephrine is released from the central nervous system and the adrenal medulla into the blood stream where it acts systemically on multiple organ systems including the gut (292). Humans possess nine adrenergic

receptors divided into three classes: α1, α2, and β. These receptors are members of the G-protein coupled receptor (GPCRs) family. Ligand (epinephrine and norepinephrine in the case of the adrenergic receptors) binding induces a conformational change which results in binding to GTP and subsequent intracellular signal transduction. Currently, two adrenergic receptors, β_1 and β_2, have been crystallized, both in the absence of agonists (44) (392). Despite the absence of an agonizing ligand, structural analysis indicated that the ligand-binding pocket of both receptors can accommodate epinephrine and norepinephrine, indicating that the ligands can share the same receptor (378).

Multiple studies have shown that enteric bacteria are responsive to epinephrine and norepinephrine. This phenomenon was first established when Lyte *et al.*, discovered that the addition of norepinephrine and epinephrine to Gram-negative bacteria, including *E. coli* and *Yersinia enterocolitica*, caused an increase in the bacterial growth rate (214). Norepinephrine was subsequently shown to increase the production of Stx in EHEC and to activate adhesin expression in enterotoxigenic *E. coli* (211). Additionally, a series of studies have indicated that norepinephrine facilitates bacterial iron acquisition (93) (94). However, it appears that norepinephrine does not aid in the actual uptake, but causes eukaryotic iron-binding proteins to release free iron (94). Norepinephrine increases the growth rate and motility of *Campylobacter jejuni* and stimulates growth and induces production of the TTSS1 in *Vibrio parahaemolyticus* (51) (249) (250). Epinephrine regulates the LEE genes and the flagellar regulon in EHEC (348). The epinephrine/norepinephrine receptor was discovered as the AI-3 sensor kinase QseC (48). QseC directly regulates the flagellar regulon through its cognate response regulator QseB, which binds to the *flhDC* promoter (50). Sensing of epinephrine/norepinephrine through

QseC is required for full virulence in rabbit models of infection (48) (294). In *S. Typhimurium, qseC* mutants were unable to colonize pigs as well as the WT strain (22). QseC homologues are present in at least 25 animal and plant pathogens including *Salmonella* sp., *Francisella tularensis*, *Pseudomonas aeuriginosa*, *Yersinia* sp., and *Vibrio* sp. (294). A second epinephrine sensing system has also been reported for EHEC. The two-component system, QseEF, can respond to epinephrine, sulfate, and phosphate and, in conjunction with QseG, an outer membrane protein, mediates EHEC pedestal formation (298). QseF, the response regulator, indirectly activates the transcription of EspFu, a secreted bacterial effector required for actin polymerization (299).

THE HISTORY OF QSEBC

The *qseBC* genes were first discovered by Sperandio *et al*. as an operon potentially regulated by the AI-2 quorum sensing system in EHEC. The AI-2 system was also thought to be involved in regulation of the flagellar genes (346). The link between these two observations was made when an EHEC *qseC* mutant was shown to express less flagellin and had decreased motility as compared to WT. This finding was substantiated when *lacZ* promoter fusions of the flagellar genes *flhD*, *fliA*, *motA* and *fliC* all showed decreased β-galactosidase activity in the *qseC* mutant. Furthermore, it appeared that QseBC was somehow acting through the FlhDC flagellar master regulator to activate expression of *fliA* (349). This study was followed by the discovery that AI-2 was not the signaling system used, but that a new signal, termed AI-3, was the QseC signal. Additionally, epinephrine was also shown to act through QseC, because epinephrine could only activate *flhDC* expression in the presence of QseC (348). Clarke *et al*. identified the transcriptional start site of the EHEC *flhDC* promoter and used this

knowledge to create a series of nested deletions of the *flhDC* promoter fused to *lacZ*. These constructs were used to compare *flhDC* activation in WT and the *qseC* mutant in order to identify promoter regions activated by QseBC. Two potential activation sites were discovered, -900 to +50 which showed a 21-fold decrease in the *qseC* mutant and -300 to +50 which showed a 3-fold decrease. Interestingly, the -650 to +50 region was repressed in all constructs indicating that this region may represent a repressor binding site. The -900 to +50 (distal) and -300 to +50 (proximal) *flhDC* promoter regions were both shown to be bound by QseB with a preference for the distal promoter, which matched the promoter fusion data (50). Similar experiments were conducted to identify the promoter regions bound by QseB to activate the *qseBC* promoter. Once again two promoter regions were bound by QseB. In both instances, DNaseI footprints were performed and the specific QseB binding sites were identified. This work led to the development of a QseB consensus sequence (49). Several genetic studies had indicated that epinephrine and AI-3 were acting on QseC to facilitate downstream gene activation, but the work by Clarke *et al.,* showed that QseC could be directly activated with epinephrine, norepinephrine, and AI-3 *in vitro*. QseC purified in lipid vesicles was able to respond to these hormones by autophosphorylation and ^{3}H-norepinephrine was shown to bind directly to QseC. Additionally, QseC could then phosphotransfer its phosphate onto its cognate response regulator QseB. This activity was specific for epinephrine, norepinephrine and AI-3 because other intestinal hormones like gastrin, secretin, and galanin had no effect in this system and because signaling could be blocked with the α-adrenergic receptor antagonist phentolamine. Finally, the *qseC* mutant was shown to be attenuated for virulence in a rabbit model of infection (48). Since, QseC is encoded in

multiple bacterial pathogens and because mutation of *qseC* leads to attenuation, small molecule inhibitors have now been developed to target QseC signaling. These compounds are effective at attenuating infection in animal models of *S. typhimurium* and *F. tularensis* (294).

THE FLAGELLAR REGULON

Generally, in the enterobacteriaceae, the flagellar regulon is composed of three classes of genes that are hierarchally expressed based on the timing of the flagellar organelle assembly (180). Genetic regulation is complex and requires the specific temporal expression of over 50 genes spread over 15 operons (45). The first class (the early genes) is comprised up of two genes, *flhD* and *flhC* transcribed as a single operon which encodes the transcriptional activation complex FlhDC, the master regulators. These proteins form a heterotetrameric complex which binds to at least 10 σ^{70}-dependent middle and late gene promoters and activates their expression (180) (411) (204) (389). The middle genes encode the proteins required for formation of the hook basal body (containing a type III secretion system), an intermediate structure in flagellar assembly, the σ^{28} alternative sigma factor (*fliA*) required for expression of the late genes, and the anti-sigma factor FlgM (145) (261) (110). σ^{28} is also involved in a feedback loop where it can activate expression of the early and middle genes from unique σ^{28}-dependent promoters (134) (177) (179) (203). After translation, the σ^{28} is unable to activate transcription due to its interaction with FlgM (262). This interaction is essential to the timing of late gene expression. FlgM dissociates from σ^{28} only after completion of the hook basal body, which secretes FlgM (178). Once the hook basal body is complete, σ^{28} with RNA

polymerase will activate expression of the late genes which encode the proteins for the motor torque generator, chemotaxis, and the flagellins (317).

Genetic regulation of *flhDC* requires a high level of complexity since it is the main checkpoint for flagellar synthesis. Multiple systems, including quorum sensing, and have been directly shown to regulate or are implicated in regulation of *flhDC* expression. These include temperature (7), osmolarity, OmpR (333), cell cycle control (257), cAMP-CRP (336) (413), RcsCDB (87), H-NS (26), IHF (414), LrhA (189), and GrlR (137). This system is made even more complex by the fact that *flhD* is also involved in regulation of cell-division (289) (288).

ACID RESISTANCE IN *E. COLI*

The extremely low infectious dose required for infection indicates that EHEC has adapted multiple mechanisms to surpass the harsh environments it encounters on its journey from the mouth to the colon. Beyond the host innate immune responses the bacterium must resist acid pH, bile salts, and high osmolarity (285). *E. coli* can survive for several hours at pH 2.5, giving it time to pass through the acidic compartments of the animal GI tract. To survive at this pH, *E. coli* has evolved three unique acid resistance systems termed AR1, AR2 and AR3. AR1, the glucose-repressed system, was the first acid resistance system discovered and the least characterized. RpoS is known to play a role in this system as is cAMP and its binding protein CRP. Since glucose represses production of cAMP, the system is called the glucose-repressed system (42). The second acid resistance system, the AR2 (the glutamate-dependent system) was discovered when bacteria grown in minimal media at pH 2.5 (which normally kills the bacteria) were able to survive for several hours upon the addition of glutamate. The AR2 is a

decarboxylase/antiporter system that relies on the function of the proteins encoded by *gadA* and *gadBC* genes. GadA and GadB are both glutamate decarboxylases and are redundant in the system (341). GadC is the antiporter (124). The decarboxylases replace the α-carboxyl group of glutamate with a proton that is recruited from the cytoplasm. The reaction creates CO_2 and γ-amino butyric acid (GABA). The antiporter exports GABA from the cell while importing another glutamate molecule, thus providing new fuel for the system. Genetic regulation of this system is complex and revolves around a general intermediary in the system GadE, a transcription factor that activates *gadA* and *gadBC* expression (Figure 1.5) (215). At least three unique regulatory cascades intersect *gadE*. The first pathway is the best characterized and most complex. This pathway begins with the repression of cAMP production by the presence of glucose and acidic conditions (217). In the absence of cAMP, CRP is unable to inhibit the expression of the sigma factor RpoS (182). This sigma factor is required for the activation of *gadX* and *gadW* (217). The transcription factors encoded by these two genes both activate *gadE* expression and act as the negative regulators to limit their own expression and expression of *gadBC* and *gadA* in order to complete the regulatory circuit (217) (366). GadW represses *rpoS*, which will repress *gadX* and *gadW*. Additionally, GadX represses *gadW*.

The second pathway involves the two-component system EvgSA. Acidic conditions activate EvgS through an unknown mechanism. EvgS activates EvgA which can activate *gadE* expression and activates expression of a transcription factor called YdeO which can also activate *gadE* expression (216). The final pathway is the least characterized but is known to require the GTPase TrmE, a protein involved in tRNA modification. TrmE has been shown to indirectly activate *gadE* (111).

The third acid resistance system, AR3, the arginine-dependent system, also uses a decarboxylase/antiporter system encoded within the *adiA* and *adiC* genes (199) (112). Genetic regulation of this system is not well understood, but the LysR-like regulator CysE has been shown to activate *adiA* and *adiC* (330).

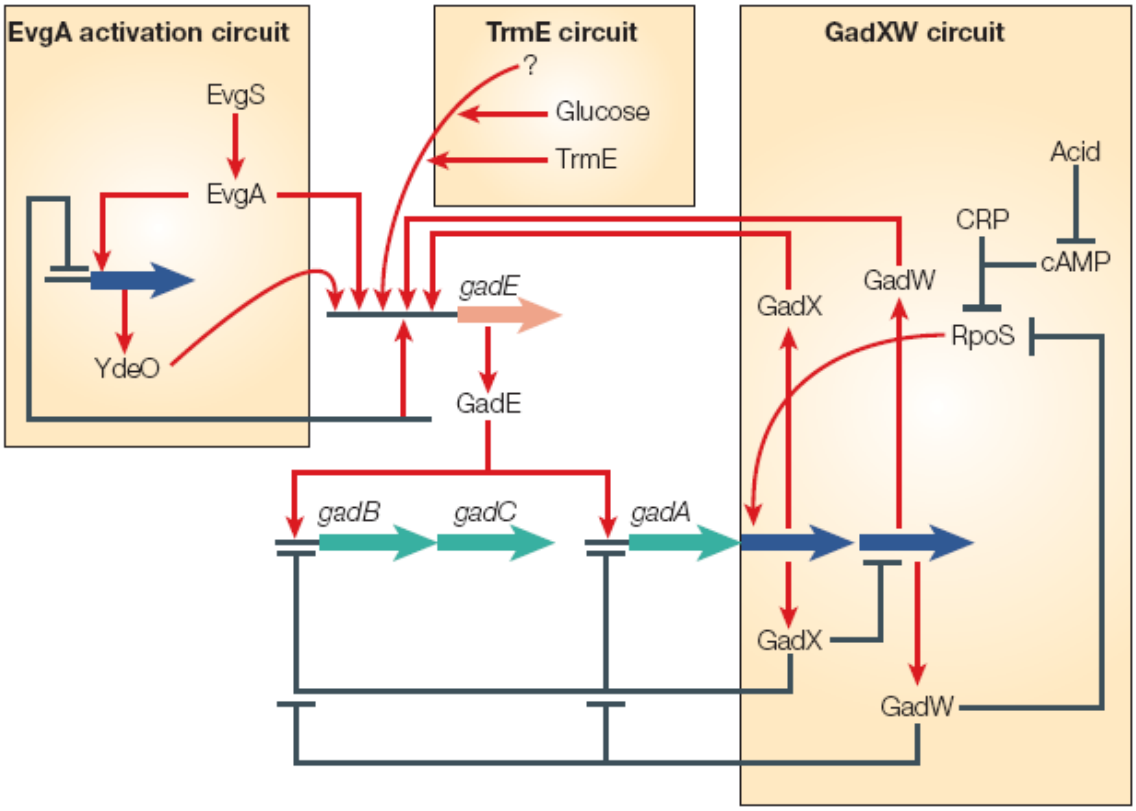

Figure 1.5. Genetic regulation of the AR2. At least three unique signaling pathways regulate the AR2, they include: the EvgA pathway, TrmE and the GadXW pathway.

CHAPTER TWO

OVERALL OBJECTIVE AND SYNOPSIS

EHEC is a human pathogen that causes hemorrhagic colitis and hemolytic uremic syndrome. EHEC infection leads to over 70,000 illnesses in the U.S.A. and is the leading cause of acute renal failure in children. EHEC uses cell-cell signaling to regulate multiple virulence mechanisms including LEE genes, the flagellar regulon, and acid resistance. These mechanisms have likely evolved to allow EHEC to sense its environmental conditions and respond accordingly. Previously, we have demonstrated that EHEC contains a cell-cell signaling receptor, QseC, which is able to respond to signals produced by other pathogens, the host microbiota, and the host hormones epinephrine and norepinephrine. This signal response led to *qseBC* autoregulation and *flhDC* regulation through a direct interaction of phosphorylated QseB with the *qseBC* and *flhDC* promoters.

The fact that QseC has evolved to interpret such global signals has led us to speculate that QseC's role in EHEC is beyond that of autoregulation and regulating the flagellar regulon. To pursue this hypothesis we undertook a series of experiments to deconstruct the regulatory cascade of QseC. Using biased and non-biased approaches we concluded that QseC plays an intricate role in the regulation of multiple signaling systems in EHEC. Genetic regulation of these pathways was facilitated, in part, by a direct phosphotransfer from QseC to QseB, and two newly identified interaction partners QseF and KdpE. Each of these transcription factors was shown to be involved in QseCs regulation of the pathways governing actin polymerization, HUS, protein export, flagellar regulation, potassium uptake and osmolarity, and AE lesion formation.

Mutation of *qseC* leads to a reduction of motility and reduced expression of genes in all three classes of the flagellar regulon. Mutation of *qseB* however has no effect on motility or any of the flagellar genes. Interestingly, overexpression of QseB leads to a reduced motility phenotype similar to the *qseC* mutant. In our attempt to interpret this phenomenon, we discovered a new QseB binding site in the *flhDC* promoter. This binding site was in the -650 to -300 region of the promoter, where, based on *lacZ* fusions to the *flhDC* promoter, we had previously hypothesized a repressor may bind. Unlike all other QseB-DNA interactions studies, this interaction did not require QseB phosphorylation. These findings indicate that QseB is bimodal in its regulation of *flhDC*. In the presence of abundant signal, QseC will phosphorylate QseB which will activate the flagellar regulon. In the absence of signal QseB will remain unphosphorylated and act as a repressor.

We have principally studied the role of cell-cell signaling in terms of pathogenesis of humans. In this dissertation, we are now beginning to address the role of cell-cell signaling in colonization. These studies are based on the finding that a cell-cell signaling protein, SdiA, was required for EHEC to successfully colonize cattle (72). SdiA is a LuxR homologue that is used by EHEC to sense the presence of AHL producing bacteria. Additionally, SdiA is required for EHEC to colonize cows. These two previous observations encouraged us to undertake a phenotypic analysis of an EHEC *sdiA* mutant in the presence and absence of ligand. To this end, we used microarray analysis of EHEC WT and *sdiA* mutants in the presence and absence of ligand to discover that SdiA represses the LEE genes in the presence of AHL and this repression can be attributed to a direct interaction of SdiA with the *ler* (the master regulator of the LEE genes) promoter.

Our array analysis coupled with a series of genetic and biochemical studies indicated that SdiA activated the glutamate-dependent acid resistance response (AR2) although the precise role of SdiA in this activation was not clear. Finally, we confirmed previous findings that *sdiA* mutants are not as fit as WT for cow colonization and that rumen extracts contain AHLs. Furthermore, these extracts could elicit repression of the LEE genes partially through SdiA and activate the AR2 genes.

Through two separate studies using both genetic and biochemical methodologies we have identified genes in EHEC that are involved in cell-cell signaling mechanisms for both its pathogenic host and its commensal host. Our understanding of mechanisms used by EHEC to infect one host and colonize another will allow us to pursue therapies to prevent EHEC from sickening an individual or prevent that individual from ever encounteringEHEC.

CHAPTER THREE

MATERIALS AND METHODS

STRAINS AND PLASMIDS

All bacterial strains, plasmids, and ologonucleotides utilized in this study are listed in Tables 3.1 and 3.2. *E. coli* strains were grown aerobically in LB or DMEM (Invitrogen) medium at 37°C unless otherwise stated. Antibiotics were added at the following concentrations: 100 μg ml^{-1} ampicillin, 30 μg ml^{-1} chloramphenicol, and 50 μg ml^{-1} kanamycin.

RECOMBINANT DNA TECHNIQUES

Standard methods were used to perform plasmid purification, PCR, ligation, restriction digests, transformation and gel electrophoresis (315).

Plasmid pDH6 was constructed by PCR amplification the *sdiA* gene, with an additional 210 base pairs upstream of the predicted start site, from the 86-24 genome using JumpStart AccuTaq LA DNA Polymerase (Sigma) with the primers sdiApromoF and sdiApromoR and cloning the resulting PCR product into the *Sma*I cloning site of the pACYC177 vector. pDH10 was generated using the same PCR product as described above but, cloned into the TOPO PCR blunt vector (Invotrogen). pDH5 was constructed by PCR amplification the *sdiA* gene

ISOGENIC MUTANT CONSTRUCTION

Construction of isogenic *kdpE* (DH11) and *qseB* (MC474) mutants was carried out as previously described (58). Briefly, 86-24 cells containing pKD46 were prepared for electroporation. A *kdpE* PCR product was generated using primers kdpEλRed-F and kdpEλRed-R and pKD3 as a template and PCR-purified (Qiagen). A *qseB* PCR product

was generated using primers qseBλRed-F and qseBλRed-R and pKD3 as a template and PCR-purified (Qiagen). Electroporation of the PCR products into these cells was performed; cells were incubated at 22°C for 16 h in SOC, and plated on media containing 30 μg ml^{-1} chloramphenicol overnight at 42°C. Resulting colonies were patched for chloramphenicol resistance and ampicillin sensitivity, and PCR-verified for the absence of the gene. The chloramphenicol cassette was then resolved from the mutants in order to create non-polar, isogenic *kdpE* and *qseB* mutants. Plasmid pCP20, encoding a resolvase, was electroporated into the mutant strains, and resulting colonies were patched for chloramphenicol sensitivity. Construction of *qseC* and *qseF* mutants has been previously published

Construction of the *sdiA* mutant (DH1) was carried out as previously described (68). The plasmid pDH10 was constructed by PCR amplification the *sdiA* gene, with an additional 210 base pairs upstream of the predicted start site, from the 86-24 genome using JumpStart AccuTaq LA DNA Polymerase (Sigma) with the primers sdiApromoF and sdiApromoR and cloning the resulting PCR product into TOPO PCR blunt vector (Invitrogen). Plasmid pDH10 was digested with *Sac*II (there is a single *Sac*II site in the middle of *sdiA* gene) and blunt ended with the Klenow fragment of DNA polymerase of *E. coli*. A chloramphenicol resistance cassette (*cat*) amplified from pACYC184 with *Pwo* polymerase (Boehringer Mannheim) and primers CmF and CmR (349) was cloned into the blunt-ended *Sac*II site of pDH10, generating plasmid pDH7, which has the cloned *sdiA* gene interrupted by *cat*. Plasmid pDH7 was digested with *Sac*I and *Eco*RV, and the *sdiA*::*cat* cassette was cloned into plasmid pCVD442 (68) digested with *Sac*I and *Sma*I, generating plasmid pDH8. The EHEC *sdiA* mutant (named DH1) was generated by allelic

exchange using vector pDH8, and the mutants were selected on plates containing chloramphenicol and 5% sucrose as previously described (68). The *sdiA* mutant (DH1) was complemented with plasmids pDH5 and pDH6, and pDH10 generating strains DH2, DH3, and DH4 respectively.

SITE-DIRECTED MUTAGENESIS

Site-directed mutagenesis was carried out using the Quick Change II site-directed mutagenesis kit (Stratagene). Mutagenesis PCR primers were constructed using the Primer X software (http://www.bioinformatics.org/primerx/) and are listed in Table 3.2 (qseBD51AF and qseBD51AR). The plasmid pVS154 was PCR amplified with the mutagenesis primers according to Stratagene's PCR protocol, generating the plasmid pDH12 (86-24 *qseB* D51A in pBADMycHis). The PCR product was digested with DpnI for 3 h at 37^{o}C in order to remove the template plasmid. After digestion, the PCR product was transformed into XL-1 Blue supercompetent cells (Stratagene) and plated on selective media. The next day, plasmid DNA was isolated and sequenced to determine if the mutation was present.

RNA EXTRACTION AND REAL-TIME RT-PCR

Cultures were grown aerobically in LB medium at 37°C overnight, diluted 1:100 in LB or DMEM (in the presence of self produced AI-3 and in the absence or presence of 10 μM epinephrine) and grown aerobically at 37°C. 0.2% arabinose was added to the media when induction was required. For the AHL studies, 10 μM N-(β-Ketocaproyl)-L-homoserine lactone (C6-oxo-HSL) or (3-Oxooctanoyl) -L-homoserine lactone (C8-oxo-HSL) (Sigma) in ethyl acetate was added to the flask and allowed to evaporate in the dark for 10 min before the addition of the DMEM. For rumen extract studies, 5 μL of the

evaporated rumen extract was added DMEM. For samples assessed without signals, the respective solvents were used at the same volumes to ensure that the solvent did not alter gene expression. RNA from three biological replicate cultures of each strain was extracted at the late exponential growth phase (OD_{600} of 1.0) using the RiboPure Bacteria RNA isolation kit (Ambion) according to the manufacturer's guidelines. The primers used in the real-time assays were designed using Primer Express v1.5 (Applied Biosystems). Real-time reverse transcription-PCR (RT-PCR) was performed in a one-step reaction using an ABI 7500 sequence detection system (Applied Biosystems). For each 20 µl reaction mixture, 10 µl 2x SYBR master mix, 0.1 µl Multi-Scribe reverse transcriptase (Applied Biosystems), and 0.1 µl RNase inhibitor (Applied Biosystems) were added. Amplification efficiency of each of the primer pairs was verified using standard curves of known RNA concentrations. Melting-curve analysis was used to ensure template specificity by heating products to 95°C for 15 s, followed by cooling to 60°C and heating to 95°C while monitoring fluorescence. Once the amplification efficiency and template specificity were determined for each primer pair, relative quantification analysis was used to analyze the unknown samples using the following conditions for cDNA generation and amplification: 1 cycle at 48°C for 30 min, 1 cycle at 95°C for 10 min, and 40 cycles at 95°C for 15 s and 60°C for 1 min. The *rpoA* (RNA polymerase subunit A) gene was used as the endogenous control. Real-time RT-PCR primers for the LEE genes and *rpoA* have been previously described (387).

ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

In order to study the binding of QseB to the *flhDC* promoter EMSAs were performed using the purified QseB protein and the *flhDC* promoter. DNA probes were then end-

labeled with [γ-32P]-ATP (NEB) using T4 polynucleotide kinase using standard procedures (315). End-labeled fragments were run on a 5% polyacrylamide gel, excised and purified using the Qiagen PCR purification kit. Electrophoretic mobility shift assays were performed by adding increasing amounts of purified QseB or QseBD51A protein (0–20 μM) to end-labeled probe (10 ng) in binding buffer [500 μg ml^{-1} BSA (NEB), 50 ng μl^{-1} poly-dIdC, 60 mM HEPES pH 7.5, 5 mM EDTA, 3 mM DTT, 300 mM KCl, 25 mM MgCl2] with or without 0.1 M acetyl phosphate for 20 min at 4°C.

In order to study the binding of SdiA to the *ler* promoter EMSAs were performed using the purified SdiA protein and the *ler* promoter. The *ler* promoter was defined as -100 bp upstream of the *ler* start site and to +202 bp downstream of the *ler* start site. This region was digested with *Eco*RI and *Bam*HI from the construct pVS219. The *kan* promoter region, used as a negative control, was amplified from the plasmid TOPO PCR blunt using primers kanF and kanPromoterR. The *ftsQ* promoter, used as a positive control, (410) was amplified using primers ftsQF and ftsQR. The *gadW* promoter was amplified using primers gadWF and gadWR. DNA probes were then end-labeled with [γ-32P]-ATP (NEB) using T4 polynucleotide kinase using standard procedures (315). End-labeled fragments were run on a 5% polyacrylamide gel, excised and purified using the Qiagen PCR purification kit. Electrophoretic mobility shift assays were performed by adding increasing amounts of purified SdiA protein (0–30 pmol) to end-labeled probe (10 ng) in binding buffer [25 μg ml^{-1} BSA, 50 ng poly-dIdC, 50 mM Tris-HCl pH 7.8, 0.1 mM EDTA, 0.1 mM dithiothreitol (DTT), 50 mM NaCl, and 3 mM magnesium acetate] for 20 min at 22°C.

Immediately before loading, a 5% Ficol solution was added to the mixtures. The reactions were electrophoresed for approximately 14 h at 65 V on a 5% polyacrylamide gel, dried and exposed to KODAK X-OMAT film.

MICROARRAYS

Microarrays and analysis were performed as previously described (160). The GeneChip *E. coli* Genome 2.0 array system of the Affymetrix system was used to compare the gene expression in strain 86-24 to various isogenic mutants. The GeneChip *E. coli* Genome 2.0 array includes approximately 10,208 probe sets for all 20,366 genes present in the following four strains of E. coli: K-12 lab strain MG1655, uropathogenic strain CFT073, O157:H7 enterohemorrhagic strain EDL933, and O157:H7 enterohemorrhagic strain Sakai (http://www.affymetrix.com/products/arrays/specific/ecoli2.affx). The RNA-processing, labeling, hybridization, and slide-scanning procedures were preformed as described in the Affymetrix Gene Expression Technical Manual (http://www.affymetrix.com/support/technical/manual/expression_manual.affx).

The output from scanning a single replicate of the Affymetrix GeneChip *E. coli* Genome 2.0 array for each of the biological conditions was obtained using GCOS v 1.4 according to the manufacturer's instructions. Data were normalized using Robust Multiarray analysis at the RMAExpress website (http://rmaexpress.bmbolstad.com/). The resulting data were compared to determine features whose expression was increased or decreased in response to inactivation of the *sdiA*, *qseC, qseB, qseF* and *kdpE* genes or in the presence of AI-3, epinephrine, or C6-oxo-HSL. Custom analysis scripts were written in Perl to complete multiple array analyses. The results of the array analyses were further confirmed using real-time RT-PCR as described. We note that the isolate used in these

studies has not been sequenced and thus is not fully contained on the array and that differences in genome content are evident. Expression data can be accessed using accession number (GSE15050) at the NCBI GEO database.

MOTILITY ASSAY

Assays were performed as previously described (50). Briefly, motility assays were performed at 37°C on 0.3% agar plates containing Tryptone media (1% tryptone and 0.25% NaCl). The motility halos were measured at 4 h and 8 h.

SDIA EXPRESSION VECTOR CONSTRUCTION AND PROTEIN PURIFICATION

Plasmid pDH5 was constructed by PCR amplification of the *sdiA* gene from the 86-24 genome using JumpStart AccuTaq LA DNA Polymerase (Sigma) with the primers sdiAHisF and sdiAHisR and cloning the resulting PCR product into the *Hind*III and *Sph*I cloning site of the pQE30 vector.

The strain DH9 was constructed by transforming pDH5 into DH5α. 4 L of DH9 were inoculated at 1:100 and grown to O.D. 0.6 at 30^{o}C in the presence of 400 μM C6-oxo-HSL (Sigma) and 400 μM N-(3-Oxooctanyl)-DL-homocysteine thiolactone (C8-oxo-HTL) (Omm Scientific). The culture temperatures were reduced to 25^{o}C, induced with 400 μM IPTG (Sigma), and grown for 18h. Cells were harvested, suspended in lysis buffer (50 mM phosphate buffer pH 8, 300 mM NaCl, 20 mM imidazole, 400 μM C6-oxo-HSL, and 400 μM C8-oxo-HTL) and lysed by homogenization. The lysed cells were centrifuged and the lysates were loaded onto to a Ni^{2+}-NTA-agarose gravity column (Qiagen). The column was washed with lysis buffer and protein was eluted with elution buffer (50 mM phosphate buffer pH 8, 300 mM NaCl, 250 mM imidazole, 400 μM C6-

oxo-HSL, and 400 μM C8-oxo-HTL). Fractions containing SdiA were confirmed by SDS-PAGE and concentrated for further use. For QseB, QseC and all other proteins purified in this manuscript, one liter of LB media was inoculated at 1:100 and grown to O.D. 0.6 at 30^{o}C. The culture temperatures were reduced to 25^{o}C, induced with 400 μM IPTG (Sigma) or 0.2% arabinose, and grown for either 3 h or 18 h. Cells were harvested, suspended in lysis buffer (50 mM phosphate buffer pH 8, 300 mM NaCl, and 20 mM imidazole) and lysed by homogenization. The lysed cells were centrifuged and the lysates were loaded onto to a Ni^{2+}- NTA-agarose gravity column (Qiagen). The column was washed with lysis buffer and protein was eluted with elution buffer (50 mM phosphate buffer pH 8, 300 mM NaCl, 250 mM imidazole). Fractions containing purified protein were confirmed by SDS-PAGE and concentrated for further use.

RECONSTITUTION OF QSEC INTO LIPOSOMES

Liposomes were reconstituted as described previously (48, 140). Briefly, 50 mg of *E. coli* phospholipids (20 mg/ml in chloroform; Avanti Polar Lipids) were evaporated and then dissolved into 5 ml of potassium phosphate buffer containing 80 mg of N-octyl-β-d-glucopyranoside. The solution was dialyzed overnight against potassium phosphate buffer. The resulting liposome suspension was subjected to freeze–thaw in liquid N2. Liposomes were then destabilized by the addition of 26.1 mg of dodecylmaltoside, and 0.625 mg of QseC-MycHis was added, followed by stirring at room temperature for 10 min. Two hundred-sixty milligrams of Biobeads (Biorad) were then added to remove the detergent, and the resulting solution was allowed to incubate at 4°C for 16 h. The supernatant was then incubated with fresh Biobeads for 1 h at 22^{o}C the next day. The

resulting liposomes containing reconstituted QseC-MycHis were frozen in liquid N2 and stored at −80°C until used.

AUTOPHOSPHORYLATION AND PHOSPHOTRANSFER ASSAYS

Assays were performed as previously described (48). Briefly, 20 μL of the liposomes containing QseC-MycHis were adjusted to 10 mM MgCl2 and 1 mM DTT, and 10 μM epinephrine, frozen and thawed rapidly in liquid N2, and kept at room temperature for 1 h (this allows for the signals to be loaded within the liposomes). [γ32P]dATP (0.625 μl) (110 TBq/mmol) was added to each reaction. To some reactions, 12.5 μg of response regulator was added. At each time point (0, 10, 30 min), 10 μl of SDS loading buffer (with 20% SDS, to completely denature the liposome) was added. For all experiments involving QseC alone, a time point of 10 min was used. The samples were run on SDS/PAGE without boiling and visualized via PhosphorImager. The bands were quantitated by using imagequant version 5.0 software (Amersham Pharmacia).

β-GALACTOSIDASE ASSAYS

Assays were performed as previously described (50). Briefly, bacteria containing *lacZ* fusions were grown overnight at 37°C in LB containing the appropriate selective antibiotic. Cultures were diluted 1:100 and grown in LB, and when necessary supplemented with 0.2% arabinose, to an OD_{600} of 1.0 at 37°C. These cultures were then assayed for β-galactosidase activity using o-nitrophenyl-beta-d-galactopyranoside (ONPG) as a substrate as described previously (238).

ACID RESISTANCE ASSAY

The glutamate-dependent acid resistance system was tested as previously described (42). Cells were grown overnight in LB with 0.4% glucose, which represses the RpoS-

dependent oxidative system (42), and diluted into E minimal media (pH 2.5) [60 mM K_2HPO_4, 20 mM $NaNH_4HPO_4$, 9.5 mM citric acid, 800 μM $MgSO_4$] with 0.4% glucose and 40 μM glutamate. Cells were incubated at 37°C without shaking and viable-cell counts were determined at 0, 1, and 2 h after the acid challenge.

WESTERN BLOTTING

Whole cell lysates and secreted proteins from EHEC strains 86-24, DH1, and DH2 were prepared as described previously (387) (142). Briefly, strains were grown to an OD_{600} of 1.0 in DMEM at 37°C. For the AHL studies, 10 μM C6-oxo-HSL in ethyl acetate was added to the flask and allowed to evaporate in the dark for 10 min before the addition of the DMEM. Cells were harvested and lysed as described previously (387). Samples were quantified using the Bio-Rad Protein Assay and separated by SDS-PAGE. The samples were then subjected to immunoblotting, as previously described (315), with rabbit polyclonal antisera to EspA (kindly provided by James Kaper) and a mouse monoclonal antibody to RpoA (Neoclone) and visualized with enhanced chemiluminescence (Bio-Rad).

RUMEN EXTRACTION AND AHL DETECTION

AHL extraction and detection were performed as previously described (327). Briefly, 50 ml of rumen fluid was extracted 2 times with dichloromethane and concentrated to 5 μL using a rotary evaporator. For analytical TLC, 5 μL of concentrated extract was applied to C_{18} reverse-phase TLC plates (200 μM layer, Whatman) and the chromatograms were developed with methanol/water (70:30, vol/vol). After development, the dried plates were overlaid with a culture of the *Agrobacterium tumefaciens traI::lacZ* (96) indicator strain and 80 μg/mL X-gal for 16 h at 30°C. For preparative plates, 50 mL of GI content

was extracted 2 times with dichloromethane and concentrated to 5 μL, the plates were spotted but no chromatography was performed before overlay. The positive controls N-hexanoyl-L-homoserine lactone (C6-HSL) (402 pmol), N-octanoyl-L-homoserine lactone (C8-HSL) (15.8 pmol), C6-oxo-HSL (2.35 pmol) C8-oxo-HSL (15 pmol) were also included. For the alkali-acid treatment, 5 μL of rumen extract in dichloromethane was evaporated and 100 μL of 0.1 M NH_4OH was added. After 3 h 50 μL of the alkali treated sample was evaporated while 50 μL of 1M HCl was added to the remaining 50 μL of alkali treated sample. After 3 h the alkali-acid sample was evaporated. This process was repeated with 15 pmol of C8-oxo-HSL. Five μL of dichloromethane was added back to all samples and analytical TLC was performed.

CATTLE INFECTION STUDIES

86-24 (Sm^R) and an isogenic Δ*sdiA* (Sm^R, Cm^R) were grown with aeration in 50 ml Luria-Bertani (LB) broth for 18 hr at 37°C. Bacterial competition assays were performed in three 1.5-year-old ruminally cannulated Charolais heifers using protocols similar to those previously described (198). We defined *in vitro* that there is no difference in the growth rates between the WT and the Δ*sdiA* strains. Briefly, 10^{10} CFU of 86-24 and *sdiA* strains were applied directly into the rumen on day zero. The 86-24/*sdiA* ratio was verified to be 1 by both absorbance at OD_{600} and by plate counts on LB agar. Samples from the rumen and the recto-anal junction (RAJ) mucosa were cultured at 0.5, 1, 1.5, 2.5 and 3 days post-infection. Samples were serially diluted and cultured on sorbitol MacConkey agar (SMAC) supplemented with 0.5mg/ml cefixime, 2.5mg/ml potassium tellurite, 40mg/ml vancomycin, and 0.1 mg/ml 4-methylumbellifryl- ß-D-glucuronide (MUG) (SMAC-CTVM). Colonies identified as *E. coli* O157 were sub-cultured to fresh

LB and LB-chloramphenicol agar. The CFU of WT bacteria was calculated by subtracting the count on LB-cm agar from that obtained on LB agar. Unpaired t-test analysis was done using SigmaPlot 11 software.

Table 3.1 Strains and plasmids used in this study

Strains	Genotyoe	Reference
86-24	Wild-type EHEC strain (serotype O157:H7)	(114)
DH5α	*supE44 lacU169 (80 lacZ M15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Stratagene
VS138	*qseC* mutant in 86-24	(349)
VS179	VS138 complemented with pVS178	(349)
DH11	*kdpE* mutant in 86-24	this study
NR01	*qseF* mutant in 86-24	(299)
MC474	*qseB* mutant in 86-24	this study
MC484	MC747 complemented with pVS154	this study
MC550	VS138 with pVS154	this study
MC471	Single-copy flhDC::lacZ (+50 bp to −900 bp) in MC1000	(50)
DH13	MC471 with pVS154	this study
SM10(λpir)	*Escherichia coli K12 thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu Km *λpir*	(239)
DH1	86-24 *sdiA* mutant	this study
DH2	DH1 with pDH6	this study
DH3	DH1 with pDH5	this study
DH4	DH1 with pDH10	this study
DH9	DH5α with pDH5	this study
Plasmids		
pBADMycHis	C-terminal Myc-His-Tag vector	Invitrogen
pBAD33	Cloning vector	(119)
pVS178	86-24 *qseBC* in pBAD33	(349)
pVS154	86-24 *qseB* in pBADMycHis	(49)
pVS155	MC1000 *qseC* in pBADMycHis	(349)
pKD46	λRed helper plasmid	(58)
pKD3	λRed template plasmid	(58)
pCP20	λRed resolvase plasmid	(58)
pDH12	86-24 *qseB* D51A in pBADMycHis	this study
pACYC177	cloning vector	NEB
pCVD442	Suicide vector	(68)
TOPO PCR blunt	PCR blunt cloning vector with topoisomerase	Invitrogen
pQE30	used for expression of 6xHis-tagged proteins	Qiagen
pDH5	*sdiA* in pQE30	this study
pDH6	*sdiA* with 210bp upstream of the predicted start site cloned into pACYC177	this study
pDH7	pDH10 with internal Cm casette	this study
pDH8	*sdiA*::Cm::from pDH7 in pCVD442	this study
pDH10	*sdiA* with 210bp upstream of the predicted start site cloned into TOPO PCR blunt	this study

Table 3.2 Oligonucleotides used in this study

Name	Oligo
kdpEλRed-F	5'-TTTTCGTGTTACACTTCCCCAGCAAACTGCCCCTGAACTT GAAGAATTTCATGAGGATATGTGTAGGCTGGAGCTGCTTC-3'
kdpEλRed-R	5'-ATTTGGCGCAGGTTTAATAATAAATTAATCACTATTTAG GCGAATTTATTGAATAAAAATCATATGAATATCCTCCTTAG-3'
flhDrtF	5'-TTTCGTCTCGGCATAAATGAAG-3'
flhDrtR	5'-TCATTCAGCAAGCGTGTTGAG-3'
nleArt549F	5'-AGCCACTACTTCGACGGTAACC-3'
nleArt624R	5'-ACGAACCACTTGAGCTGTTAATCC-3'
stx2ArtF	5'-ACCCCACCGGGCAGTT-3'
stx2ArtR	5'-GGTCAAAACGCGCCTGATA-3'
recArtF	5'-CAGGCGCGTGGTACAGCTA-3'
recArtR	5'-CAGCCAGGCAGTTGCATTC-3'
kdpArtF	5'-GCAGCATCAATATGGAAGGTAAAGA-3'
kdpArtR	5'-ACGACCGCAAACAGGTACTGA-3'
qseBD51AF	5’-GATGCGGTGATCCTGGCTTTAACCTTACCAGG-3’
qseBD51AR	5’-CCTGGTAAGGTTAAAGCCAGGATCACCGCATC-3’
qseBλRed-F	5’ -GTCCTTAACAACTTCTTAAGGGAAAAAAATAAAATT TAGTGCTGTACAGAGCGCGTTACAACACGGTTTACTG GCAGCGTGTAGGCTGGAGCTGCTTCG - 3’
qseBλRed-R	5’ -AAAAGATTAGCGTCAGCCTGACGCGCAGACTAAGAC GTTGGGTAAATTTCATTTCTCACCTAATGTGTAACCAATA CCATGCACCATATGAATATCCTCCTTA - 3’
sdiApromoF	5'-CCCCGACGATTCGACAGTCG-3'
sdiApromoR	5'-GATAGCGTGCCTTTCAGCC-3'
sdiAHisF	5'-CTCGAGGCAGGATACGGATTTTTTCAGC-3'
sdiAHisR	5'-AAGCTTAATTAAGCCAGTAGCGGCCG-3'
kanF	5'-CCGGAATTGCCAGCTGGGGCG-3'
kanPromoterR	5'-TCTTGTTCAATCATGCGAAACGATCC-3'
gadXrtF	5'-TGCGCAACTATCGCAGAATAA-3'
gadXrtR	5'-ACAACGCAAAGATTAATGCTCTTTT-3'
gadWrtF	5'-TGCCCAAACGTTGGTATCTG-3'
gadWrtR	5'-CCTGCAACTTTTTTTTGATGAGACT-3'
gadErtF	5'-CCTTGATGAAGAAGCGATTAAATTT-3'
gadErtR	5'-CGCTTTAGCTTTTAGTTTACTGATGTG-3'
gadArtF	5'-TCGTCGCGGCTTCGAA-3'
gadArtR	5'-TGAGATATTTCAGGGAGGCTTTG-3'
gadBrtF	5'-CGACAAAGAAGAATATCCGCAAT-3'
gadBrtR	5'-CCACAGATCGGCAACCATATTT-3'
gadCrtF	5'-GCTCTGTTGGCGGTTTGTCTA-3'
gadCrtR	5'-CCCTGCGGAGAGGTTGATT-3'

CHAPTER FOUR

QseC Regulatory Cascades

Introduction

The survival of an organism lies within its intrinsic ability to detect and efficiently respond to stress cues. Stress responses play a key role in adaptation to environmental, psychosocial and physical insults. Hence it comes as no surprise that stress responses require synchronization and coordination of an organism's resources to ensure that metabolic substrates are available to meet the increasing energy demands of an effective stress response. Stress responses are generally termed "fight or flight" responses in higher animals, because they rely on the ability of an organism to assess whether it has a better chance of survival when facing or avoiding an environmental insult. The hormones epinephrine and norepinephrine are at the core of stress responses (242).

Most of the knowledge of epinephrine/norepinephrine-mediated signaling has been derived from studies in mammalian systems. However, bacteria do not express homologues of mammalian adrenergic receptors. These signals are sensed through histidine sensor kinases (HKs) (48, 298). HKs constitute the predominant family of signaling proteins in bacteria. HKs usually act in concert with a response regulator (RR) protein constituting a two-component system. Upon sensing a defined environmental cue the HK autophosphorylates a conserved histidine residue, and then transfers this phosphate to an aspartate residue in the receiver domain of a cognate RR. The majority of the RRs are transcription factors, which are activated upon phosphorylation (351).

Two HKs, QseC and QseE, characterized in *E. coli* have been reported to sense epinephrine and norepinephrine (48, 298). QseC binds to and increases its

autophosphorylation in response to epinephrine, norepinephrine, and a bacterial signaling molecule termed autoinducer-3 (AI-3) (48). QseE increases its autophosphorylation in response to epinephrine, phosphate, and sulfate (298). QseC acts upstream of QseE, given that transcription of *qseE* is activated by QseC (299). The cognate RR for QseC is QseB (48), and the genes encoding this two-component system are co-transcribed constituting an operon (49). The cognate RR for QseE is QseF, with the *qseF* gene also being co-transcribed with *qseE* within the same operon (49). QseF, however, is also phosphorylated by four other non-cognate HKs: UhpB, BaeS, EnvZ and RstB (409). QseC homologues exist in at least 25 bacterial species (294), while QseE homologues can only be found in enterics. This distribution of receptors may play a role in colonization or virulence with increased levels of epinephrine/norepinephrine.

The majority of the studies assessing adrenergic regulation of bacterial gene expression, have been conducted in bacteria that inhabit the human gastrointestinal (GI) tract (16, 23, 48, 212, 213, 297, 347). Norepinephrine is present in the GI tract, being synthesized by adrenergic neurons of the enteric nervous system (ENS) (100). Epinephrine is synthesized in the central nervous system and the adrenal medulla, and reaches the intestine in a systemic manner after being released into the bloodstream (291). Norepinephrine is found at a nanomolar range in sera, while it is at a micromolar range in the intestine (77). Both hormones have important roles in intestinal homeostasis regulating peristalsis, blood flow, chloride and potassium secretion (128, 291). Both epinephrine and norepinephrine are recognized by the same adrenergic GPCRs in mammalian cells, and the ligand-binding site for these hormones is largely similar (89).

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 is a GI pathogen that exploits adrenergic signaling to regulate virulence gene expression (347). EHEC colonizes the human intestine and leads to the development of hemorrhagic colitis and hemolytic uremic syndrome (HUS). In the colon, EHEC forms attachment and effacement (AE) lesions on the intestinal epithelial cells, which cause extensive rearrangement of the host cell cytoskeleton resulting in the formation of a pedestal-like structure underneath the bacterial cell (148). The genes required for AE lesion formation are located in the chromosomal pathogenicity island termed the locus of enterocyte effacement (LEE) (228). The first operon in the island (named *LEE1*), encodes Ler, the master regulator of the LEE genes (233). The remaining genes encode the type-three secretion system (TTSS) (142), which forms a syringe-like apparatus that the bacteria use to translocate effector molecules to the host cells. Many of these effectors mimic mammalian signaling proteins having profound effects in the host cell signal transduction culminating in diarrheal disease (103). Seven of these effectors are encoded within the LEE region (103), while many others are scattered throughout the genome (64, 364). The first secreted effector discovered outside of the LEE was NleA (117). NleA is known to inhibit cellular protein secretion by disrupting mammalian COPII function and mutation of the *nleA* gene resulted in attenuation in mouse model of infection (117, 246). EHEC also produces a potent Shiga toxin (Stx) that is responsible for the major symptoms of hemorrhagic colitis and HUS (153).

Expression of LEE, Shiga toxin and the flagella and motility genes in EHEC are regulated by the signals AI-3, epinephrine and norepinephrine through QseC (48, 294). This regulation is important for EHEC virulence, given that *qseC* mutants are attenuated

for infection in animal models of disease (48, 294). QseC activates transcription of the *flhDC* genes, which encode the master regulators of the flagellar regulon, directly through QseB binding to the *flhDC* promoter. Importantly, this interaction is dependent on QseB's phosphorylation state (50), whereas, expression of the LEE and Shiga toxin genes are not regulated by QseB. Here we report a global analysis of EHEC gene expression in response to adrenergic signals, and map the QseC signaling cascade.

In this chapter, we unravel the adrenergic response of a bacterial cell at the genetic and biochemical levels, and demonstrate that adrenergic signaling has a profound effect on cell homeostasis, cell-to-cell signaling, and bacterial pathogenesis.

Results

Global assessment of QseC gene regulation in EHEC. We had previously reported that inactivation of the *qseC* gene results in reduced flagella expression and motility, and reduced auto-activation (49, 50). To further characterize the role of QseC in EHEC, Affymetrix *E. coli* 2.0 microarrays were used to compare expression profiles of the WT and Δ*qseC* strains in the presence and absence of the signals AI-3 and epinephrine in Dulbecco's modified eagle media (DMEM), which is optimal for expression of the LEE genes, and LB, which is optimal for expression of the flagella regulon. These arrays contain ~10,000 probe sets (array genes), covering all genes in the genomes of the two sequenced EHEC strains (EDL933 and Sakai), K-12 strain MG1655, uropathogenic *E. coli* (UPEC) strain CFT073, and 700 probes to intergenic regions (which can encode non-annotated small ORFs, or small regulatory RNAs). Expression data can be accessed using accession number (GSE15050) at the NCBI GEO database. During growth in LB, 126

probe sets were down-regulated (28 specific to EHEC), and 708 were up-regulated (232 EHEC specific) in the *qseC* mutant (Table 4.1).

	Increased	Marginal Increase	Decreased	Marginal Decrease	No Change
Δ*qseC* LB	708	130	126	112	9132
Δ*qseC* DMEM AI-3	106	562	273	206	9061
Δ*qseC* DMEM Epi	70	432	311	224	9171

Table 4.1. Δ*qseC* Array Analysis. Increased and decreased are at least two-fold changes in the expression levels.Marginally Increased or decreased are changes that are either less than two-fold or designated as "marginally increased or decreased" by the Affymetrix analysis software GCOSv1.4. "Δ*qseC* LB" reflects the difference between WT and Δ*qseC* grown in LB media. "Δ*qseC* DMEM AI-3" reflects the difference between WT and Δ*qseC* (AI3 is produced endogenously in both WT and Δ*qseC*) in DMEM. "Δ*qseC* DMEM Epi" reflects the difference between WT and Δ*qseC* upon the addition of 10 μM Epi.

Δ*qseC* LB	MG1655	EDL933	Sakai	CFT073	Intergenic
Decreased	56	25	3	34	3
Marginal Decrease	45	35	2	24	3
Increase	266	194	38	121	83
Marginal Increase	56	37	7	14	16
No Change	3647	1496	323	2293	1192

Δ*qseC* DMEM Epi	MG1655	EDL933	Sakai	CFT073	Intergenic
Decreased	75	144	23	44	25
Marginal Decrease	115	62	10	12	5
Increase	36	7	5	14	2
Marginal Increase	239	62	14	65	28
No Change	3605	1512	321	2351	1237

Δ*qseC* DMEM AI-3	MG1655	EDL933	Sakai	CFT073	Intergenic
Decreased	118	109	13	22	11
Marginal Decrease	135	42	8	6	15
Increase	46	18	5	23	13
Marginal Increase	245	156	30	59	49
No Change	3526	1462	317	2376	1209

Table 4.2. Δ*qseC* Array Analysis, Separated by Strain. Increased and decreased are at least two fold changes in the expression levels. Marginally Increased or decreased are changes that are either less than two fold or designated as "marginally increased or decreased" by the Affymetrix analysis software GCOSv1.4. (see Table 4.1 for comparison definitions)

The majority of the genes with an altered profile were derived from the *E. coli* K-12 strain MG1655 (68%), which represent a common *E. coli* backbone conserved among all *E. coli* pathovars (296), (Table 4.2). Many of these genes are associated with metabolism, and they also include the flagella regulon (Figure 4.1 and Figures 4.4C and D). The EHEC specific genes (32%) include several prophage-encoded genes and *stxAB* encoding Shiga toxin. These studies revealed that QseC not only activates transcription of the flagella regulon, but also of the genes encoding Shiga toxin.

Transcriptome comparisons between WT and the *qseC* mutant grown in DMEM, a condition conducive to LEE and virulence gene expression, in the presence of AI-3 alone (both WT and the *qseC* mutant produce AI-3 when grown to late exponential phase in DMEM) or AI-3 plus epinephrine also revealed a global role for QseC regulation of virulence genes. In the presence of AI-3 alone, expression of 106 genes was increased and 273 decreased in the *qseC* mutant compared to WT. In the presence of endogenous AI-3 and epinephrine, expression of 70 genes was increased and 311 decreased in the *qseC* mutant compared to WT (Table 4.1). AI-3 and epinephrine have been reported to act as agonistic signals (387). This agonistic relationship in signaling can be further illustrated by the observation that while AI-3 is only sensed through QseC, epinephrine is sensed by both QseC and QseE (48, 298). However, it is worth mentioning that QseC acts upstream of QseE, given that transcription of *qseE* is activated by QseC (299). These data suggest that both signals tend to activate global gene expression in a *qseC*-dependent fashion more frequently than repress expression.

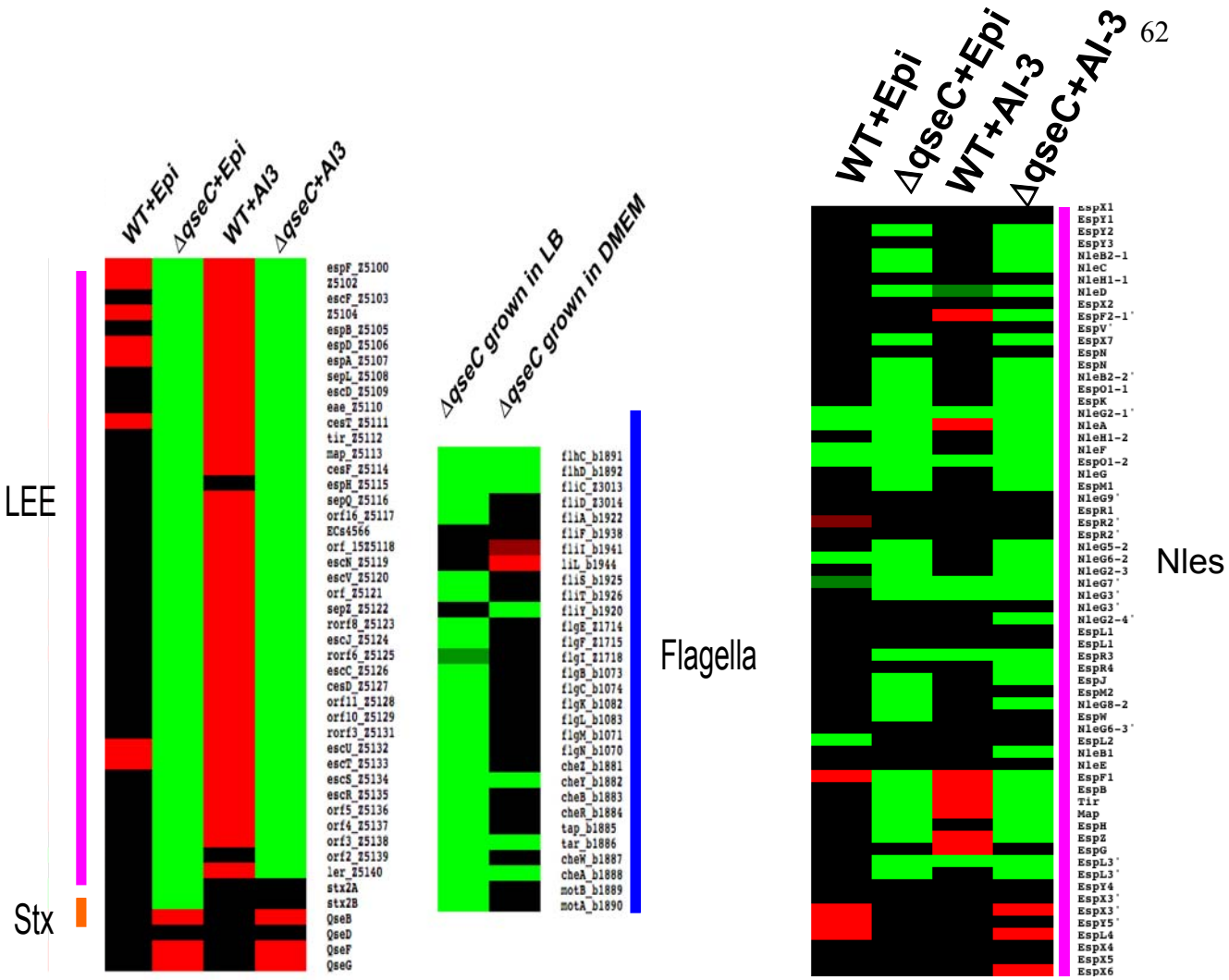

Figure 4.1. Heat maps showing regulation of the LEE genes, the flagellar regulon and the non-LEE encoded secreted effectors in WT vs Δ*qseC* under multiple growth conditions. Green color means there is greater expression of the gene in the WT baseline.

Among the genes activated in a *qseC*-dependent manner are the LEE (through activation of *ler* transcription, within the *LEE1* operon, encoding the Ler activator of all other LEE genes) and *stxAB* (Shiga toxin) genes (Figures 4.1 and 4.2A and B).

The genes encoding Stx are located within the late genes of a λ- bacteriophage and are transcribed when the phage enters its lytic cycle upon induction of an SOS response in the bacterial cell (255). Upon the induction of an SOS response, *recA* is upregulated and cleaves the λ cI repressor allowing transcription of the middle and late

genes to proceed, and together with them the *stxAB* genes. QseC-induction of *stxAB* transcription occurs through induction of *recA* expression (Figure 4.2B), suggesting that QseC mediates SOS induction in bacterial cells. In addition to activating expression of the LEE-encoded TTSS, the majority of the genes encoding effectors translocated through this TTSS are also regulated by QseC (Figure 4.1). Of note, transcription of the gene encoding the NleA effector is strongly repressed by QseC in LB, while its expression is slightly (non-statistically significant) decreased in the *qseC* mutant in DMEM (Figure 4.2C and D). These analyses confirmed QseC's activation of the flagellar genes and revealed several new regulatory targets, including: LEE (through *ler*), *nleA*, genes of the SOS response and Shiga toxin. Altogether, these data suggest that QseC is at the top of the signaling cascade activated by AI-3, epinephrine and norepinephrine, initiating regulation of all EHEC virulence genes.

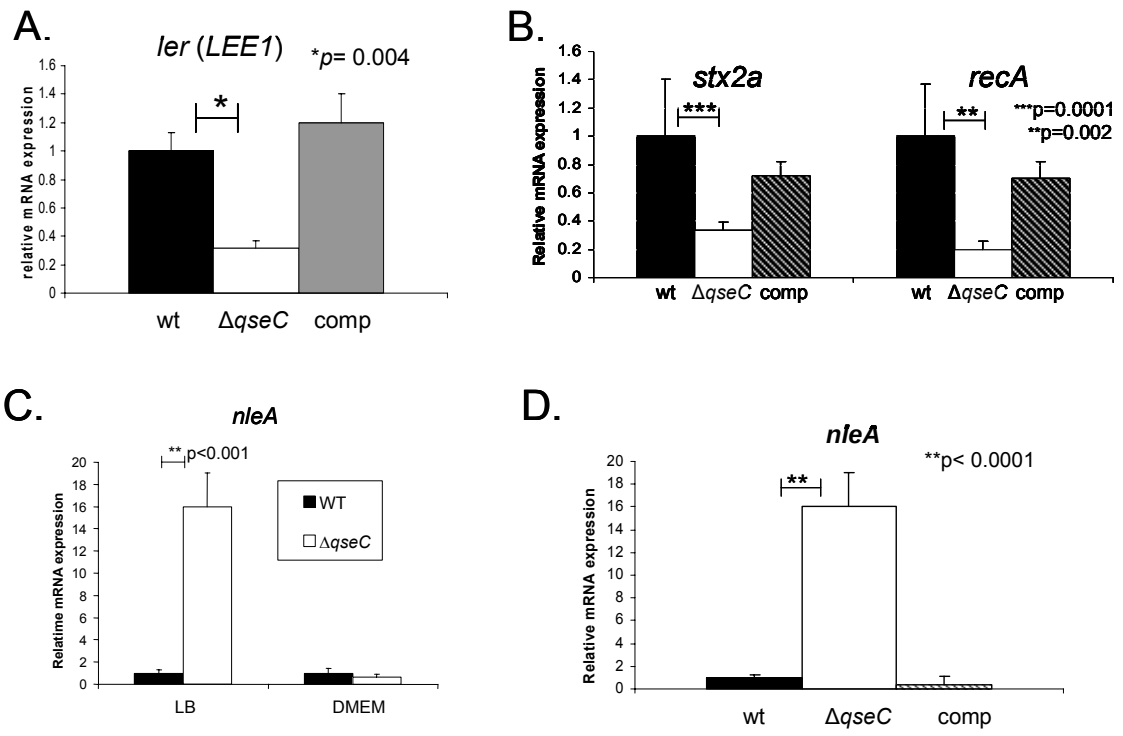

Figure 4.2. qPCR comparing WT and Δ*qseC.* expression of (A) *ler* (the master regulator of the LEE genes), (B) *recA* (an SOS response protein involved in Stx expression), and *stx* (the causative agent of HUS), and (C, D) *nleA* (a secreted effector involved in inhibition of protein secretion)

The QseC signaling transduction pathway. Through QseC, EHEC senses AI-3, epinephrine and norepinephrine to activate flagella and motility, AE lesion formation and Shiga toxin expression. Given that these are expensive biological processes that have to occur in concert, the kinetics of expression of these genes has to be exquisitely fine-tuned. We have previously reported that a Δ*qseC* EHEC had reduced motility, expressed less flagella, and presented reduced transcription of the flagella regulon (349). The cognate RR of the QseC HK is QseB, which is phosphorylated at a conserved aspartate residue by QseC (48) (Figure 4.3).

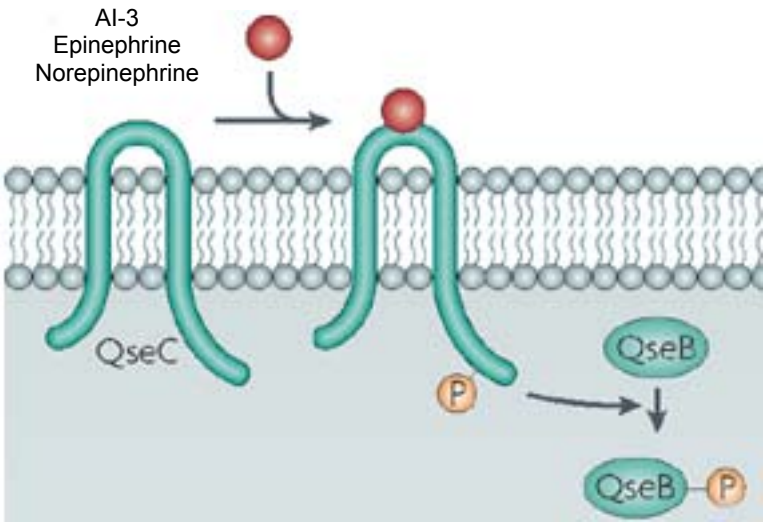

Figure 4.3. QseC is activated by AI-3, Epinephrine, and Norepinephrine. Acitvation leads to autophosphorylation and subsequent phosphotransfer to QseB. Adapted from (132).

In this study we deleted the cognate response regulator *qseB*. Since we had previously shown that QseC regulated the flagellar genes through a direct interaction of QseB and the *flhDC* promoter (FlhDC are the master activators of the flagella regulon) (50), we hypothesized that mutation of *qseB* would result in decreased motility. However, a Δ*qseB* mutant has no motility defect (Figure 4.4A), and expresses flagellin at the same levels as the WT strain (Figure 4.4B).

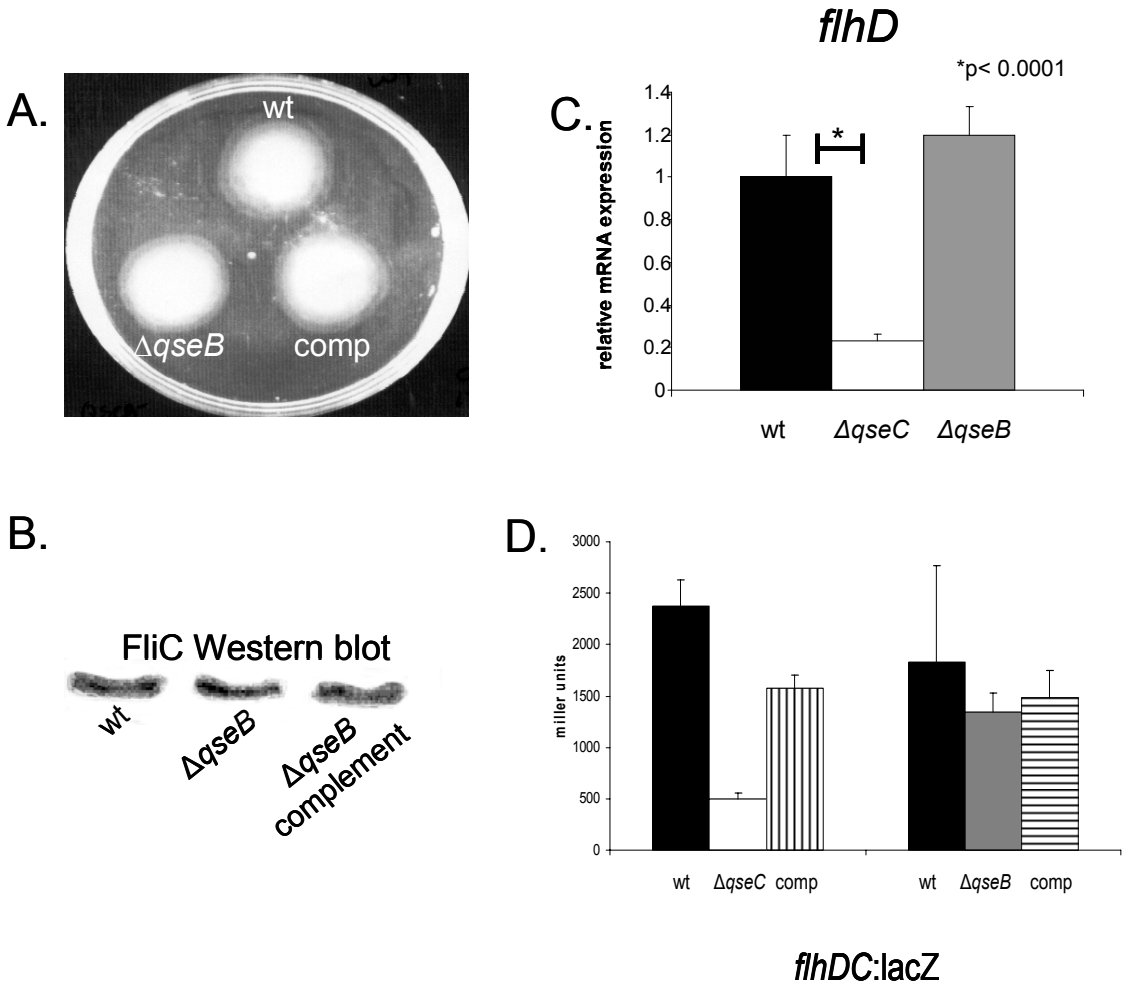

Figure 4.4. Δ*qseC* and Δ*qseB* do not have the same phenotype. **(A)** Motility plate of wt EHEC, Δ*qseB,* and the Δ*qseB* complement strain (complemented with plasmid pVS178, *qseBC* in pBAD33 (349)) (in the presence of self produced AI-3) **(B)** Western blot of FliC in wt EHEC, Δ*qseB,* and the Δ*qseB* complement strain (complemented with plasmid pVS178, *qseBC* in pBAD33) (in the presence of self produced AI-3) **(C)** QPCR of *flhD* in wt EHEC, Δ*qseC,* and Δ*qseB* in LB (OD_{600} 1.0) (in the presence of self produced AI-3) **(D)** β-galactosidase assay of the *flhDC* promoter controlling *lacZ* expression in wt EHEC, Δ*qseC,* the Δ*qseC* complement strain, Δ*qseB,* and the Δ*qseB* complement strain (complemented with plasmid pVS178, *qseBC* in pBAD33) in LB (OD_{600} 1.0) (in the presence of self produced AI-3).

To confirm these results, we assessed transcription of *flhD* by real-time RT-PCR in WT, Δ*qseC,* and Δ*qseB* mutants. Relative expression levels of *flhD* in these three strains indicated that transcription of *flhD* is decreased in Δ*qseC* but is unaltered in Δ*qseB* (Figure 4.4C). We then performed β-galactosidase assays with the -900 to +50bp region

of the *flhDC* promoter fused to a promoterless *lacZ* gene as a reporter. We found that in Δ*qseC* there was five-fold less β-galactosidase activity as compared to WT (Figure 4.4D), but there was no difference in β-galactosidase activity between the WT and Δ*qseB*. Because QseB and QseC constitute a cognate two-component system, we expected that the *qseC* and *qseB* mutants would have similar phenotypes. However, while the *qseC* mutant has decreased motility and expression of the flagellar regulon, the *qseB* mutant shows similar levels of *flhDC* expression and motility as the WT strain. These results led us to develop two potential hypotheses for the differential effects of knocking out a HK (QseC) and its cognate RR (QseB) on *flhDC* transcription. First, QseB can bind to different DNA sequences according to its phosphorylation state, acting as a repressor or activator depending on which site it is bound to. Second, QseC could be a promiscuous HK and can phosphorylate non-cognate RRs that acts on the *flhDC* promoter.

To test the first hypothesis we overexpressed QseB in a Δ*qseC* background. We assumed that this strain would have an overabundance of unphosphorylated QseB. We found that this strain was less motile than Δ*qseC*, indicating that unphosphorylated QseB can act as a repressor of the flagellar gene expression (Figure 4.5A). We also complemented the Δ*qseB* strain with a plasmid expressing QseB, and observed that the complemented strain had decreased motility; again suggesting that overabundance of unphosphorylated QseB has a repressive role in motility (Figure 4.5B).

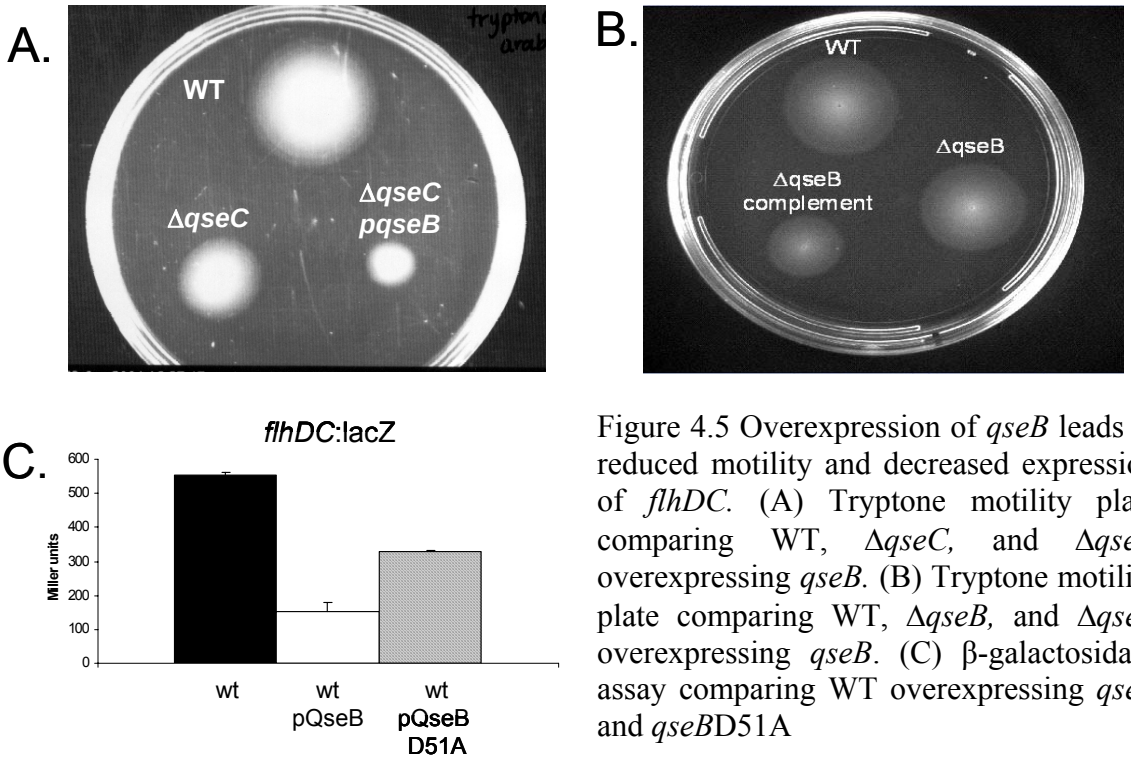

Figure 4.5 Overexpression of *qseB* leads to reduced motility and decreased expression of *flhDC*. (A) Tryptone motility plate comparing WT, Δ*qseC,* and Δ*qseC* overexpressing *qseB*. (B) Tryptone motility plate comparing WT, Δ*qseB,* and Δ*qseB* overexpressing *qseB*. (C) β-galactosidase assay comparing WT overexpressing *qseB* and *qseB*D51A

However, when we complemented the Δ*qseB* strain with a plasmid expressing *qseBC* (Figure 4.4A), we did not observe any differences in motility, probably because the levels of QseB and QseC were balanced in this strain. Next, we overexpressed *qseB*, in a strain containing the -900 to +50bp region of the *flhDC* promoter upstream of a promoterless *lacZ.* We found that in the strain overexpressing *qseB* there was a five-fold decrease in β-galactosidase activity (Figure 4.5C). We also observed decreased *flhDC* transcription in a strain overexpressing a QseB site-directed mutant (QseB D51A) that cannot be phosphorylated (the conserved aspartate phosphorylated residue has been changed to an alanine) (Figure 4.5C), further indicating that an abundance of unphosphorylated QseB represses expression of *flhDC*.

We had previously shown that QseB can bind to two regions of the *flhDC* promoter, -300 to +50bp and -900 to -650bp (50). We demonstrated that this binding

required QseB to be phosphorylated (50) (Figure 4.6A), which can be achieved by providing a small phosphate donor, acetyl phosphate, to QseB *in vitro*. QseB will only bind to the -300 to +50bp *flhDC* region in the presence of acetyl phosphate (Figure 4.6A), and the QseB D51A mutant is also unable to bind to this region of *flhDC* (Figure 4.6A). We have discovered a new QseB binding site in the *flhDC* promoter from -650 to -300bp to which QseB can bind in the absence of phosphorylation. QseB binds to this -650 to -300bp site in the absence of acetyl phosphate, and QseB D51A can also bind to this site (Figure 4.6B and 4.6C).

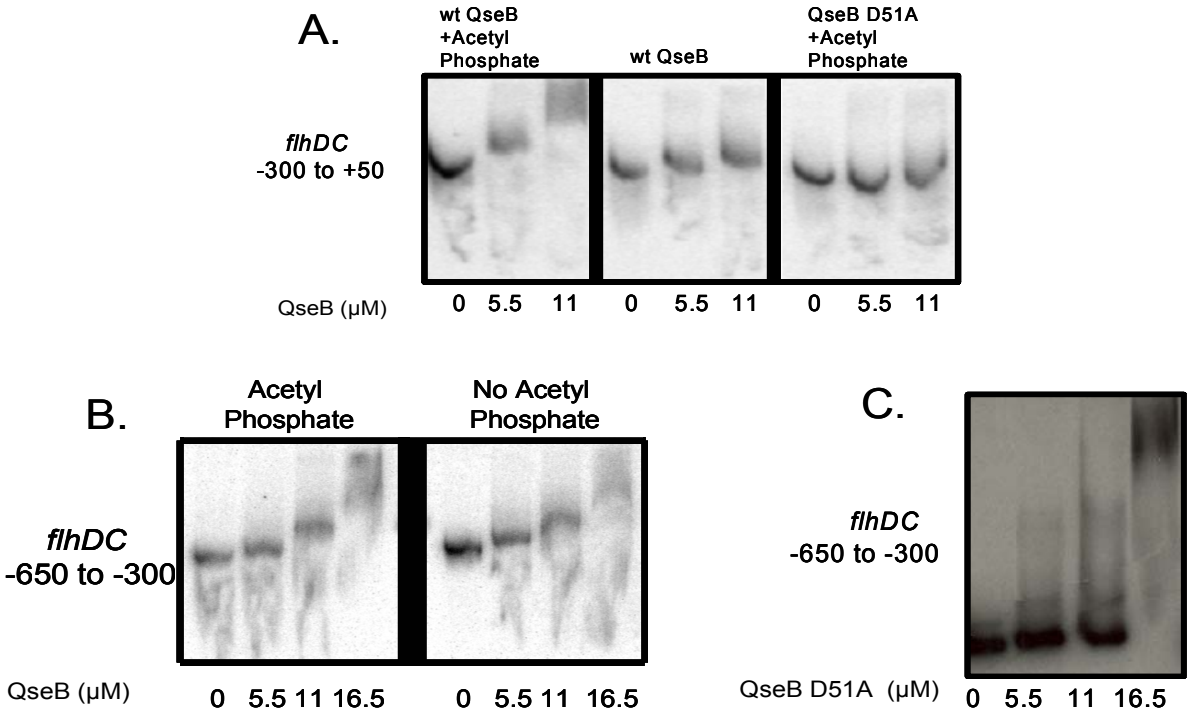

Figure 4.6 The *flhDC* promoter contains a QseB binding site that does not require QseB phosphorylation. (A) The interaction of QseB with its proximal binding site on the *flhDC* promoter requires QseB to be phosphorylated. (B) QseB can bind to the -650 to -300 region of the *flhDC* promoter in the absence of phosphorylation. (C) QseB that us unable to be phosphorylated can bind to the -650 to -300 region of the *flhDC* promoter.

The presence of this new binding site provides further evidence for a dual role of QseB in the regulation of the *flhDC* promoter. At low signal concentration there is low QseC activation and thus low QseB phosphorylation. In this case only the -650 to -300bp site of the *flhDC* promoter will be occupied by non-phosphorylated-QseB and this binding may lead to repression. When the signal is high the opposite is true. The -300 to +50bp and -900 to -650bp sites will be occupied by phosphorylated QseB and *flhDC* will be activated (Figure 4.7B).

In further support of this model, a nested deletion analyses of the *flhDC* promoter fused to *lacZ* shows that the full length fusion (-900 to +50bp) is activated by QseC (Figure 4.7A). This fusion contains all three QseB binding sites, and in the presence of QseC, phosphorylated QseB will occupy the activating sites from -950 to -650bp and -300 to +50bp, increasing transcription. In the -650 to +50bp fusion, transcription of *flhDC* is repressed in the absence or presence of QseC, probably because of non-phosphorylated QseB binding to the -650 to -300bp site, which represses *flhDC* transcription. Unphosphorylated QseB binding to the -650 to -300bp region is probably "locked" in the absence of the upstream (-900 to -650) site. When both upstream sites are removed (-300 to +50bp fusion), phospho-QseB bound to this proximal site will activate *flhDC* transcription (Figure 4.7A). In the complete absence of QseB, as in a Δ*qseB*, there will be QseC-independent expression of *flhDC* transcription, without any repression or activation (de-repression) by QseB (Figure 4.4). These data indicate that regulation of *flhDC* transcription by QseC occurs through its cognate RR QseB, and that QseB plays a dual role in this regulation according to its phosphorylated state.

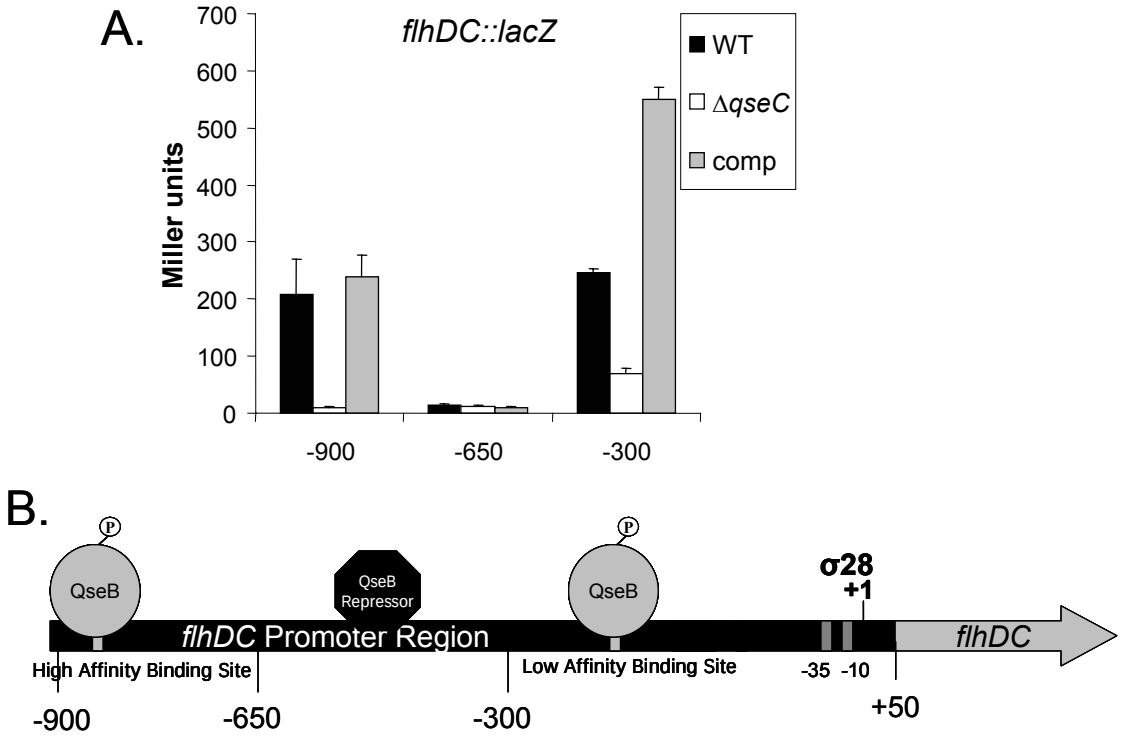

Figure 4.7 The -650 to -300 region of the flhDC promoter is involved in *flhDC* repression. (A) A β-galactosidase nested deletion promoter analysis of the *flhDC* promoter comparing WT, Δ*qseC,* and Δ*qseC* complement. (B) Representation of the flhDC promoter showing the three QseB binding sites.

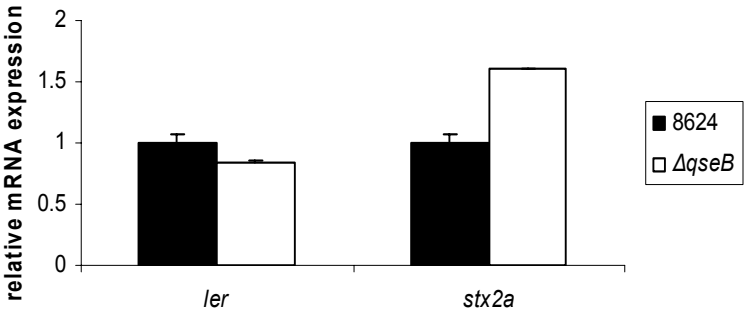

Figure 4.8. QseB does not regulate *ler* or *stx2a.* qPCR of *ler* and *stx2a* in wt EHEC and Δ*qseB* in DMEM (OD_{600} 1.0) (in the presence of self produced AI-3) showing no regulation of *ler* or *stx* in Δ*qseB*

QseB, however, does not seem to play a role in QseC-dependent activation of LEE and *stxAB* transcription (Figure 4.8), suggesting that this regulation may occur through phosphorylation of other RRs. In addition to QseB there are at least 31 other RR in *E. coli* that could be activated via QseC (409). There is minimal cross-talk (cross-phosphorylation) between different two-component systems ensuring faithful transmission of information through distinct signaling pathways (184, 340). Indeed, the incidence of cross-phosphorylation between non-cognate HKs and RRs is low in *E. coli*, Yamamoto *et al.* showed that phosphorylation of non-cognate response regulators by HKs is rare and occurs in only 22 of 692 possible combinations (409). However, in this same study, Yamamoto noticed that a distinct few HKs are more prone to also signal through non-congate RRs.

We have previously reported that QseC autophosphorylates in response to AI-3, epinephrine, and norepinephrine in an *in vitro* liposome assay and can phosphotransfer onto its cognate RR, QseB (48). In order to test QseC's ability to phosphotransfer onto non-cognate RRs, we purified 31 *E. coli* RRs and performed phosphotransfer assays with QseC in liposomes. Of note all of these RRs were soluble and correctly folded upon purification, and have been previously shown by Yamamoto et al. to be active in phosphotransfer reactions with their cognate HKs (409). Through this assay, we found only two additional QseC phosphorylation targets: KdpE and QseF (Appendix A, Figures 4.9A and 4.9B).

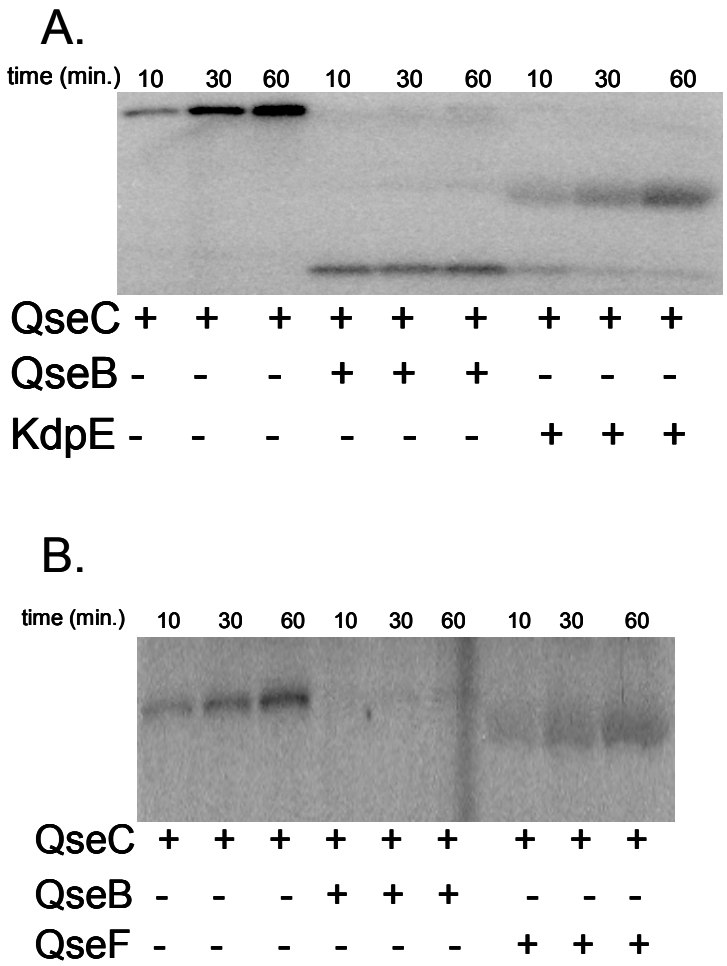

Figure 4.9. QseC phosphotransfers to the response regulators QseB, QseF and KdpE **(A)** autoradiograph of QseC autophosphorylation in lipid vesicles in the presence of 10μM epinephrine and phosphotransfer onto QseB and KdpE **(B)** autoradiograph of QseC autophosphorylation in lipid vesicles in the presence of 10μM epinephrine and phosphotransfer onto QseB and QseF.

KdpE has been shown to regulate potassium uptake and medium osmolarity (251). We found that *kdpA,* one of the genes regulated by KdpE, is also down-regulated in the Δ*qseC* (Figure 4.10A), indicating that cross-phosphorylation between QseC and KdpE results in QseC regulation of KdpE-dependent targets. To assess the contribution of KdpE to QseC's signaling transduction pathway, we deleted *kdpE* but found no motility defect (Figure 4.10C) or decreased *flhDC* expression (Figure 4.10B) in the *kdpE*

mutant, indicating that KdpE is not regulating *flhDC*. When we assessed transcription of *ler* (LEE) and *stx*, we observed that KdpE activates transcription of the LEE genes, but not *stx*, suggesting that through the KdpE RR, QseC activates expression of the LEE genes (Figure. 4.10D).

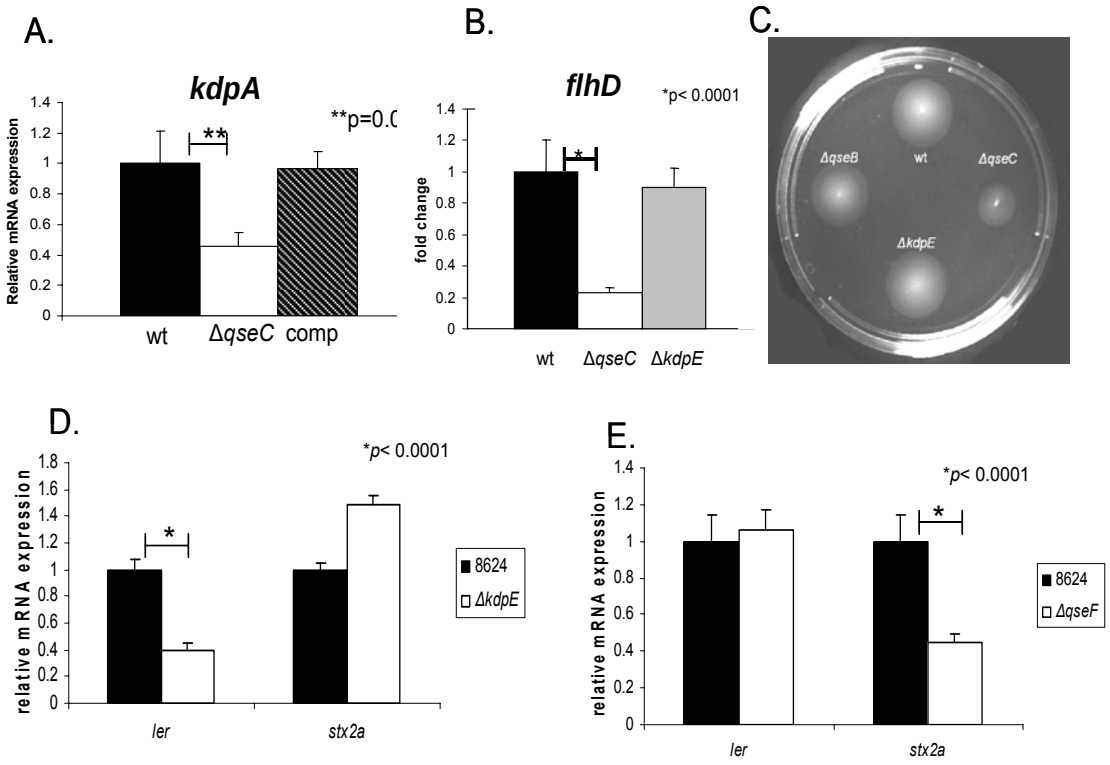

Figure 4.10 KdpE and QseF regulatory targets. QseC, KdpE and QseF regulatory targets. **(A)** QPCR of *kdpA* in wt EHEC, Δ*qseC,* and Δ*qseC* complement strain in DMEM (OD_{600} 1.0) (in the presence of self produced AI-3) **(B)** QPCR of *flhD* in wt EHEC, Δ*qseC,* and Δ*kdpE* in DMEM (OD_{600} 1.0) (in the presence of self produced AI-3) **(C)** Motility plate of wt EHEC, Δ*qseB,* Δ*kdpE,* and Δ*qseC* **(D)** QPCR of *ler* and *stx2a* in wt EHEC and Δ*kdpE* in DMEM (OD_{600} 1.0) (in the presence of self produced AI-3) **(E)** QPCR of *ler* and *stx2a* in wt EHEC and Δ*qseF* in DMEM (OD_{600} 1.0) (in the presence of self produced AI-3).

The second non-cognate RR phosphorylated by QseC, QseF, is responsible for aiding in AE lesion formation by activating expression of the phage-encoded gene *espFu* (299). EspFu is a secreted effector, translocated to epithelial cells by the LEE-encoded

TTSS, and it is involved in host actin nucleation and polymerization for AE lesion formation (39, 104). QseF, however is not involved in regulation of LEE gene expression (Figure 4.10E) (299), nor in flagella and motility regulation (299). However, a *qseF* knockout presented diminished expression of the *stx* gene (Figure 4.10E), suggesting that QseC activation of Shiga toxin expression occurs through the QseF RR. The QseF cognate HK is QseE (409), which is a second bacterial adrenergic receptor that senses epinephrine, phosphate and sulfate (297). The addition of epinephrine to EHEC activates expression of *qseEF*, and this regulation is eliminated in the Δ*qseC* mutant, indicating that QseC activates transcription of *qseEF* (299). Transcriptional regulation of *qseEF* by QseC, in addition to cross-phosphorylation of QseF by QseC and QseE may fine tune the timing for switching from motility, to AE lesion formation to Shiga toxin production during infection.

QseC phosphorylates three RRs: QseB, KdpE and QseF. Through QseB the flagella regulon is regulated. KdpE activates expression of *ler*, and consequently all the LEE genes. QseF plays a role in inducing an SOS response and Shiga toxin production, as well as activating expression of *espFu* (299), which encodes an effector essential for AE lesion formation. To search globally which sets of QseC-dependent genes are regulated through each RR we performed transcriptome assays (GEO series GSE15050). These comparisons were performed with gene arrays hybridized with cDNA from RNA extracted from WT, Δ*qseC*, Δ*qseB*, Δ*kdpE,* and Δ*qseF* strains grown in DMEM to an OD_{600} of 1.0, conditions known to yield maximal endogenous AI-3 production in these strains (386). We avoided using epinephrine in these comparisons, because epinephrine is also sensed by the QseE HK (48, 297). Transcription of 324 genes was increased, and

344 decreased in the Δ*qseC* mutant compared to WT (Figure 4.11). Of the 324 genes increased in the Δ*qseC*, 15 were also increased in Δ*qseB*, 13 in Δ*qseF*, and 63 in Δ*kdpE* (Figure 4.11). These data suggest that 91 of these 324 genes repressed by QseC are under the control of the QseB, KdpE and QseF RRs. These leaves 233 genes repressed through QseC unaccounted for. A possible explanation could be that these genes may be activated and repressed by QseB in a similar fashion to *flhDC* (Figures 4.5 and 4.7), and these genes would not appear as transcriptionally regulated through QseB using gene arrays. QseC activates transcription of 344 genes, with 205 being activated through QseB, 44 through QseF and 87 through KdpE (Figure 4.11). These three RRs activate transcription of 336 of the 344 QseC-dependent genes, giving almost 100% coverage of QseC-activated genes.

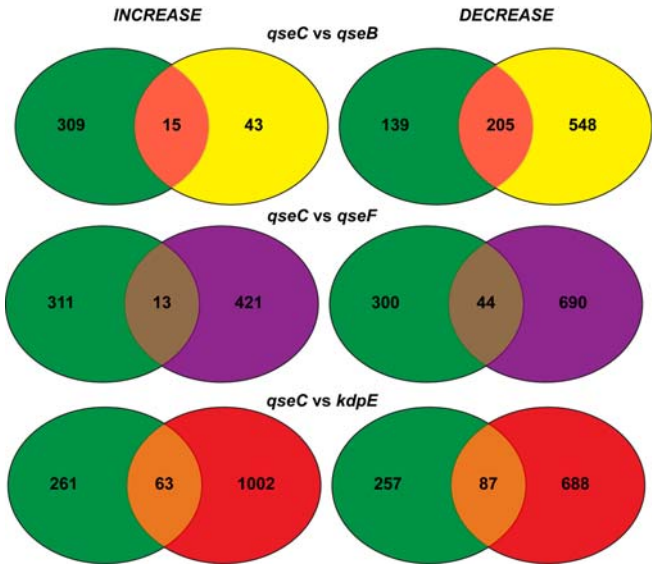

Figure 4.11 Microarray comparisons of Δ*qseC* and Δ*qseB*, Δ*qseF*, and Δ*kdpE*. Transcription of 324 genes was increased, and 344 decreased in the Δ*qseC* mutant compared to WT (Figure 4.11). Of the 324 genes increased in the Δ*qseC*, 15 were also increased in Δ*qseB*, 13 in Δ*qseF*, and 63 in Δ*kdpE* (Figure 4.11). These data suggest that 91 of these 324 genes repressed by QseC are under the control of the QseB, KdpE and QseF RRs. These leaves 233 genes repressed through QseC unaccounted for.

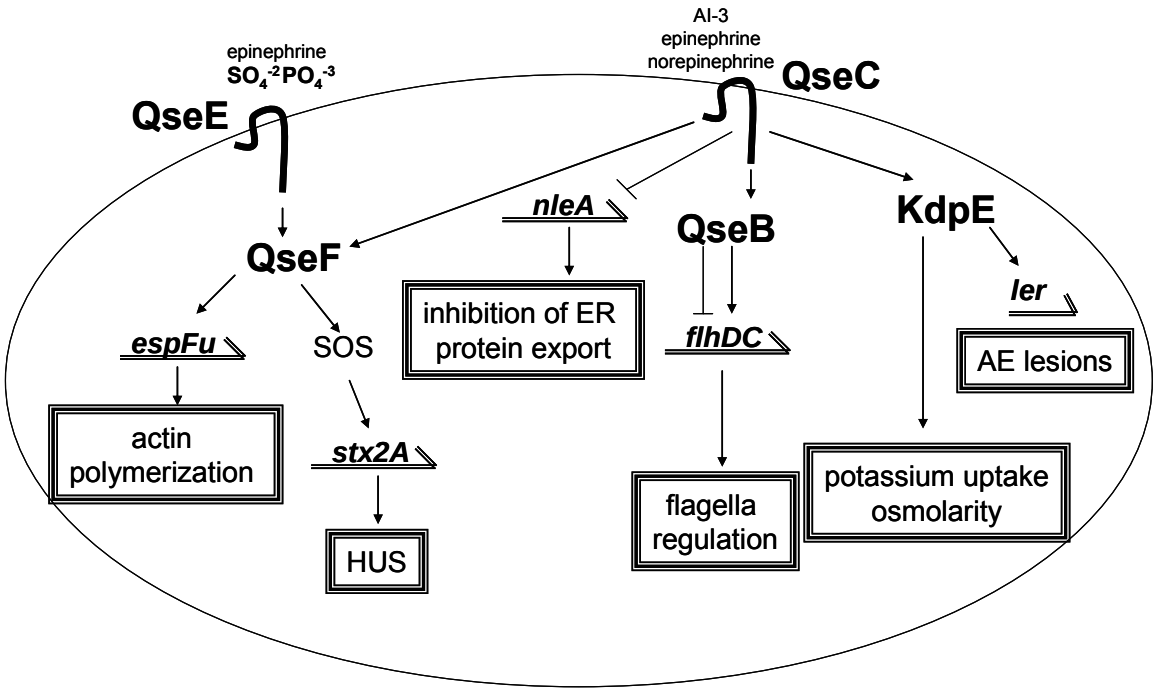

Figure 4.12. The QseC global regulatory cascade.

Discussion

Chemical signaling between cells underlies the basis of multi-cellularity. Although bacteria are unicellular, bacterial populations also utilize chemical signaling, through hormone-like compounds named autoinducers, to achieve cell-cell communication and coordination of behavior (98). Chemical signaling is also essential for an organism to survive, successfully adapt to ever changing environments, and protect themselves from insults, which can be collectively considered stress. Successful stress responses require energy input, and the coordination of many complex signaling pathways within the cell. Co-evolution of prokaryotic species and their respective eukaryotic host have exposed bacteria to hormones and eukaryotic cells to autoinducers. Therefore, it is not surprising that bacteria can respond to host hormones, and that some pathogenic species have high-jacked these signaling systems to promote disease states (131).

One example of a pathogen that senses host hormones to regulate virulence is EHEC (347). Upon reaching the human colon, EHEC senses the autoinducer-3 (AI-3) produced by the microbial gastrointestinal flora, and epinephrine and norepinephrine produced by the host through the HK QseC (48, 347). This signal transduction activates transcription of virulence genes in a coordinated fashion leading to the formation of AE lesions on intestinal cells by the locus of enterocyte effacement (LEE) genes, the flagella regulon for enhanced motility, and Shiga toxin production which is responsible for HUS. EHEC probably first encounters the AI-3 signal produced by the microbial flora that inhabits the intestinal lumen (347). Because the infectious dose of EHEC is very low (estimated to be 50 CFUs) (148), it is unlikely that it responds to self-produced signal to initiate infection. Upon sensing AI-3, QseC initiates the signaling cascade that will activate the flagella regulon leading to swimming motility, which may aid EHEC to come closer to the intestinal epithelial layer. As EHEC approaches the epithelium and starts forming AE lesions it is probably then exposed to epinephrine and/or norepinephrine. Norepinephrine is synthesized within the adrenergic neurons of the enteric nervous system (ENS) that innervates the basolateral layer of the intestine (100). Epinephrine is synthesized in the central nervous system (CNS) and in the adrenal medulla; it acts systemically after being released into the bloodstream, when it can reach the intestine (291). AE lesion formation and the commencement of bloody diarrhea may increase EHEC exposure to epinephrine and norepinephrine, further upregulating expression of virulence genes in EHEC. This coordinated regulation involves a number of two-component regulatory systems composed of HKs and RRs that result in cascades of gene expression.

Recognition of AI-3/epinephrine/NE by QseC can be specifically blocked by the administration of the α-adrenergic antagonist phentolamine (48), and a synthetic compound called LED209 (294). Using two different rabbit infection models it has been demonstrated that QseC plays an important role in pathogenesis *in vivo*, since *qse*C mutants were attenuated for virulence in these animals (48, 294). Recently, a novel two-component system, the QseEF system (299), where QseE is the HK and QseF is the RR was shown to also regulate virulence in EHEC. QseE can also respond to the host hormone epinephrine like QseC, but in contrast, does not sense the bacterial signal AI-3. QseE is downstream from QseC in this signaling cascade, given that *qseEF* transcription is activated by epinephrine via QseC. The QseEF system is not involved in regulation of flagella and motility, but plays an important role in activating genes necessary for AE lesion formation (299) and also activates expression of Shiga toxin (Figure 4.10E).

The AI-3/epinephrine/NE signaling system is not restricted to EHEC. *In silico* analysis showed homologues of QseC in other bacterial species such as *Salmonella* sp, *Shigella flexneri*, *Francisella tularensis*, *Haemophilus influenzae*, *Erwinia carotovora*, and many others (294). *In vivo* studies provided evidence that the QseC HK is important in *Salmonella typhimurium* (22, 294) and *Francisella tularensis* (396) pathogenesis, since *qseC* mutants of these strains are attenuated in animal models of infection and *in vivo* inhibition of QseC by LED209 results in attenuation of infection by these organisms (294).

Because QseC is central for sensing adrenergic signals, and the effect these signals have in basic biological processes, a complete understanding of the QseC signaling transduction pathway in bacteria will offer clues on how eukaryotic stress

responses affect a prokaryotic cell. We demonstrate that QseC acts promiscuously through three RRs (Figure 4.9) to initiate a complex signaling cascade that affects both metabolism and pathogenesis. QseC controls the expression of all of these features, either directly or indirectly and must be considered to be at or near the top of the signaling cascade. The fact that more that one kinase can activate multiple response regulators suggests that there is a hierarchy of signaling, beginning with QseC. It is currently unclear if the regulation by the associated HK and RR overrides the signal employed by a non-cognate HK or if they work in synergy to amplify the initial signal. This additional level of control may be the fine-tuning that is observed in EHEC where the motility, formation of lesions, and secretion of toxin must be exquisitely choreographed to have an effective infection occur.

An additional level of complexity included in this signaling cascade is that QseB, binds to different sites on target promoters according to its phosphorylation state (Figure 4.6). This allows further modulation of gene expression by the spatial arrangement of these sites in the regulatory region of genes, allowing the same RR to both repress and activate transcription of the same gene. In the non-activated form (non-phosphorylated) QseB forms an additional regulatory barrier to the expression of *flhDC*. Only under conditions where QseB is both phosphorylated and in sufficient concentration is there full activation of the flagella regulon. Thus this two-step process provides additional levels of control for this energetically expensive appendage. These types of mechanisms ensure that only under conditions which are favorable the resources are devoted to this response. The DNA binding domain of QseB shares similarities with the DNA binding domain of

the OmpR RR, which also recognizes different sites on DNA according to its phosphorylation state (25, 122).

Because epinephrine and norepinephrine exert a profound effect in the host physiology and immune system, the ability to sense these hormones by bacteria may facilitate gauging the fitness of the host. Inter-kingdom chemical signaling plays an important role in the relationships forged between bacteria and animals. Chemical communication within kingdoms has been studied for many decades, however, the interception of these languages between different kingdoms has only been appreciated recently.

CHAPTER FIVE

SdiA is a bacterial sensor of the rumen environment in cattle

Introduction

Bacteria live in complex multi-species communities within the GI tract of mammals (109). EHEC is an example of a bacterium that behaves as a commensal or a pathogen depending on its host. EHEC is a commensal in the GI tract of cattle, its main reservoir, but is a pathogen of humans (148). EHEC causes bloody diarrhea and colonizes the large intestine of humans forming attaching and effacing (AE) lesions, thought to be largely responsible for promoting disease (148). The genes for AE lesion formation are encoded within the locus of enterocyte effacement (LEE) (148). The LEE and AE lesion formation are also necessary for EHEC colonization of the recto-anal-junction (RAJ) of cattle, facilitating shedding of this pathogen in the environment (329). Hence, it is logical that there exists signaling systems which connect EHEC with the flora and the host (131).

EHEC uses several cell-to-cell chemical signaling systems to modulate gene expression including the AI-2/*luxS* (354), indole (388), AI-3/epinephrine/norepinephrine (347) systems, and the LuxR homolog SdiA (72, 147). Although the *luxS*, AI-3/epinephrine/norepinephrine and indole systems are important for EHEC virulence (14, 295), the role of the SdiA system in EHEC pathogenesis has not been established. In the LuxR/I–type of cell-to-cell signaling systems the signaling molecule is an AHL. The LuxI-type proteins are the AHL synthases. AHLs have a conserved homoserine lactone ring connected through an amide bond to a variable acyl-chain. Acyl chains vary among 4 to 18 carbons and the third position may or not be modified (carbonyl group, hydroxyl or fully reduced). Different acyl-chains ensure that different AHLs will be recognized by

different LuxR-type proteins. Upon binding to AHLs, these proteins regulate transcription of their target genes. AHL binding to most of these proteins stabilizes them; otherwise, in the absence of signal, they are degraded (268, 420, 421).

EHEC encodes a LuxR homologue, SdiA, but does not contain a *luxI* gene, and does not produce AHLs (236). Expression of SdiA from a high-copy number plasmid in EHEC caused reduced expression of the LEE genes (147). However, no *sdiA* mutant was constructed and tested. Additionally, it was found that addition of C6-oxo-HSL led to an increase in one of the genes required for acid resistance in EHEC.

Because no *E. coli* genes have yet been demonstrated to be regulated by the single chromosomal copy of *sdiA*, it has been recently concluded that there are no confirmed members of a SdiA regulon in this species (10). The precise role of SdiA in cell-to-cell signaling was elusive for several years until SdiA was reported not to sense self-produced AHLs, but rather AHLs produced by other bacterial species (236). These authors also reported that SdiA-dependent phenotypes could only be observed in the presence of AHLs. These results are consistent with LuxR-type proteins using AHL as a folding switch (268, 420, 421). Indeed the NMR structure of SdiA shows that AHL binding allows proper protein folding (412). Signature tagged mutagenesis (STM) indicated that EHEC Δ*sdiA* is deficient for colonization of the bovine GI tract (72). This is intriguing given that cattle rumen fluid harbors AHLs (85).

In this chapter, we show that AHLs can lead to repression of the LEE genes through SdiA. This repression is the product of a direct interaction of SdiA with the promoter of *ler* (the master regulator of the LEE genes). Additionally, we show that SdiA is an activator of the glutamate-dependent acid response (AR2), which is required for

EHEC to colonize cows. To this end, we show that rumen extracts contain AHLs and these extracts can modulate expression of the LEE and AR2 genes. Finally, we show that Δ*sdiA* is less fit than WT EHEC at colonizing cows.

Results

The EHEC SdiA regulon. To investigate SdiA-AHL signaling in EHEC, transcriptome studies were performed. AHL signaling changed expression of 323 genes (Table 5.1). Within these genes are the LEE and the *gad* acid resistance system, which has been shown to be essential for the survival of EHEC in cows (285).

	Increased	Decreased	Marginally Increased	Marginally Decreased	No Change
WT vs WT C6-oxo-HSL	260	35	422	762	8729
WT vs Δ*sdiA*	176	61	291	632	9048
WT vs Δ*sdiA* C6-oxo-HSL	171	76	301	680	8980
WT C6-oxo-HSL vs Δ*sdiA* C6-oxo-HSL	179	144	484	583	8818
Δ*sdiA* vs Δ*sdiA* C6-oxo-HSL	62	13	235	330	9568

Table 5.1 Increased and decreased are at least two fold changes in the expression levels. Marginally Increased or decreased are changes that are either less than two fold or designated as “marginally increased or decreased” by the Affymetrix analysis software GCOSv1.4. (see Table 4.1 for comparison definitions)

We then assessed expression of the LEE genes by qRT-PCR (Figure 5.1A and B). There is no difference in transcription of the LEE operons between WT and Δ*sdiA* in the absence of AHLs (EHEC produces no endogenous AHLs). However, when the AHL signal C6-oxo-homoserine lactone (HSL) was added, transcription of the *LEE1* and *LEE4* operons were diminished 8.3 and 24-fold respectively in the WT strain, but not in Δ*sdiA* (which is unable to regulate LEE operons through SdiA in response to AHLs) (Figure 5.1A and B).

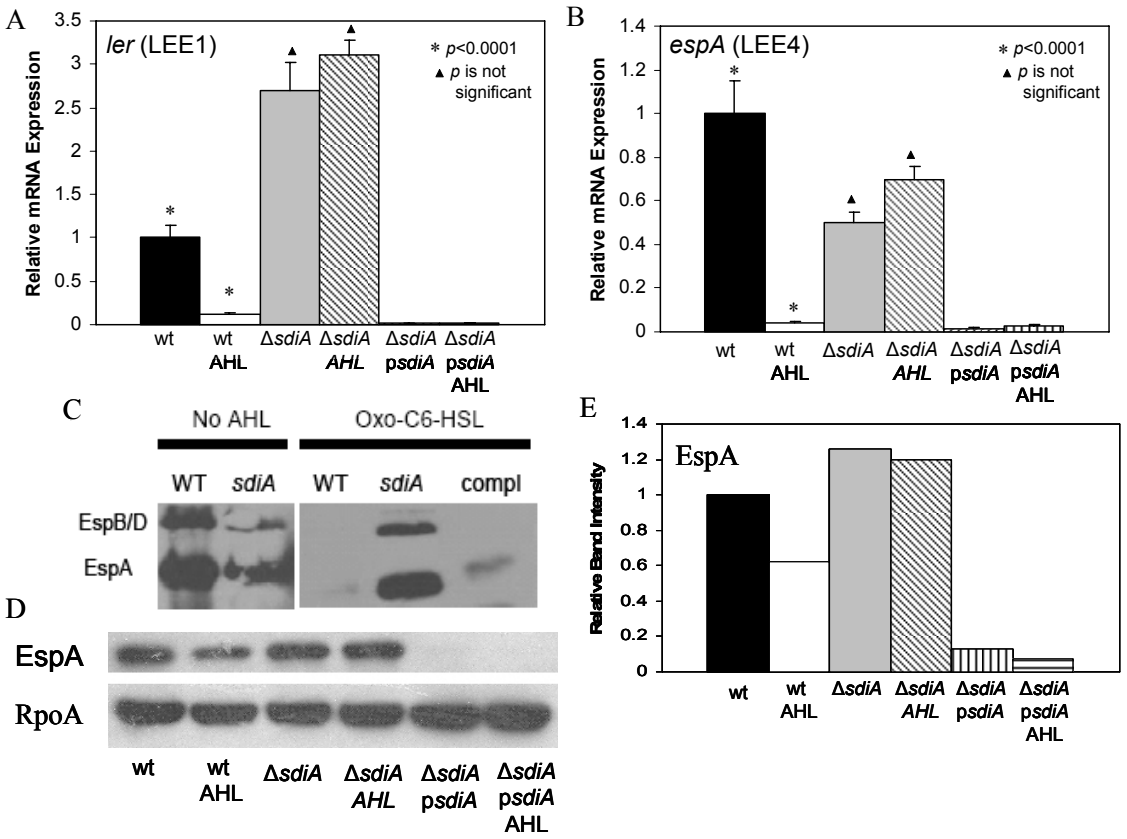

Figure 5.1 AHL repression of the LEE genes is dependent on SdiA. (A) qPCR showing repression of ler (the master regulator of the LEE genes) upon the addition of AHLs. This effect is absent in Δ*sdiA*. (B) qPCR showing repression of *espA* upon the addition of AHLs. This effect is absent in Δ*sdiA*. (C) Secreted protein western blot of EspA and EspB/D (D) Whole-cell lysate western blot of EspA and RpoA (E) Quantification of the band intensities from (D) relative to RpoA expression.

These results suggest that AHLs repress transcription of the LEE genes, and that this repression is mediated through SdiA. These results are also congruent with the role of AHLs in stabilizing the SdiA protein, given that like TraR, in the absence of AHLs SdiA will misfold and be targeted for degradation (412). Transcription of the LEE genes is repressed in the complemented strain both in the presence or absence of AHLs, suggesting that through even mild plasmid over-expression (pACYC177 is a low copy number vector that has 5-7 copies per cell), enough SdiA is made to overcome the lack of the AHL.

The LEE encodes for a type III secretion system (TTSS), a needle-like structure used to inject bacterial effectors into host cells, as well as the translocon of this system comprised of the EspA, EspB and EspD proteins, which are themselves secreted through this TTSS (148). There is no difference in the expression of EspA between the WT and Δ*sdiA* in the absence of AHLs (Figure 5.1D and E). However, expression of EspA is reduced in the WT in the presence of AHL, and decreased in the complemented strain in both the presence and absence of signal (Figure 5.1D and E). These results are consistent with the SdiA-AHL repression of LEE transcription (Figure 5.1A and B). Type III secretion of EspB/D and EspA on WT, Δ*sdiA* and complemented strains in the presence of AHL is reduced in WT and complemented strains but not the *sdiA* mutant (Figure 5.1C). SdiA-AHL controls the expression of all LEE genes, including the genes encoding the TTSS. Hence, one would expect a more striking phenotype on type III secretion of EspA, where one observes defects in secretion coupled to lesser expression of *espA*.

SdiA represses transcription of the *LEE1* operon (Figure 5.1A). *LEE1* encodes the LEE-encoded regulator (Ler), which is required for the expression of the other LEE

genes (232). Eletrophoretic mobility shift assays (EMSAs) of *LEE1* with increasing amounts of SdiA-AHL shows that SdiA directly binds to the *LEE1* promoter (Figure 5.2A.), but not to the promoters of other LEE operons (Figure 5.2B). These data demonstrate that SdiA directly binds to *LEE1*, which represses transcription of *ler* and consequently transcription of the other LEE operons.

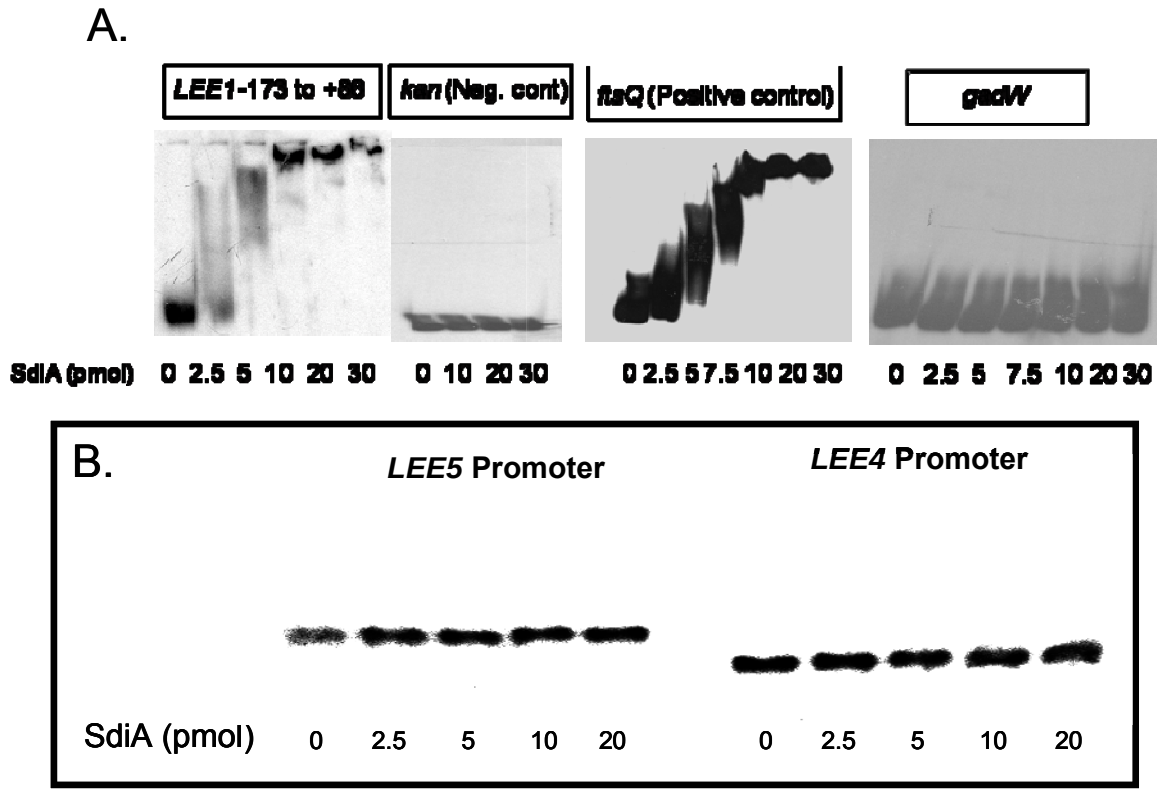

Figure 5.2 (A) SdiA EMSAs. EMSA of the *ler*, *kan* (no shift), *ftsQ* (SdiA has been shown to bind to the *ftsQ* promoter (410)), and *gadW* (no shift) promoters with purified SdiA protein. (B) EMSA of the LEE5 and LEE4 (no shift) promoters with purified SdiA protein.

As indicated by our transcriptome studies, AHLs activated expression of the *gad* acid resistance genes (Figure 5.3A and B). Expression of *gadW* and *gadX* was enhanced by AHLs (Figure 5.3A and B). However, the expression of *gadW* and *gadX* was drastically decreased in Δ*sdiA* even in the absence of AHLs (Figure 5.3A and B).

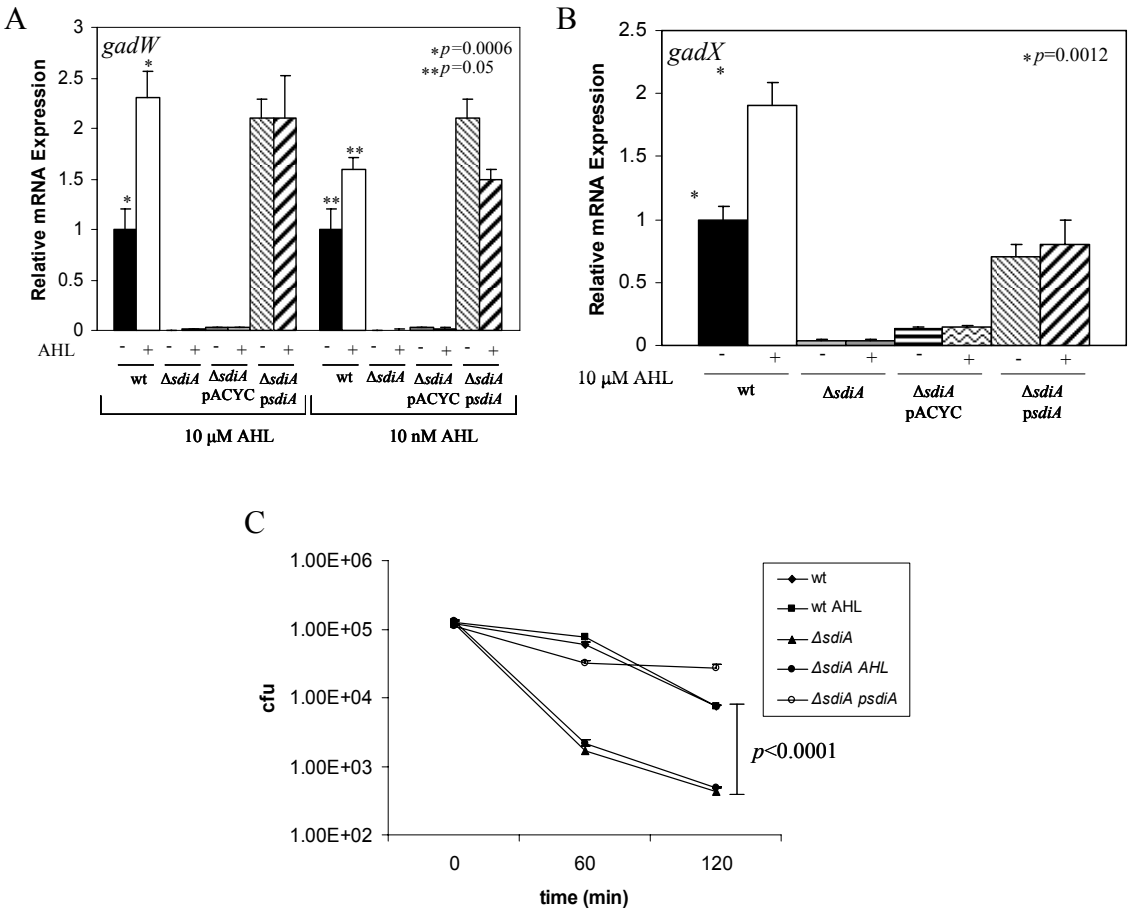

Figure 5.3 SdiA activates acid resistance in EHEC through the glutamate-dependent acid resistance response. (A and B) qPCR showing that the addition of AHLs can activate the *gad* genes. Additionally, there is a drastic decrease in gad gene transcription in Δ*sdiA*. (B) glutamate-dependent acid resistance assay showing that WT is more acid resistant that Δ*sdiA*

SdiA does not directly activate transcription of the *gad* system (Figure 5.2A), suggesting that SdiA-AHL activates expression of a yet unidentified transcription factor that directly activates transcription of the *gad* system. Because of the strong indirect regulation of the *gad* system by SdiA, we then tested whether the *sdiA* mutant was less acid resistant than WT. As predicted, the Δ*sdiA* strain is less resistant to acidic environments than WT EHEC, and this diminished acid resistance is rescued upon complementation of Δ*sdiA* (Figure 5.3B). The effect on acid resistance was not increased by AHLs (Figure 5.3B), suggesting that although AHLs transcriptionally activate expression of the *gad* system (Figure 5.3A), the lack of *sdiA* and the cellular consequences of this mutation, are epistatic to AHL signaling in the regulation of the *gad* system.

Cattle rumen fluid extracts contain AHLs and can modulate gene expression in EHEC. Because SdiA is active in the presence of AHLs (412), and to confirm previous reports of AHLs within the cattle rumen (85), we first tested for the presence of AHLs in rumen fluid. AHLs were extracted from the rumen fluid of cannulated cattle, and tested with an *Agrobacterium tumefaciens* (96) reporter strain for their presence. This strain has the *traI* gene (which is activated by TraR-AHL) fused to *lacZ*, which in the presence of AHLs and its substrate turns blue on a reverse phase TLC overlay assay. This detection strain was chosen because it detects a wide range of AHLs, with acyl chains ranging from 4 to 16 carbons, and containing 3-oxo, 3-hydroxy, or 3-unsubstituted side chains (96). To confirm that the signals detected were AHLs we performed alkalinization of the rumen extract, which will hydrolyze the lactone ring of AHLs, and indeed lost the presence of some rumen signals (Figure 5.4A). The presence of these signals was restored in this

rumen sample upon acidification of this reaction (Figure 5.4A). We detected AHLs in rumen extracts (Figure 5.4B), confirming that AHLs are present within the cow rumen. AHLs were not detected in other portions of the ruminant GI tract, suggesting that these signals may be restricted to the rumen (Figure 5.4C). These results are consistent with the chemistry of AHLs. In neutral environments (rumen) AHLs are stable. However, in alkaline environments (intestine) the AHL lactone ring is hydrolyzed leading to inactivation.

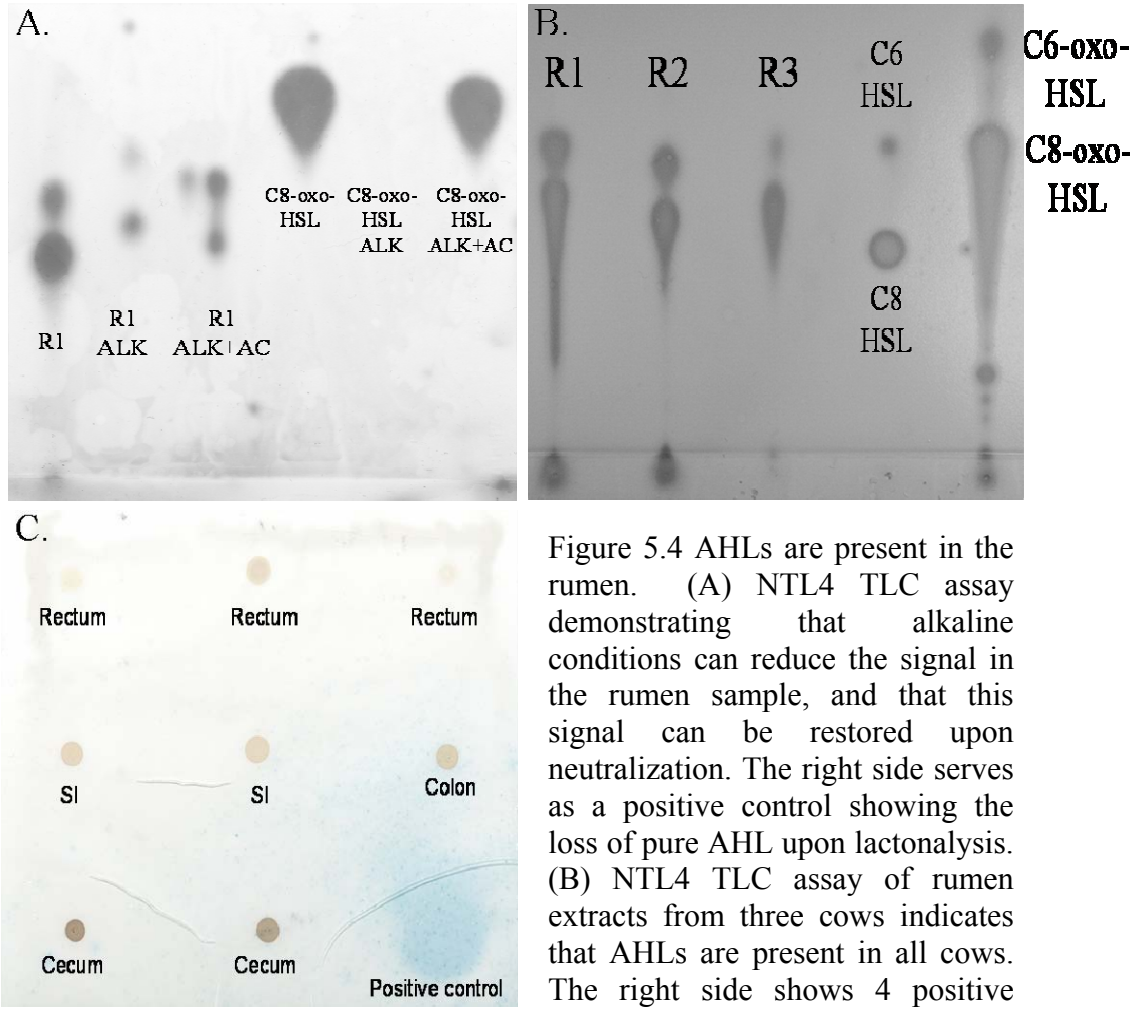

Figure 5.4 AHLs are present in the rumen. (A) NTL4 TLC assay demonstrating that alkaline conditions can reduce the signal in the rumen sample, and that this signal can be restored upon neutralization. The right side serves as a positive control showing the loss of pure AHL upon lactonalysis. (B) NTL4 TLC assay of rumen extracts from three cows indicates that AHLs are present in all cows. The right side shows 4 positive AHL controls. (C) AHLs could not be detected in sections of the lower GI tract. The positive control is C8-oxo-HSL

Given that AHLs repressed expression of the LEE, activated expression of the *gad* genes (Figures 5.1 and 5.3), and that the rumen harbors AHLs (Figure 5.4), we then tested whether AHLs extracted from the rumen could mimic the effect of purified AHLs on transcription. The AHLs extracted from the rumen repressed transcription of the LEE (Figure 5.5A and B) and activated expression of the *gad* genes (Figure 5.5C). However, not all rumen AHL effects occurred through SdiA (Figures 5.5A and B). A potential explanation for this observation is presence of other molecule(s) besides AHLs in these extracts that are responsible for this further regulation.

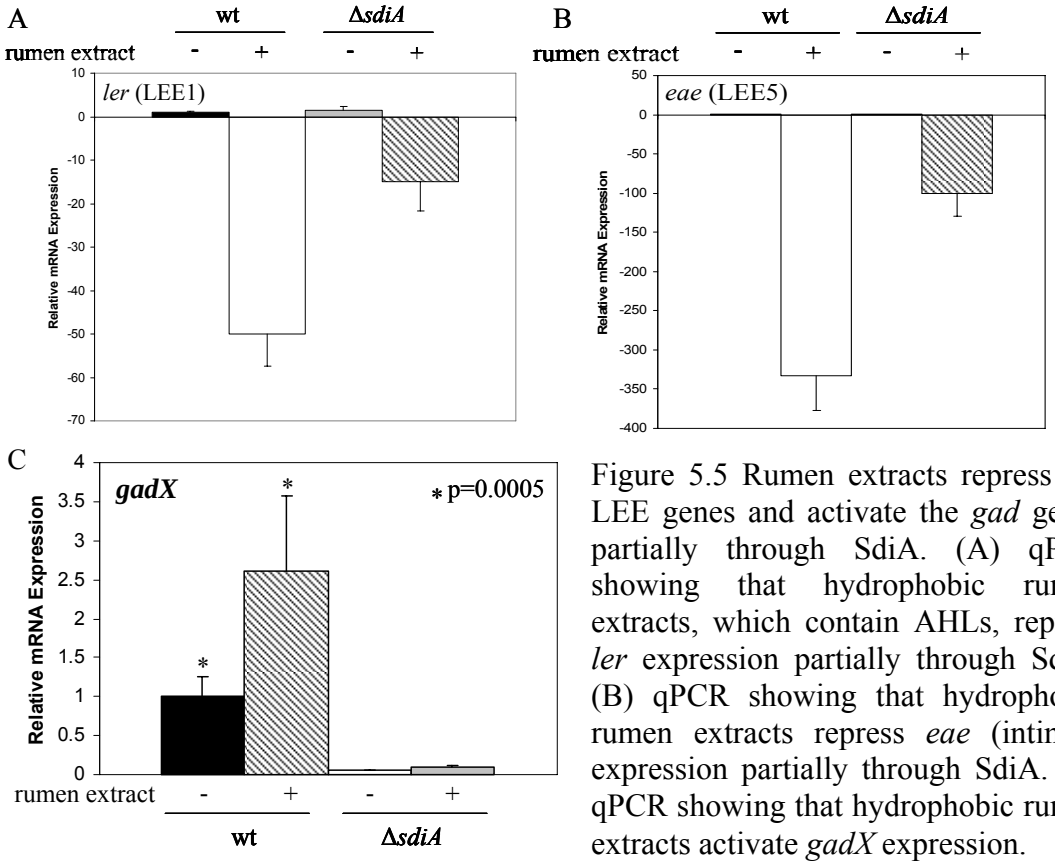

Figure 5.5 Rumen extracts repress the LEE genes and activate the *gad* genes partially through SdiA. (A) qPCR showing that hydrophobic rumen extracts, which contain AHLs, repress *ler* expression partially through SdiA. (B) qPCR showing that hydrophobic rumen extracts repress *eae* (intimin) expression partially through SdiA. (C) qPCR showing that hydrophobic rumen extracts activate *gadX* expression.

SdiA is involved in effective EHEC passage through the acidic stomachs. A STM study, where EHEC was sampled from the RAJ, reported that an *sdiA* mutant is deficient for the colonization of the bovine GI tract (72). To confirm the STM studies (72) and

differentiate colonization at the RAJ mucosa from survival/passage through the GI tract, a competition trial was done in ruminally cannulated heifers. Competition studies were performed to avoid issues concerning individual variation between different heifers in scoring the ability of these strains to establish themselves in the GI tract. As an initial control, an *in vitro* competition experiment between the WT and the *sdiA* mutant was performed to ensure that there were no growth defects in the *sdiA* mutant (Figure 5.6C). Figure 5.6A shows that the *sdiA* mutant out-competed WT in the rumen but this advantage was not continued at the RAJ where the WT outcompeted the *sdiA* mutant (Figure 5.6B). Measurements were taken during the first three days post challenge, a time that measures inoculum passage rather than colonization. Together these observations indicate that the *sdiA* mutant did not survive passage through the GI tract as well as WT, supporting our hypothesis that decreased activation of the *gad* genes in Δ*sdiA* decreases distal stomach survival (Figure 5.6D).

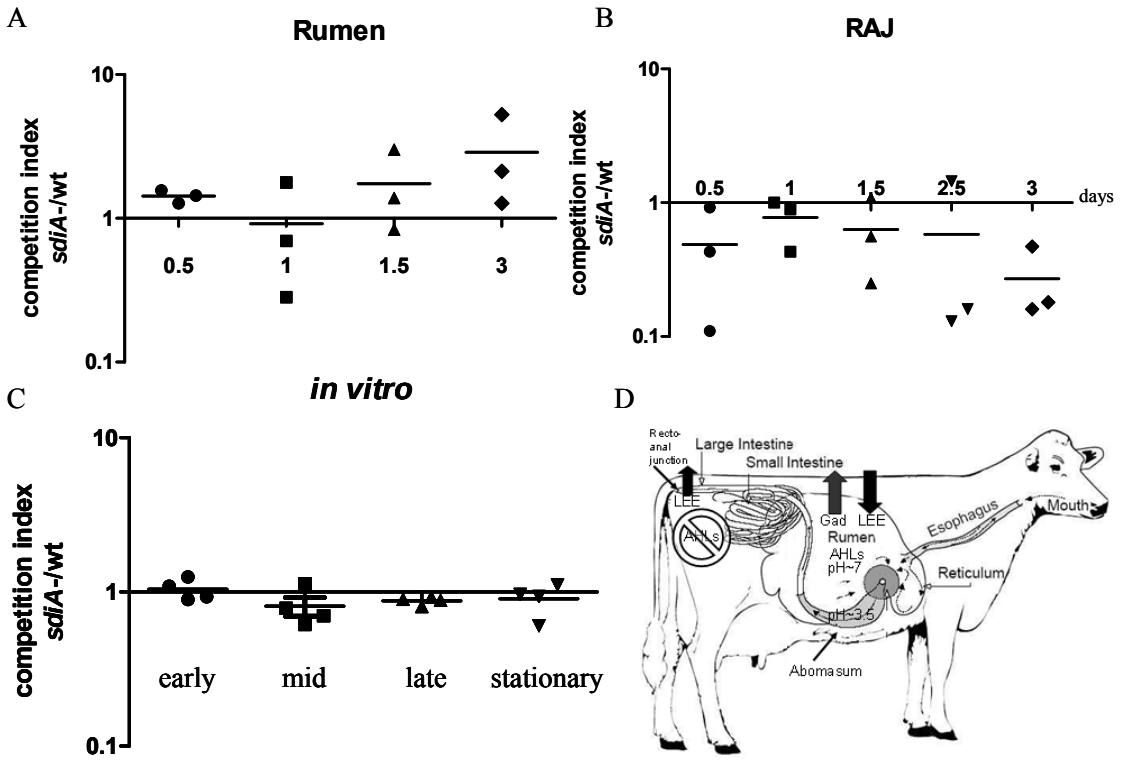

Figure 5.6 Δ*sdiA* is not as fit as WT at cow colonization. (A) WT and Δ*sdiA* were passaged through a cow in competition and samples were isolated from the rumen periodically (B) WT and Δ*sdiA* were passaged through a cow in competition and samples were isolated from the rectal anal junction periodically (C) WT and Δ*sdiA* were grown together in DMEM for 6 hours samples were isolated periodically (D) model for the role of AHLs and SdiA in EHEC during passage through the cow

Discussion

This study demonstrates for the first time a function for the chromosomally encoded SdiA in EHEC, and also defines the SdiA regulon (Table 5.1). Previous studies on *sdiA* in EHEC utilized overexpression of this gene cloned in high copy number plasmids (147). Given that SdiA uses AHL as a folding switch (412), and EHEC does not produce an AHL, we hypothesized that SdiA-dependent gene regulation could only be observed in the presence of exogenous AHLs. In agreement to this hypothesis, we show that SdiA directly represses expression of the LEE genes only in the presence of AHLs (Figure 5.1).

Although AHLs activate expression of the *gad* genes (Figure 5.3), an *sdiA* mutation strikingly decreases expression of these genes in an AHL independent manner. A potential explanation for this AHL independent effect of an *sdiA* mutation in the expression of the *gad* system, and acid resistance, could be due to coupled post-translational regulation of the levels of RpoS and SdiA by the ClpXP system (218).

We also show that rumen extracts containing AHLs, partially through SdiA, down-regulate expression of the LEE genes, whose expression in this GI compartment would constitute a wasted expenditure of energy (Figure 5.5A and B). However, expression of the LEE, is necessary for colonization of the RAJ (72), where AHLs are absent (Figure 5.4C), allowing for LEE expression and AE lesion formation. While AE lesion formation leads to disease in humans, it seems to be innocuous for cattle. One hypothesis is that location plays a role in the outcome. While AE lesions occurs in the large intestine in humans, leading to diarrheal disease, AE lesion formation in the RAJ may not compromise the electrolyte balance in the gut. Conversely, rumen AHLs activate expression of the *gad* system (Figure 5.5C). The rumen serves as a fermentor in cattle, and immediately downstream of this compartment, the nutrients are digested in the acidic distal stomachs. We hypothesized that EHEC activates the *gad* system in the neutral rumen, so it is primed for survival through the acidic environment of the distal stomachs. Indeed our competition studies show that while the *sdiA* mutant out-competed WT in the rumen, this advantage was not continued at the RAJ (Figure 5.6B), indicating that the *sdiA* mutant did not survive passage through the GI tract as well as WT.

EHEC is carried by an estimated 70-80% of the cattle herds in the US, hence, interference with AHL signaling in cattle colonization may constitute an alternative to

diminish shedding, and consequently human disease. Two of the other cell-to-cell signaling systems in EHEC (the indole and AI-3/epinephrine/norepinephrine systems) (14, 295) contribute to pathogenesis, while SdiA seems to adapt EHEC to a commensal life-style in the ruminant host.

CHAPTER SIX

Discussion and Future Directions

EHEC is a human pathogen that causes major outbreaks of hemorrhagic colitis and hemolytic syndrome. Annually, in the USA, over 70,000 people are sickened by EHEC and it is the number one cause of acute renal failure in children (210), (151). EHEC is present in over 70% of cattle herds in the USA (76). The presence of EHEC in our cattle population has led to outbreaks not only from processed beef, but also anything that has the potential to come in contact with ruminant fecal matter, including produce and water sources.

EHEC must coordinate the expression of a myriad of genes at appropriate time points and in proper locations not only to infect humans but to colonize its commensal host. Through natural selection, the most successful pathogens and colonizers have balanced energy conservation with the appropriate energy expenditure required for the bacterium to collect nutrients and resist threats. Expression of the flagellar regulon is a classic example of this evolution. Making a flagellum consumes large amounts of energy, but the ability of a bacterium to swim toward an energy source or away from danger is critical to successful pathogenesis and colonization. Achieving this balance is vital for EHEC since, in humans and cows, it must survive the gastric stomach, the innate immune response, and contend with at least 10^8 bacterial competitors.

The broad objective of this body of work was to examine how EHEC has evolved the ability to sense its environment and govern energy expenditure through cell-cell signaling. EHEC can sense signals produced by its competitors as well as signals

produced by its host. EHEC uses AI-3, epinephrine, and norepinephrine to regulate the expression of two expensive processes: making a flagellum and the LEE encoded type III secretion system and translocon. Additionally, this same signaling mechanism is used to activate expression of Stx, the causative agent of HUS. In its natural reservoir, cows, EHEC uses cell-cell signaling to activate acid resistance and the LEE genes when they will be most beneficial to the bacterium.

Before this study we knew that QseC was an activator of the flagellar regulon and epinephrine activated the flagellar genes, but only in the presence of QseC and that epinephrine, norepinephrine, and AI-3 could facilitate QseC autophosphorylation (349), (348), (48). This phosphate could be transferred to QseB, QseC's cognate response regulator and phospho-QseB could activate its own expression and expression of the flagellar regulon through a direct interaction of phospho-QseB with the *qseBC* and *flhDC* promoters (48), (49), (50).

We proposed that, since EHEC has evolved a system to sense regulatory signals produced in two different kingdoms it is possible that QseC is involved in more than autoregulation and activation of the flagellar regulon. To this end, we used microarrays in an attempt to find new QseC regulatory targets. Through our comparison of Δ*qseC* and WT we confirmed QseC's regulation of the flagellar genes and discovered that QseC was able to activate expression of the LEE genes. Additionally, we discovered that QseC activated the genes governing the SOS response and *stx*. Furthermore, we found that QseC was a repressor of the secreted effector NleA.

To further test our hypothesis, we used a biased approach to identify proteins that directly interact with QseC. Using purified full-length QseC in lipid vesicles we

performed phosphotransfer experiments between QseC and 31 predicted response regulators from the *E. coli* genome. With this approach we discovered two new QseC interaction partners: QseF and KdpE. We then analyzed the EHEC *qseF* and *kdpE* mutants to determine if they were the intermediates between QseC and activation of the LEE genes and *stx*. We found that KdpE could activate *ler* and therefore activate all of the LEE genes and that QseF could activate *stx* expression.

Continued examination of the microarray results would be a logical next step in the investigation of the role of QseC in EHEC. We used the array data to identify known virulence pathways regulated by QseC, but a large amount of array data remains unanalyzed because the regulated genes have unknown functions and determining their functions would have been outside the scope of our investigation. Looking for the most highly QseC-regulated genes would be a good place to start. Preliminary analysis of these genes reveals that, in DMEM and LB, the same gene, Z5934 is at the top of the list of genes activated by QseC. qPCR of this gene revealed that it is down regulated by nearly 400 fold in the *qseC* mutant. Additionally, the gene directly downstream of Z5934, Z5933, is down regulated by nearly 20-fold (Hughes and Sperandio unpublished data). Blast search reveals that Z5934 is homologus to genes encoded on the *Salmonella* virulence plasmid that are activated by SdiA, but no function has been assigned to these genes. DAS membrane protein prediction reveals Z5934 is likely an inner membrane protein. Z5933 shares homology with proteins of the Major Facilitator Superfamily (MFS) which contains a large and diverse group of secondary transporters that includes uniporters, symporters, and antiporters. MFS proteins facilitate the transport, across the inner membrane, of a variety of substrates including ions, sugar phosphates, drugs,

neurotransmitters, nucleosides, amino acids, and peptides (225). Considering the fact that QseC binds small molecule signals, it is plausible that this system is involved in transporting AI-3 across the inner membrane. Mutational analysis of these genes could confirm their role in QseC-dependent gene regulation. Precedence for a regulator being involved in transport of its signal occurs in the AI-2/Lsr cascade. In this system phospho-AI-2 binds to LsrR to derepress activation of the AI-2 uptake system (357). Additionally, using the QseB consensus sequence we found a predicted QseB binding site upstream of Z5932, a predicted secreted effector, which is directly upstream of Z5933 (364).

The array analysis indicated that several putative and confirmed secreted effectors were differentially regulated in the *qseC* mutant. qPCR was performed to confirm these hits (Hughes and Sperandio unpublished data), but only *nleA* was differentially regulated (repressed) in a *qseC* mutant. NleA is secreted into a host cell, through the LEE encoded type III secretion system, where it inhibits protein export (169). Interestingly, the *nleA* promoter contains a putative QseB binding site, but preliminary studies indicated that QseB did not bind to this region (Hughes and Sperandio unpublished data). With the knowledge that QseC can activate KdpE and QseF it would be interesting to see if either of these response regulators could regulate *nleA* repression. If not, further analysis (beginning with the microarray) could reveal an *nleA* repressor that is activated through QseC. Since QseC activates the flagellar regulon and the cell does not want to "park and drive" at the same time, it makes sense that it would want to repress a gene that can only be used after EHEC has activated the LEE-encoded type III secretion system. This seems contradictory, because QseC also activates the LEE genes,

but *nleA* is only repressed by QseC in LB media (Figure 4.2C). The flagellar genes are also differentially regulated by QseC only in LB media, while QseC has no effect on the LEE genes in these growth conditions. This indicates that the specific environment where QseC is activating the flagellar regulon and repressing *nleA* is different than the environment the LEE genes are activated in. It would be interesting to discern what factors mediate LB versus DMEM gene regulation by QseC.

The fact that QseC is so intimately involved in regulation of multiple EHEC virulence mechanisms makes it an appealing target for antibiotic development. Since QseC has no effect on the growth of EHEC the bacterium would be under less pressure to develop resistance. Additionally, use of current antibiotic therapies on EHEC infected patients increases the likelihood of HUS development (266). Since QseC is an activator of *stx* (Figure 4.2B) drugs targeting it would likely reduce the incidence of HUS. Inhibition of QseC has been undertaken in our laboratory with the development of the drug LED209 which can inhibit EHEC, *S. typhimurium*, and *F. tularensis* virulence, through an inhibition of QseC signaling (294). Although, LED209 protected mice during *S. typhimurium* and *F. tularensis* infections, infant rabbits were not protected from EHEC infection. This is likely due to the fact that LED209 is rapidly absorbed into the blood (Sperandio *et al*. unpublished data). Rapid absorption is efficacious for invasive pathogens like *S. typhimurium* and *F. tularensis*, but this phenomenon is detrimental in subverting EHEC infections. EHEC resides in the lumen and does not penetrate past the epithelium. Current strategies are underway to modify LED209 to increase its effectiveness against EHEC infections but a new drug has yet to be discovered. These efforts would benefit from structural knowledge of the QseC periplasmic domain where

LED209 is though to bind. Primary structure alignment of all known QseC periplasmic domain homologues revealed four residues that are completely conserved: F117, W129, R130, and Q147. Mutational analysis of these residues would be a good starting point for a structural analysis of the periplasmic binding domain. Obviously a 3-D structure would be the most beneficial tool for discerning the nature QseC-ligand interaction. The size of the periplasmic domain, 13.8 kDa, suggests that NMR analysis could be used. This approach has been attempted by our laboratory (Hughes and Sperandio *et al*. unpublished data) but the construct appeared unfolded in the NMR spectra. Addition of epinephrine or norepinephrine had no effect on the folding. Varying the length of the periplasmic domain as well as linking the termini of the periplasmic domain to mimic a transmembrane confirmation would be useful alternative approaches to getting a structured product. Crystallization of QseC has also been attempted in our lab with mixed success (Parker and Sperandio unpublished data). QseC has crystallized but the crystals were not large enough for diffraction.

In this study we also discovered that KdpE, a sensor kinase that activates potassium uptake in response to osmotic stress, appears to activate expression of *ler* (Figure 4.10D). We are assuming that since QseC activates *ler* (Figure 4.2A) and since QseC activates KdpE (Figure 4.9A), that KdpE's activation of *ler* is QseC dependent. The fact that QseC activates *kdpA* (Figure 4.10A), one of the genes activated by KdpE, further substantiated this claim, as did the regulatory overlap seen in our Δ*qseC*-Δ*kdpE* array comparison. Although, further work is required to completely understand the QseC-KdpE relationship. Understanding the role of KdpE itself in regulation of the LEE genes would also be interesting. The high osmolarity of the intestinal lumen is a signal for *S.*

typhimurium to activate its invasion genes and therefore could be a signal for EHEC to activate the LEE genes (17). In fact, SepL a LEE-encoded regulator of type III secretion is activated in high osmolarity, through an unknown mechanism (175). Further analysis of the LEE genes and their activation in the *kdpE* mutant would begin to shed light on this phenomenon. Studies on regulation of the LEE genes in high osmotic situations in the presence and absence of *qseC* and *kdpE,* would be a good place to start.

In this dissertation, we presented evidence that QseF, a response regulator involved in actin pedestal formation, is involved in QseC dependent activation of *stx*. This finding was intriguing, because QseF is also responsible for the activation of EspFu, a gene required for EHEC to form pedestals. EspFu, as well as several other EHEC secreted effectors, is encoded within a lambdoid prophage, the same type of phage *stx* is encoded within. The genetic regulation of EspFu has yet to be uncovered, but it is likely modulated by the same factors that mediate *stx* expression, because members of the λ phage family share a common regulatory scheme (385). The EspFu promoter is known to contain a σ^{70} promoter element, but no other reports about the nature of its promoter exist (299). It would be intriguing to determine if the SOS response also activates expression of the prophage encoded secreted effectors. Analysis of *espFu* expression in a *lexA* (repressor of the SOS response) background would be a good place to start this investigation. Additionally, it would be interesting to determine why QseF seems to activate genes that are involved in the SOS response. Does QseF activate the SOS response? Why would the bacterium have proteins to activate its own lysis? The possibility also exists that QseC/QseF is activated by a phage or SOS element. In this

case QseF does not mediate the response but once the response has started QseF becomes involved in a positive feedback loop.

We compared microarray analysis of Δ*qseC* versus Δ*qseB,* Δ*qseF,* and Δ*kdpE* in order to determine which regulatory pathways shared overlap (Figure 4.11). Activation of genes by QseC seemed to occur through the three response regulators. However 233 of the genes repressed by QseC remained unaccounted for. This phenomenon can be explained by the fact that QseB can act as a repressor in the absence of phosphorylation. In the instance of the *flhDC* promoter phospho-QseB acted as an activator while unmodified QseB acted as a repressor. If unphosphorylated QseB could act as a repressor on other promoters then we would predict that mutation of *qseC* would lead to increased repression QseB repressed genes. This repression would be lost in the *qseB* mutant. Hence, in the *qseC* mutant any QseB repressed genes would show up as repressed while in the *qseB* mutant they would be unchanged or activated. If this hypothesis were true, then we would expect a *qseBC* double mutant to behave like a *qseB* mutant (no QseB and no phospho-QseB). This double mutant has been made in EHEC and in uropathogenic *E. coli* and preliminary analyses indicate that it behaves like Δ*qseB* (Njoroge and Sperandio unpublished data and Hultgren S.J. 2009 American Society for Microbiology abstract). Comparing expression profiles of *qseC* and *qseB* overexpressing the non-phosphorylatable QseB D51A would also help to identify genes repressed by QseB since all the QseB in both strains would be acting as a repressor. Mutagenesis of all the combinations involving the QseBC and QseEF signaling cascades is currently underway in our laboratory. The results from these studies will help to fill in many of the gaps currently left in our understanding of the adrenergic signaling cascade in EHEC.

Our work also uncovered a cell-cell signaling regulatory cascade in EHEC that mediates colonization of its natural reservoir, cows. We discovered that the EHEC LuxR homologue SdiA was regulating two systems, the glutamate-dependent acid resistance response (AR2, *gad* genes) and the LEE genes, both of which are required for successful colonization of the cow (285), (72) . We showed that, through SdiA, AHLs could repress the LEE genes. We also found that AHLs could activate the AR2, but were unable to fully determine if they were interacting through SdiA, because the *sdiA* knockout had a profound loss of *gad* gene expression. SdiA's ability to activate the *gad* genes was consistent with our findings that Δ*sdiA* was attenuated for survival at pH 2.5.

Signature tagged mutagenesis looking for EHEC genes required for cow colonization revealed that *sdiA* was required for EHEC to successfully colonize the cow (72). We followed up this study with a comparison of WT EHEC vs Δ*sdiA* in the cow and showed that in competition the WT strain was more fit than Δ*sdiA* at colonization. We then confirmed a previous report that AHLs are present in the rumen (85). These AHL containing rumen extracts were able to repress *ler* and *eae* partially through *sdiA* and activate the *gad* genes. We hypothesize that EHEC has evolved the SdiA cell-cell signaling mechanism in order to regulate pathways that are necessary for survival in the cow. In the rumen, the LEE genes are repressed, through SdiA, because EHEC cannot use these genes until it reaches the rectal-anal junction (RAJ). Additionally, EHEC must have the *gad* genes activated before it reaches the abomasum, the first acidic stomach downstream of the rumen. Finally, our most recent studies indicate that some of the SdiA regulation of the *gad* is occurring in the absence of AHLs, indicating that either SdiA can

act in the absence of AHLs or that the presence of unfolded SdiA is activating a response that leads to *gad* activation.

Understanding AHL independent SdiA activation of the *gad* genes would be a logical follow up to the work presented in this dissertation. One of our hypotheses to explain this phenomenon is based on the fact that RpoS can activate expression of *gadX* (218). In WT EHEC, in the absence of AHLs, we hypothesize, based on NMR studies, that SdiA is unfolded. Abundant, unfolded SdiA, we assume, would be destroyed by the ClpXP bacterial protease system. This system is also responsible for the degradation of RpoS (323). If the ClpXP system is engaging SdiA, then it would be less likely to destroy RpoS. Increased levels of RpoS could then activate *gadX* and facilitate increased acid resistance. The addition of AHLs leads to a folded SdiA protein which we believe can activate the *gad* genes through an unknown mechanism. We propose that in the WT strain with AHL condition that there is a balance of activation through SdiA and repression through the absence of RpoS, but that there is an overall activation phenotype. Finally, in the absence of SdiA, there is no AHL-dependent activation and no degraded SdiA to engage ClpXP. In this case, there is a loss of activation coupled with a gain of repression and the *gad* genes are down regulated and EHEC loses some of its acid resistance capabilities. Testing this hypothesis would be difficult, because mutation of either *rpoS* or *clpXP* would likely have broad pleiotropic effects. Finding the AHL-dependent activator would be key in understanding this phenomenon. This process could begin by examining the microarray analyses for transcriptional regulators that are activated in an AHL- and SdiA-dependent manner. Finally, the fact that there is some AHL-independent regulation of the *gad* genes through SdiA indicates that EHEC may

have evolved a mechanism to cope with acid resistance in humans, where AHLs are not believed to exist.

In this body of work we confirmed that AHLs are present in the rumen, but did not determine their source. Current research indicates that mammals do not produce AHLs. We propose that the AHLs are the products of the bacterial species residing in the rumen. We also believe that the cow's diet affects the production of AHLs. We have shown that AHLs are abundant in foraging cows but are less prevalent in cows on grain diets (Hughes and Sperandio unpublished data). Although finding the specific species that is producing these signals would be exceedingly difficult because the majority of ruminant species are unable to be cultured, a better knowledge of the bacterial species present in the rumen in both diets would aid our pursuit of these hypotheses (33). We propose that a more detailed sequencing analysis be completed to compare not only the microbial populations on forage and grain diets but also to determine if *luxI* homologues exist in the rumen bacterial population. Pyrosequencing could be used to address both of these questions. Cows fed grain diets shed more EHEC than cows on forage diet (66). It is possible that these changes in shedding are due to the presence or absence of AHLs in the rumen. The pyrosequencing data would be a starting point to specifically study the role of diet on the presence of potential AHL producers and the role of their presence on the shedding of EHEC. If AHL producers do in fact play a role in EHEC shedding, then it is possible that SdiA also plays a role in this phenomenon.

Since we know that SdiA plays a role in the colonization of cows and that AHLs in the rumen can affect EHEC cow colonization factors, it may be possible to use AHLs or AHL mimics in cattle diets to reduce EHEC colonization and shedding. Once we

achieve a better understanding of the AHL-SdiA-cow relationship we can begin to develop strategies to modify this signaling in order to inhibit EHEC colonization. If we discover that the presence of AHLs are aiding EHEC in cow colonization, then we could either use AHL signaling inhibitors or enzymatically destroy the AHLs. Eukaryotes have developed the ability to subvert AHL signaling through the use of AHL mimics. The halogenated furanones naturally produced by the Australian red alga *Delisea pulchra* are probably the best characterized of these compounds. These furanones are structurally similar to AHLs and have been shown to inhibit cell-cell signaling through destabilization of LuxR homologues (220). Inhibitory furanones have also been used as a backbone to make novel inhibitors of cell-cell signaling. These synthetic furanones have been used to show prolonged mouse survival and increased lung clearance in a *P. aeruginosa* mouse model of infection. Additionally, these compounds had no effect on bacterial growth which decreasing the bacterial selective pressure to become resistant. These molecules could be used as a food additive, but once again cost would likely be an inhibiting factor. L-canavanine an arginine analog found in some leguminous seeds is also believed to inhibit AHL dependent cell-cell signaling (165). Use of this compound could be more cost effective than the furanones since the legumes it is produced in could be fed to the cows.

Enzymatic degradation of AHLs in the cow is also a potential strategy. Generally, two classes of AHL inactivating enzymes exist in nature: Lactonases and acylases. AiiA from *Bacillus* sp. was the first AHL lactonase discovered and it has been shown to inactivate cell-cell signaling in *E. carotovora* (67), *P. aeruginosa* (391), *Sinorhizobium meliloti* (102), and others. An AHL acylase, termed AiiD, from *Ralstonia*

strain XJ12B was expressed in *P. aeruginosa* and shown to reduce several cell-cell signaling dependent mechanisms including virulence in a nematode model of infection (200). Either of these enzymes could be cloned into a culturable predominant cow rumen species and inoculated back into the cow to elicit AHL degradation. They could also be cloned into a plant that is fed to the cows.

In this work we also reported that AHLs inhibited the LEE genes through SdiA. We showed that repression may be occurring through a direct interaction of SdiA with the *ler* promoter. Characterizing the SdiA-*ler* interaction would aid in our understanding of EHEC's regulation of virulence mechanisms and thus move us closer to subverting them. The *ler* promoter does not contain the classic *lux*-box motif found upstream of many LuxR regulated genes. In fact, only one of these motifs even exists in *E. coli,* it is not an exact lux-box match (Rasko D. personal communication), and AHL addition and *sdiA* mutation had no effect on the regulation of the gene downstream of this promoter (Hughes and Sperandio unpublished data). This finding indicates that some LuxR transcriptional repressors may bind to a unique DNA motif. A nested deletion analysis of the *ler* promoter fused to *lacZ* in the presence of AHLs would aid in determining if there is more than one SdiA binding site and would help pinpoint potential binding sites. DNaseI footprints of putative binding regions could then be used to determine its binding motif. This motif could then be used to find other SdiA dependent genes. Array analysis could then be used to determine if genes downstream of these promoters were differentially regulated in the presence of AHLs or absence of *sdiA*.

We have demonstrated that EHEC uses two unique cell-cell signaling systems to govern pathogenesis and colonization. In both instances EHEC has adapted quorum

sensing systems to mediate this signaling. EHEC has redefined these quorum sensing signals to meet its own needs. This adaptation should facilitate a reevaluation of the quorum sensing field. When quorum sensing signals are detected in a bacterial population are they being used to determine population density? It is possible that, like EHEC these species simply want to know who and what is around them and govern gene regulation accordingly.

Since, pathogens uses small molecule signals to govern processes required for pathogenesis and colonization, we should be able to develop novel classes of small molecule drugs to subvert these processes. We can now exploit to our advantage the systems that EHEC exploited to its advantage. Not only can we limit EHEC pathogenesis, we may be able to limit EHEC's ability to colonize its host, potentially preventing it from ever reaching humans.

response regulator	Phosphorylated by QseC
ArcA	No
AtoC	No
BaeR	No
BasR	No
CheB	No
CheY	No
CitB	No
CpxR	No
CreB	No
CusR	No
DcuR	No
EvgA	No
HydG	No
KdpE	**Yes**
NarL	No
NarP	No
NtrC	No
PhoB	No
PhoP	No
QseB	**Yes**
QseF	**Yes**
RcsB	No
RssB	No
RstA	No
TorR	No
UhpA	No
UvrY	No
YedW	No
YehT	No
YfhK	No
YhjB	No
YpdB	No
UvrY	No

Appendix A: Phosphotranfer studies were performed with purified QseC inserted in a liposome in the presence of 50μM epinephrine and each purified response regulator. Yes indicates that QseC phosphotransfers to that response regulator (positive responses are bolded), No indicates the absence of phosphotransfer.

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