THE CHARACTERIZATION OF QUORUM SENSING *E. COLI* REGULATORS E, F, AND G (QseEFG) AND THEIR ROLE IN PATHOGENESIS OF ENTEROHEMORRHAGIC *ESCHERICHIA COLI*

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DEDICATION

To my Beloit College professor Ken Yasukawa. To my boyfriend Andrew Mans. To my family: Catherine Reading, Melvyn Reading, Alex Reading Meyer, Marc Meyer, Rosalind, and Madeline.

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by

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DISSERTATION

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by

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THE CHARACTERIZATION OF QUORUM SENSING *E. COLI* REGULATORS E, F, AND G (QseEFG) AND THEIR ROLE IN PATHOGENESIS OF ENTEROHEMORRHAGIC *ESCHERICHIA COLI.*

Nicola C. Reading, Ph.D.

The University of Texas Southwestern Medical Center at Dallas, 2008

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Escherichia coli O157:H7 (EHEC) causes hemorrhagic colitis and life-threatening hemolytic uremic syndrome (HUS) worldwide. EHEC colonizes the large intestine and adheres to intestinal epithelial cells by forming attaching and effacing lesions (AE). These lesions result in the rearrangement of the actin cytoskeleton to pedestal-like structures, which cup each bacterium. The genes necessary for formation of pedestals are encoded in the Locus of Enterocyte

Effacement (LEE), including a type III secretion system, an effector protein, Tir, and the outer membrane protein, Intimin. Also, a prophage encoded effector protein, EspFu, is required. EHEC regulates many of its virulence genes including the AE lesion genes in response to environmental signals. Utilizing these signals allows EHEC to colonize the intestine efficiently and effectively. Environmental signals are often recognized by bacterial sensor kinases. In response to cognate signals, sensor kinases autophosphorylate and transfer the phosphate to a response regulator. The regulator then binds downstream genes to regulate transcription. This pathway for flagellation and motility, which allows EHEC to be motile, has been well-characterized and involves the two-component sytem QseBC. Less is known about the signaling towards EHEC's AE lesion formation capability. Here, we describe a unique signaling system important for EHEC pedestal formation. In contrast to conventional two-component signaling systems, this one consists of three-components. Quorum sensing *E.coli* regulators E (*qseE*), *qseG*, and *qseF*, encode a sensor kinase, membrane protein, and response regulator respectively. *qseF* and *qseG* mutant strains cannot form pedestals on epithelial cells. We have shown that QseF transcriptionally regulates *espFu*. When *espFu* is expressed on a plasmid, pedestal formation is restored to the *qseF* mutant. Microarray analysis comparing *qseE, qseF, and qseG* mutants to wild-type revealed that these genes may also play a role in metabolism and stress. The similar profiles of these mutants in the microarray

indicate that these proteins may work together. QseE is able to autophosphorylate and this activity is stimulated by epinephrine, phosphate, and sulfate sources. These data indicate that QseEFG is a three-component system involved in regulation of virulence and metabolism in EHEC. The following study undertakes a genetic and functional analysis of these proteins.

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Prior Publications

Reading NC, Rasko DR, Torres AG, Sperandio, V. "The outer membrane protein QseG is the third member of a three component signaling system important for EHEC pathogenesis." *Manuscript in preparation.*

David A. Rasko, Cristiano Moreira, DeRun Li, **Nicola C. Reading**, Jennifer M. Ritchie, Matthew K. Waldor, Noelle Williams, Ron Taussig, Shuguang We, Michael Roth, David T. Hughes, Jason F. Huntley, Maggie W. Fina, John R. Falck, Vanessa Sperandio. "A New Strategy for Development of Antimicrobial Agents: Inhibition of Inter-Kingdom Communication." *Submitted to Science.*

Reading NC, Torres AG, Kendall MM, Hughes DT, Yamamoto K, Sperandio V. "A novel two-component signaling system that activates transcription of an enterohemorrhagic *Escherichia Coli* effector involved in remodeling of host actin." J. Bacteriol. 2007 Mar; 189(6):2468-76.

Reading NC, Sperandio V. "Quorum sensing: the many languages of bacteria." FEMS Microbiol Lett. 2006. Jan; 254(1):1-11.

Marino-Ramirez L, Minor JL, **Reading N**, Hu JC. "Identification and mapping of self-assembling protein domains encoded by the *Escherichia coli* K-12 genome by use of lambda repressor fusions." J. Bacteriol. 2004 Mar; 186(5):1311-9.

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86-24	Enterohemorrhagic *E. coli* wild-type strain
AE	Attaching and Effacing
AHL	Acyl homoserine lactone
AI	Autoinducer
AI-2	Autoinducer – 2
AI-3	Autoinducer – 3
bp	Basepair
cAMP	cyclic adenosine monophosphate
cyaA	adenylate cyclase
CDC	Center for Disease Control
CFU	Colony Forming Units
CNS	Central Nevous System
DAEC	Diffusely adherent *E. coli*
DEPC	Diethyl pyrocarbonate
DMEM	Dulbecos modified Eagle's medium
DNA	Deoxyribonucleic acid
DPD	AI-2 precursor; 4, 5-dihydroxy-2,3-pentanedione
E. coli	*Escherichia coli*
EAEC	Enteroaggregative *E. coli*
EBP	Enhancer binding protein
EDTA	Ethylenediamine tetraacetic acid

EHEC	Enterohemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EMSA	Electrophoretic mobility shift assay
ENS	Enteric nervous system
EPEC	Enterpathogenic *E. coli*
Esps	*E. coli* secreted proteins
ETEC	Enterotoxigenic *E. coli*
FAS	Fluorescence Actin Staining
FBS	Fetal Bovine Serum
FCS	Fetal Calf Serum
FITC	Fluorescein isothiocyanate
g	gram
G	gravity
Gb3	Globotriaosylceramide
GBD	GTPase binding domain
gDNA	Genomic DNA
GI	Gastrointestinal
GTP	Guanosine Triphosphate
h	Hour(s)
His	Histidine
HUS	Hemolytic Uremic Syndrom
IgG	Immunoglobulin G
IL	Interleukin

IPTG	Isopropyl-β-d-thiogalactopyranoside
kDa	kiloDalton
LB	Luria-Bertani
LEE	Locus of Enterocyte Effacement
ler	*LEE*-encoded regulator
LPS	Lipopolysaccharide
M	Molar
Map	Mitochondrial-associated protein
MG1655	*E. coli* K-12
ml	Milliliter
Nle	Non-LEE-encoded
N-WASP	Neronal Wiskott-Aldrich Syndrome Protein
OD	Optical Density
PAI	Pathogenicity island
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phentolamine
PFGE	Pulsed-field gel electrophoresis
PMN	Polymorphonuclear leukocytes
PMSF	Phenylmethylsulfonyl fluoride
PO	Propranolol
QS	Quorum sensing
qse	*quorum sensing E. coli regulator*

RNA	Ribonucleic acid
rpm	Revolutions per minute
RT-PCR	Reverse transcriptase polymerase chain reaction
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SOC	Super optimal catabolite medium
sRNAs	small RNAs
Stx	Shiga toxin
Tir	Translocated intimin receptor
TNF-α	Tumor Necrosis factor alpha
TRITC	Tetramethyl rhodamine isothiocyanate
TTSS	Type III secretion system
UTR	Untranslated region
VS94	Enterohemorrhagic *E. coli luxS* mutant
WCL	Whole-cell lysates
WT	Wild Type
X-gal	5-bromo-4-chloro-3-indolyl β-galactopyranoside
µl	Microliter

CHAPTER ONE

LITERATURE REVIEW OF EHEC

Classification

Escherichia coli (*E. coli*) is a gram negative, motile, rod-shaped facultative anaerobe that inhabits the intestine of humans and animals (Fig. 1.1). It was originally identified and named for the Austrian doctor Theodor von Escherich in the latter half of the 19th century. *Escherichia coli* belongs to the family Enterobacteriaceae, which includes most enteric bacteria. This organism colonizes the intestinal tract within forty hours of birth and is usually beneficial to the host by preventing establishment of pathogenic bacteria, breaking down cellulose, and taking part in the adsorption of vitamin K. Enterohemorrhagic *E. coli* O157:H7 (EHEC) is a serotype of *E. coli* that has acquired extra genetic material as described later, which allows it to persist within the intestine and cause disease in some animals. O157:H7 refers to its expression of the 157th known "O" antigen from LPS chains and 7th flagellar "H" antigen (194).

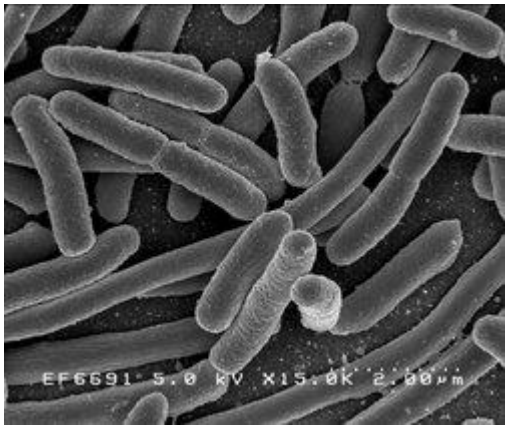


Fig 1.1 – Electron micrograph depicting *Escherichia coli* rods. (http://www.answers.com/topic/escherichia-coli?cat=health)

History and Epidemiology

E. coli is an important beneficial organism that is the most abundant facultative anerobe in the human instestinal microflora. Despite the large numbers of bacteria in the intestine, *E. coli* is still able to successfully occupy its niche at the mucous layer of the mammalian colon. This organism is extremely diverse and aside from its natural role within the intestine, it is also widely used in scientific research as a cloning tool and host. As implied by the fact that humans are colonized by *E. coli* shortly after birth, typically humans and this organism co-exist well throughout life. However, several strains of *E. coli* have been able to acquire extra genetic elements that allow them to cause disease and illness in humans. These so-called virulence factors can be encoded on either mobile genetic units such as prophages or genetic elements that become part of the *E. coli* genome (130). Different combinations of these virulence traits and factors have

lead to numerous pathogenic strains of *E. coli* that lead to several types of disease, including, urinary tract infection, sepsis/meningitis, and enteric diarrheal disease (194). Among the strains that cause enteric diarrheal disease are enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), diffuse adherent *E. coli* (DAEC) and enteroaggregative *E. coli* (EAEC). Each one of these organisms has obtained a slightly different combination of extra genetic elements leading to differences in virulence mechanisms as well as clinical outcomes.

EHEC causes hemorrhagic colitis and hemolytic uremic syndrome (HUS), the most common cause of renal failure in children, throughout the world. Outbreaks have been documented not only in the United States, but also in: Japan, Europe, South America, Australia, and South Africa. Interestingly, in developing countries EHEC is isolated with much less frequency than other strains of diarrheal causing *E. coli* such as EPEC (194).

EHEC was initially identified as a pathogen by Riley et al. in 1983 following two outbreaks that occurred in 1982. In this study, Riley, *et al*. linked an illness characterized by bloody diarhhea and no fever to the organism *E. coli* O157:H7, which was cultured from the stool of those affected after consuming undercooked hamburger meat at a fast-food chain restaurant (222). The same year, Karmali *et al*. also associated a number of cases of HUS with an *E. coli* produced cytotoxin (134). Interestingly, this newly emerged enteric pathogen

seemed to be a true emerging pathogen rather than one which had been undiagnosed previously. In 1983 at the time of Riley's report, The Center for Disease Control (CDC) went over the serotypes of over 3,000 *E. coli* strains collected between 1973 and 1983, and identified only one strain of *E. coli* O157:H7 (222). This was confirmed by public health groups in the United Kingdom and in Canada (53, 100, 124).

In the last two decades, EHEC has become one of the major food borne pathogens in the United States. It is the most frequently isolated pathogen from stool samples after *Campylobacter* and *Salmonella* and the most frequently isolated pathogen from stool samples containing blood (194). Although initially identified by the two scientific studies noted above, EHEC did not draw large attention until a massive outbreak occurred in 1993 in the western United States. This outbreak involved 732 people, of which, 195 were hospitalized and 4 died. The outbreak was caused by consumption of hamburgers from a fast-food restaurant (194). Following this outbreak, in 1994, EHEC became a nationally notifiable infection, and in 2000, reporting became mandatory in most states (218).

In the United States, it is estimated that EHEC causes 73,000 illnesses annually. Between 1982 and 2002, the CDC received reports of 350 outbreaks from 49 states for a total of approximately 8,500 cases. From these cases,

approximately 1400 people were hospitalized, 300 developed HUS, and 40 died. The distribution of these cases by year is shown in Fig. 1.2.

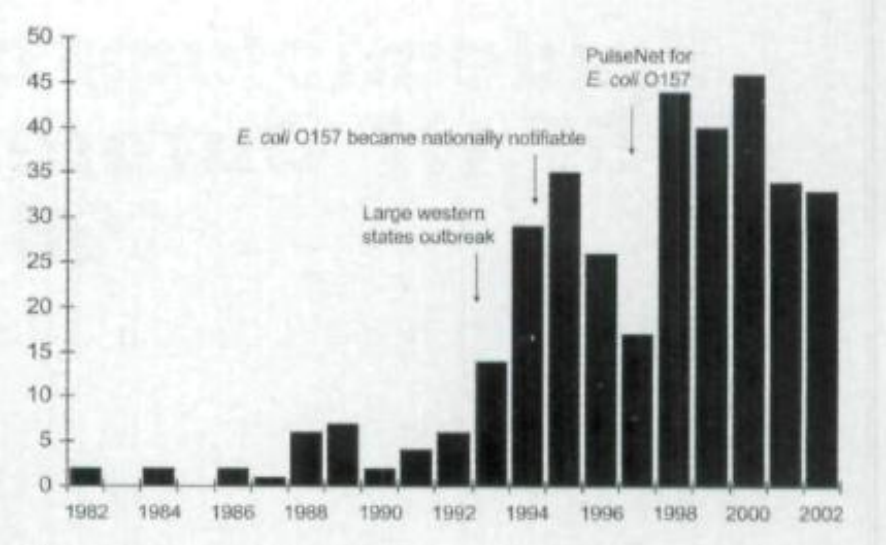


Figure 1.2 – Outbreaks of *E. coli* O157:H7 per year from 1982-2002. Figure from Rangel, *et al.*, 2005 (218).

EHEC's natural reservoir is cattle and it naturally colonizes the intestine of these animals without making them sick. EHEC is estimated to colonize approximately 25% of bovine populations, but up to 60% has been reported (100, 105, 298). The high occurrence of cattle colonized with EHEC makes it not surprising that EHEC arises in our food sources. For example, one study showed the isolation of EHEC from: 3.7% of market beef, 1.5% pork, 1.5% chicken, and 2.0% lamb (63). EHEC was originally dubbed the "hamburger *E. coli*" based on causing illness that was associated with the consumption of undercooked hamburger meat at fast-food restaurants. Ground beef is still the most common

form of EHEC transmission; however, EHEC outbreaks have been documented from numerous other sources, including produce (lettuce, apple cider and juice, salad, coleslaw, melons, sprouts and grapes) and dairy products (218). In addition, EHEC is estimated to have an extremely low infectious dose (from 1-200 colony forming units), making person to person contact also a mode of transmission (100). Two early outbreaks involving a nursing home and a child-care center were indicative of person to person transmission when caretakers at the nursing home and families of the children in the child-care center also became ill (132) The low infectious dose of EHEC has also made transmission by contact with contaminated water possible (137, 194, 218). The largest outbreak of EHEC in the United States involved consumption of contaminated drinking water at a county fair in 1999. This outbreak involved 781 people. Finally, outbreaks have occurred from animal contact primarily at fairs and petting zoos. This type of transmission is quite new and was not recognized until 1996 (218). Figure 1.3 illustrates the distribution of infection source from 1982 through 2002.

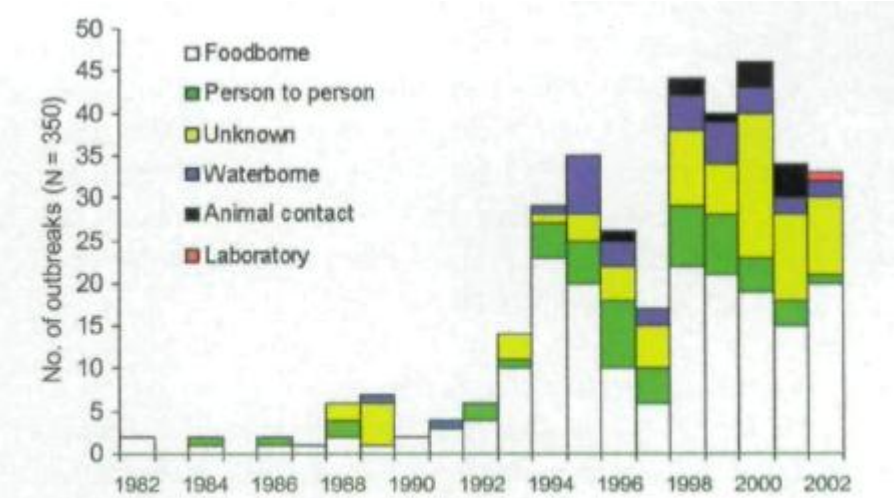


Figure 1.3 – Routes of EHEC transmission by year, from 1982-2002. Figure from Rangel, *et al*., 2005 (218).

Since the initial EHEC outbreaks, many changes have taken place in food monitoring, processing, and handling in order to prevent the extent of future outbreaks. In addition, due to mandatory reporting set in place in 1994, public awareness concerning this problem has increased leading to safer practices to avoid food contamination and person to person transmission. After the 1993 outbreaks, the Food and Drug Administration updated the Model Food Code for restaurants ensuring that meat would be fully cooked. The National Livestock and Meat Board's Blue Ribbon Task Force follow suit in 1994 by encouraging the use of automated cookers. These changes were effective and no fast food outbreaks have been seen since 1995 with the exception of a recent outbreak at Taco Bell (2006) involving produce. In addition, traceback investigations of outbreaks have led to the recall of millions of pounds of beef further preventing

new infections. Any juices in the United States are now required to be pasteurized or contain a warning label. Due to the most recently uncovered method of transmission, contact with animals, public education has increased and events like fairs and petting zoos are much better equipped with hand sanitizers and washing hands stations (218). Finally, research is underway to reduce the number of cattle hosting EHEC and prevent EHEC from ever reaching food sources by switching these cattle from feedlot grain diets to foraging diets (27).

During traceback studies designed to identify the source of an outbreak, currently, the most utilized method in order to "fingerprint" strains of EHEC is Pulse Field Gel Electrophoresis (PFGE). This allows comparison with the PulseNet databank, which contains information about thousands of isolates from numerous outbreaks. The technology has greatly improved epidemiological studies to find the source of outbreaks. Recently, a new methodology has been introduced, Multi-Locus Variable number tandem repeat Analysis (MLVA), which could improve these studies even further as it is quicker and able to better distinguish individual strains and with more reproducibility (47).

Even with many new measures in place, EHEC still remains a major emerging pathogen and significant health problem in the United States. In the past two years alone, in the United States, three major multi-state outbreaks occurred involving produce and beef. These outbreaks involved hundreds of people and led to several deaths (286). Ongoing occurrences of numerous

outbreaks re-affirm how crucial advancement in research of prevention and treatment of EHEC infection is and will be in the coming years.

Presentation, Diagnosis, and Treatment

The incubation period for EHEC is on average three to four days. The initial symptoms may include a fever and non-bloody diarrhea sometimes combined with vomiting and abdominal pain. This is followed by bloody diarrhea and increased abdominal pain over the next one to two days. Most infections will clear naturally; however, about 10% of patients go on to develop hemolytic uremic syndrome (HUS). These patients are typically under 10 years old or elderly. HUS is characterized by hemolytic anemia, thrombocytopenia, and renal failure. Its initial symptoms can include: oligouria, anuria, edema, pallor, and occasionally seizures, which begin a few days after the initial onset of diarrhea (194).

EHEC infection is commonly misdiagnosed as one of the following: appendicitis, intussusceptions, primary inflammatory bowel disease, and ischemic colitis. It is recommended by numerous groups including the CDC that all stool specimens containing blood be cultured for *E. coli* O157:H7. There are three main methods for diagnosing infection by EHEC: 1. Isolation of the bacteria from stool samples, 2. detection of Shiga toxin (Stx) producing organisms, or 3.

detection of antibodies against O157 LPS or alternative EHEC antigens in the blood. Diagnosis usually occurs during the bloody diarrhea stage of illness. However, often, patients don't seek evaluation or treatment until they already have HUS symptoms. EHEC is isolated using sorbital MacConkey medium (SMAC) taking advantage of the fact that in contrast to other strains of *E. coli*, EHEC does not rapidly ferment sorbital. On these plates, EHEC will appear colorless. Polymerase Chain Reaction (PCR) or DNA probes against the genes that produce Shiga toxin or other antigenic EHEC proteins (described in subsequent sections) can also be used to identify EHEC (194). Since there are numerous laboratories in the United States, but particularly in the Southern regions that do not routinely screen for EHEC, the actual number of infections with this organism may be much higher than reported (21).

No treatment for EHEC has been identified so far. Because of this, treatment remains for the most part supportive, including fluid replacement and electrolyte replacement. The use of antibiotics against EHEC has been controversial and studies have reported contrasting results showing antibiotic treatment increasing the risk of HUS in some cases and lowering the risk in other cases (32, 194, 210, 216). It has been considered risky to treat patients with antibiotics due to the fact this might promote further release and subsequent uptake of Shiga toxin. Treatment of HUS resulting from infection with EHEC may include: dialysis, transfusion, and in severe cases renal transplant (194).

Some potential treatments for the future may include antitoxins and toxin-binding drugs (21, 133, 297) as well as drugs that might interfere with EHEC's cell-to-cell signaling as described in subsequent sections (Rasko and Sperandio, unpublished data).

Pathology

One of the most characteristic features of EHEC infection and histopathology is the attaching and effacing lesion (A/E) (see figure 1.4) (130). Once EHEC overcomes the gastric acid layer and passes through the small intestine, it reaches the large intestine and arrives in close proximity to the intestinal

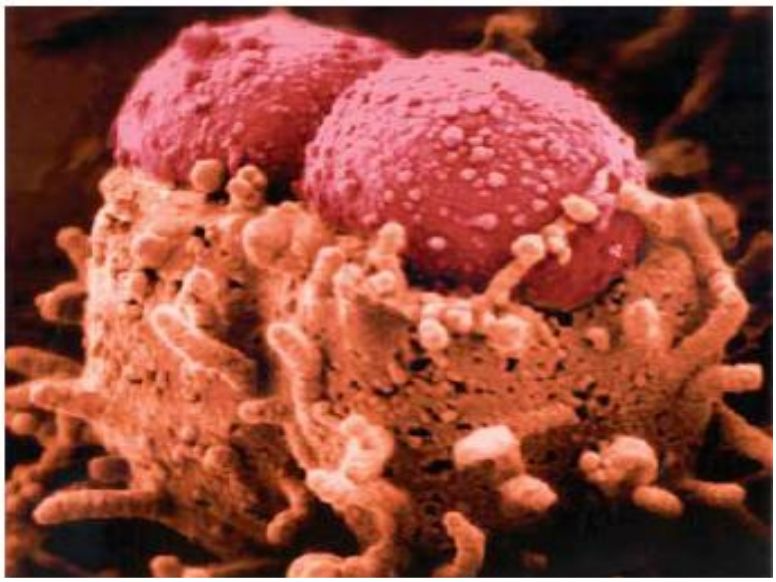


Figure 1.4 – Histology of the attaching and effacing lesion. This shows two bacteria sitting atop two actin pedestals formed by epithelial cell actin nucleation. Figure from Kaper, *et al*., 2004.

epithelial layer. Here, the AE lesion results in the effacement of the microvilli allowing close attachment of the bacterium to the epithelial cell (149). In addition, actin accumulation and nucleation leads to the formation of pedestal like structures that raise the bacteria above the surface of the epithelium (149). The histology of the AE lesion has been described and observed in animal models such as the gnotobiotic piglets and the rabbit model as well as culture epithelial cells (see figure 1.3); however, it has never been observed in a clinical specimen due to the timing and sensitivity of intestinal biopsy (186, 194).

Sweeping effects that are seen in EHEC infected patients are: mucosal ulceration, submucosal hemorrhage and edema in the lamina propria (98, 194). An immune response linked to the AE lesion is also seen with neutrophil infiltration and IL-8 seen at the site of AE lesions (138) (125). Additionally, there is a high concentration of polymerized actin (148) and increased levels of inositol triphosphate and intracellular calcium. It is proposed that the calcium levels lead to actin depolymerization and also eventually decreased adsorption, leading to diarrhea (9).

Genetic Content

The acquisition of foreign DNA is what allows EHEC to be pathogenic as opposed to commensal strains of *E. coli*, which are highly beneficial to human hosts. The extra DNA obtained by EHEC was thought to be a small number of

pathogenicity islands. However, in 2001, the genomes of two EHEC O157:H7 strains, EDL933 and Sakai, were sequenced revealing the large extent of DNA transfer that had actually occurred in EHEC. EDL933 was isolated from ground meat associated with the original EHEC outbreaks in 1982 (211, 222). The Sakai strain was isolated from a patient affected by the primary school outbreaks in Sakai, Japan in 1996 (107, 296). While the pathogenic strains have 4.1 Mb of backbone DNA in common with the laboratory strain K-12 (98.3% conserved), the pathogenic strains contain approximately 1.34 Mb of extra DNA not present in K-12. This composes about 1,400 genes that are found in approximately 177 different O-island regions in the EHEC genome. The extra DNA contains numerous putative virulence factors (a type three secretion system, fimbrial genes, and Stx), prophages, and genes implicated in alternative metabolic functions. 33% of the genes contained in the O-islands are of unknown function. Most surprisingly, EHEC is lacking 0.5 Mb or approximately 528 genes, of DNA that is present in the K-12 laboratory strain (107, 211). Fig. 1.5 shows a comparison of the genetic content of K-12 versus the EDL933 strain of EHEC.

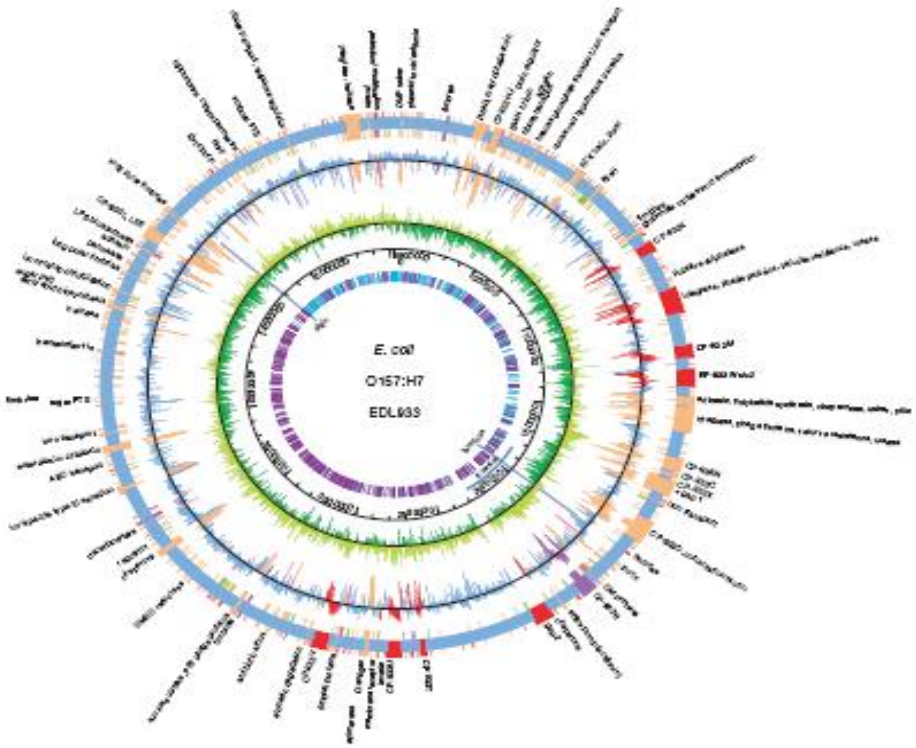


Figure 1.5 – Circular chromosome map of EDL933 EHEC compared to MG1655 K-12 *E. coli.* The outer circle blue shows the shared backbone between the two strains, red shows EHEC specific O-islands, green shows the K-12 specific islands, and tan shows areas where K-12 and EHEC specific islands lie in the same location. Figure from Perna, *et al.*, 2001 (211).

Pathogenesis

Colonization of the Intestine

Once EHEC surpasses the gastric acidity of the stomach and passes through the small intestine, it is able to colonize the large intestine. It has been shown that *E. coli* is able to tolerate highly acidic conditions and this trait is important for its ability to colonize its niche, the large intestine (97, 160). Once it reaches the large intestine, it is thought that EHEC is able to swim across the

mucus layer to get in close proximity with the intestinal wall lining. The use of flagella in order to be motile is important for EHEC pathogenesis and *fliA* mutants, which cannot produce flagella, are deficient in colonization of cattle (249). In addition, a *qseC* mutant is attenuated for virulence in rabbits. As discussed in subsequent sections, QseC activates the master regulator of flagella and motility, *flhDC* (39, 41). Once near the intestinal epithelium, EHEC must adhere to these cells. While multiple adherence factors and fimbriae have been implicated in EHEC's adherence (130), the most well-characterized and thus far important method of adherence is through attaching and effacing lesion formation (AE). The AE lesion is characterized by effacement of the intestinal microvilli and tight adherence of the bacteria to the intestinal epithelial cells. The interaction of the bacteria with host cells induces actin nucleation and rearrangement so that actin accumulates beneath the bacteria and forms a pedestal-like structure (see Fig. 1.4).

The Locus of Enterocyte Effacement (LEE) Region

Most of the genes required for the formation of AE lesions on epithelial cells are encoded in a pathogenicity island known as the Locus of Enterocyte Effacement (LEE) region. This region, acquired by horizontal gene transfer, is composed of 41 open reading frames organized into five major operons (*LEE1,*

LEE2, LEE3, LEE5, LEE4) (figure 1.6) and is highly conserved among all pathogenic *E. coli* that make AE lesions (72, 73, 121, 175, 178).

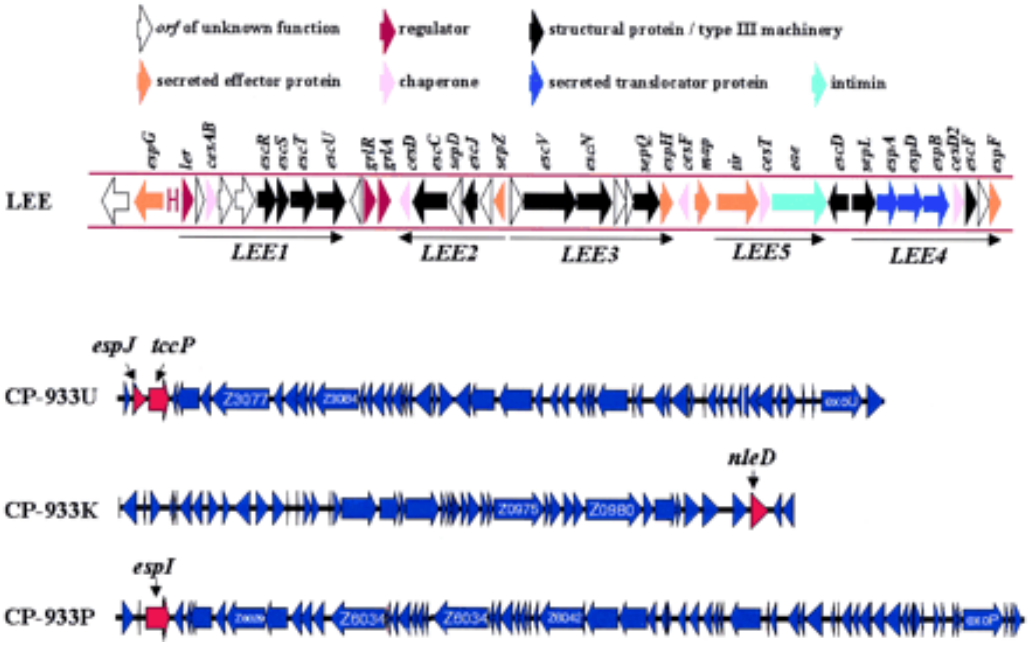


Figure 1.6 - Genetic organization of the EHEC/EPEC *LEE* region and EHEC prophages: CP-933U (encodes *espJ* and *espFu*), CP-933K, CP-933P. Figure from Garmendia, *et al.*, 2005 (92).

Included in the LEE region are the components of a structural type three secretion system (TTSS), an outer membrane adhesin named intimin, and a translocated effector that acts as a receptor for intimin from within eukaryotic cells (Translocated intimin receptor, Tir) (121, 123, 141). The TTSS is a large needle-like apparatus that is used to translocate bacterial effector proteins into host cells. Once inside host cells, these proteins act in numerous ways to either mimic or manipulate the host proteins and signaling pathways to the benefit of the

bacteria. In some cases this leads to the uptake of bacterial cells by eukaryotic cells, while in others such as EHEC, it leads to intimate adherence (44, 95).

The EHEC TTSS system is composed of approximately 20 proteins (see Figure 1.7), which when assembled, have a high degree of conservation with both virulence TTSS from other organisms as well as the flagellar apparatus from other organisms. The main components of the TTSS from EHEC have counterparts with high homology in *Salmonella, Shigella,* and *Yersinia* as well as *Pseudomonas* (2, 169, 270). Figure 1.7 shows a detailed depiction of EHEC's TTSS. The entire complex is assembled beginning with membrane-bound components that are targeted to the membrane using the Sec secretion pathway (92).

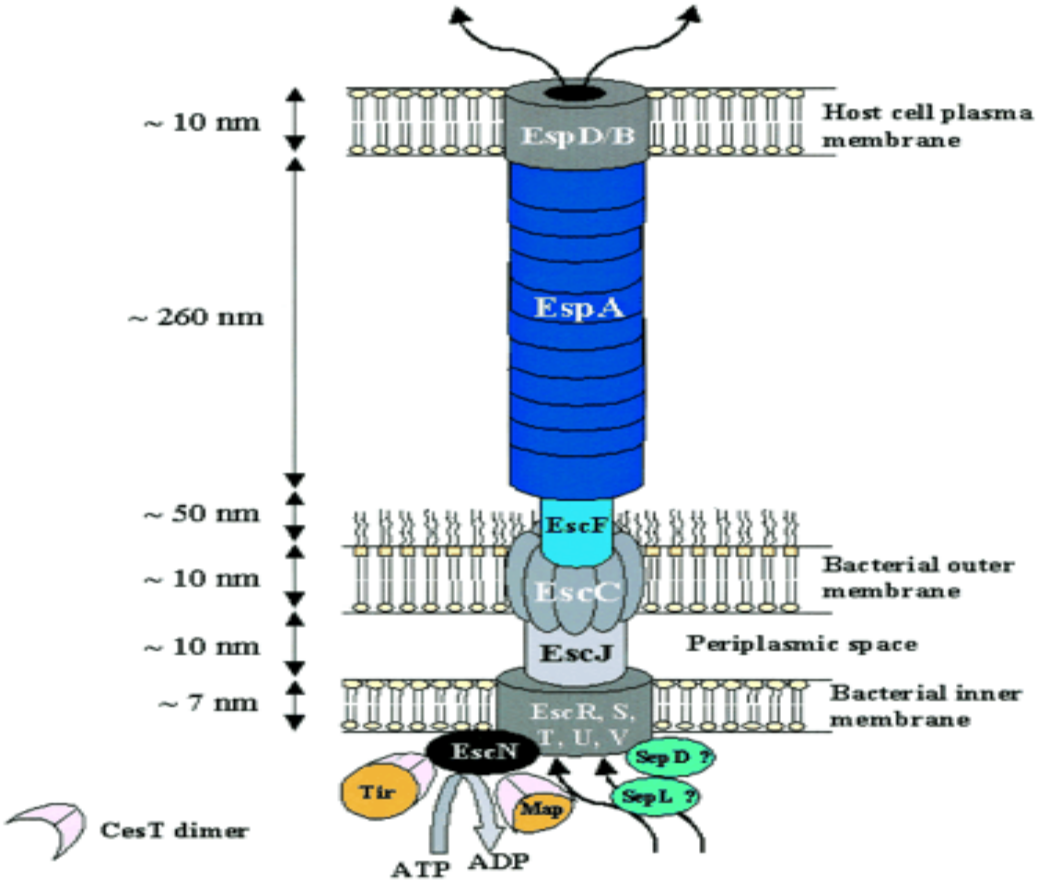


Figure 1.7 - Schematic of the type three secretion system (TTSS) from EHEC. The basal body portion of the TTSS spans the bacterial inner and outer membrane and is composed of the proteins: EscC, EscR, EscS, EscT, EscU, EscV, and EscJ, which connects the inner and outer membrane spanning portions. The needle is composed of EscF and EspA subunits polymerize to form the EspA filament. EscN is a cytoplasmic ATPase that provides the energy required for the system to translocate effectors. Figure from Garmendia, *et al.*, 2005 (92).

EscV is a 72 kDa protein encoded in *LEE3* that makes up the bacterial inner membrane ring structure of the apparatus. This protein contains a Sec sequence to target it to the inner membrane, where its' seven transmembrane

domains make up the inner ring of the structure (94, 152, 153). EscC, encoded in *LEE2*, is a 54 kDa protein from the secretin family that allows molecules of the TTSS and effector proteins to be translocated across the bacterial outer membrane. Although this protein contains a Sec sequence, it is believed that it uses both the Sec system and the initial factors of the type three secretion system to reach the outer membrane. This is based on the observation that in an *escN* mutant, EscC localizes to the periplasm rather than the outer membrane (94).

EscJ, encoded in *LEE2,* is predicted to be a cylinder-like apparatus that spans the periplasm between EscV and EscC. This protein is also Sec-dependent for its target location and has been shown to be required for the secretion of the outermost portions of the TTSS structure (49, 60). EscF, encoded in *LEE4*, is the main needle like structure of the TTSS. A mutant in *escF* is unable to translocate the components of the tranlocation pore as well as any effector proteins (240, 303).

Numerous filaments of EspA, encoded in *LEE4*, make up an extension of the EscF needle (50, 57) . This protein seems to be unique to EPEC and EHEC and seems to play a role in adherence to host cells (43, 66). EspA filaments have been shown to bind directly to EscF (302). Both the EscF needle apparatus and the EspA filamentous tube are removed after translocation of effector proteins so that tight adhesion between the host cell and the bacterium can be achieved (81, 150).

The final and most distal components of the TTSS are EspB and EspD, both encoded in *LEE4*. Together, these proteins make a trans location pore in the host cell membrane, through which effector proteins will pass (26). EspD is able to interact with itself and with EspB and makes up the major component of the pore. Although these proteins share little homology with analogous proteins from other organisms, they do share the following features: hydrophobic transmembrane domains, predicted coil-coil domains, and the ability to homo or hetero-dimerize in order to form the pore (92) (115, 150).

It is not known how the initiation of the formation of the TTSS system is triggered, especially considering that direct contact with host cells is not thought to be required. However, numerous theories exist such as increased levels of ions such as Ca^{2+} or pressure encountered by the cell. Once the formation of the type three secretion system is complete, effector molecule translocation is essential for pedestal formation and tight adherence of the bacterium to the cell. Two proteins that are suspected to have a role in the transition from secretion of translocation machinery to translocation of effector proteins are SepL (*LEE4)* and SepD (*LEE2).* These proteins are both predicted to be cytoplasmic; however, SepD is thought to be a part of the TTSS. A *sepD* mutant is unable to secrete any translocators or effector proteins. On the other hand, a *sepL* mutant is able to secrete effectors, but has reduced secretion of translocators with no EspA filament synthesis and no ability to form AE lesions. SepD has been shown to interact

with SepL indicating that these two proteins may act together to form a key switch in type three secretion (58, 203).

Translocation of effectors is thought to be an ATP-dependent process. The ATPase EscN is required for secretion of proteins (226). In addition, most of the effector molecules and secreted proteins require chaperones for proper translocation and stability. Chaperones have been described for: EspF, Tir, Map, EspB, EspA, and EspD (121, 142, 291). The translocated intimin receptor (Tir) is absolutely required for attaching and effacing lesion formation in EHEC and EPEC. Mutant strains containing deletions in Tir are drastically attenuated for virulence. Tir is translocated into host cells once the type three secretion apparatus is formed. Once in the host cell, Tir is involved in two functions. First, Tir embeds itself in the host plasma membrane in a hairpin loop conformation serving as a receptor for the bacterial outer membrane protein intimin (141). Intimin's N-terminal serves as an anchor in the bacterial outer membrane forming a β-barrel like structure (282) and mediates dimerization, while the C-terminus of intimin extends from the bacterial cell and interacts with receptors on the host cell membrane and Tir (79). In addition to binding Tir, many studies have suggested that intimin also binds the host cell proteins nucleolin and β-1 integrin (80, 244, 245) .

The cytoplasmic portion of Tir serves in Tir's second function in initiating a signaling cascade in order to nucleate actin polymerization. Herein

lies one of the major differences in pedestal formation by EPEC and EHEC. In EPEC, Tir is tyrosine phosphorylated at Tyr^{474} by the eukaryotic proteins Src, Arg, and Abl (212, 266). This creates a binding site for the SH2/SH3 adaptor protein Nck, which in turn activates N-WASP and recruits the Arp2/3 complex to initiate actin nucleation. EHEC, in contrast, does not contain a corresponding Tyr residue, and instead utilizes a non-LEE encoded protein similar to Nck to connect it with N-WASP and Arp2/3. EspFu/TccP is encoded on prophage CPU933, but is secreted into host cells via the LEE TTSS. This protein binds the GTPase-binding domain (GBD) domain of N-WASP and is recruited to Tir, enabling actin nucleation (Figure 1.8) (28, 82, 93). While EspFu is generally considered an EHEC specific protein, it has been identified in atypical strains of EPEC that are able to utilize two pathways to form pedestals (82). The role of EspFu in pathogenesis and enhancing EHEC's ability to make pedestals has been highly debated. A recent report showed that EspFu may play a role in EHEC persistence and stability in attachment to the epithelial cytoskeleton after initial attachment

(224).

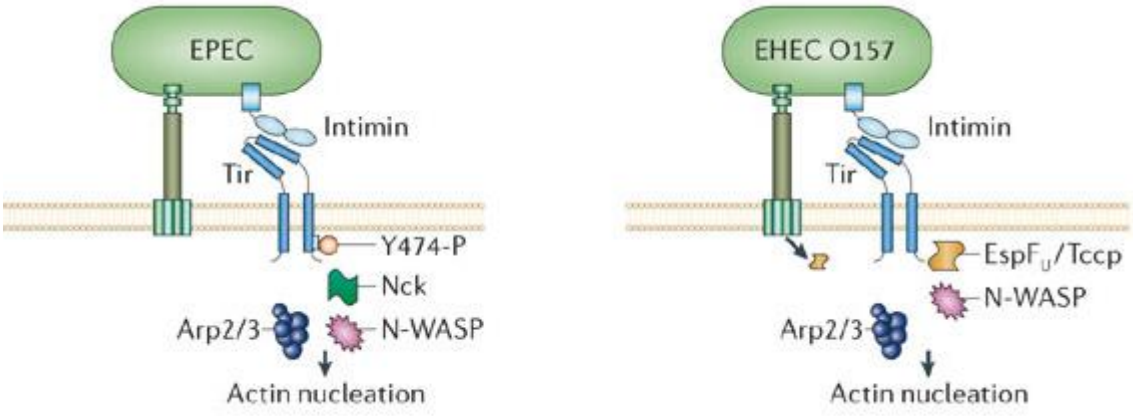


Figure 1.8 – Pedestal formation in EHEC vs. EPEC. Depiction of the host and bacterial cell signaling that leads to pedestal formation in EHEC (right) and EPEC (left). EHEC tir is phosphorylated on tyrosine 474 leading to Nck binding and recruitment of the Arp2/3 complex and N-WASP. In contrast, EHEC Tir is not tyrosine phosphorylated, but interacts with the translocated effector protein EspFu to recruit N-WASP and Arp 2/3. Figure from Stevens, *et al.*, 2006 (261).

LEE Encoded Effector Proteins

In addition to Tir and the components of the TTSS, there are numerous other effectors encoded within the LEE region, which are translocated into host cells where they target specific host pathways and proteins. Many of these have been characterized and the following is a brief summary.

Mitochondrial-associated protein (MAP) has been implicated in several major functions. As its name implies, it targets the mitochondria and interferes with mitochondrial membrane potential. It is also characterized by the formation of filopodium-like structures at the sites of bacterial infection. Finally, MAP has

an important role in altering tight junctions of epithelial cells (140, 143). Although mutant strains for this gene can still colonize, it is thought that MAP plays a role in persistence of the organism in the host (59, 190).

EspF is a proline-rich protein that also plays a role in tight junctions; however, its role is thought to be separate from MAP. It is known that this protein is required for loss of transepithelial resistance, increased monolayer permeability, and redistribution of the protein occludin, which is involved in tight junctions. This protein is thought to be responsible for blocking the uptake of EHEC by macrophages (176, 177). Recently, EspF was found to initiate a cascade involving eukaryotic sorting nexin 9 (SNX9) and neuronal Wiskott-Aldrich syndrome protein (N-WASP) in order to elicit membrane remodeling. The same study showed that EspF localizes to membrane-trafficking organelles (4, 5).

EspG shows similarity to the protein VirA from *Shigella*. In Shigella, VirA plays a role in bacterial uptake by cells, and EspG is able to complement this mutant (70, 311). EspG seems to trigger the formation of actin stress fibers and disrupt microtubule networks underneath the site of bacterial adherence (174). Little is known about EspH; however, it seems to also act on the eukaryotic actin cytoskeleton (283).

While EspB is part of the TTSS translocation machinery, it also has been shown to have an effector function. This protein affects cell morphology when

He-La cells are transfected with it. EspB localizes to the cytosol as an effector and binds the eukaryotic protein, β-catenin, which is associated with the cell cytoskeleton (151, 274, 275).

SepZ is a recently characterized *LEE* encoded effector. Although originally implicated in uptake of bacteria into host cells, EPEC and EHEC are not considered intracellular pathogens. SepZ is shown to accumulate beneath the site of bacterial infection or pedestals in eukaryotic cells (127) .

Non-LEE Encoded Effector Proteins

Numerous proteins have also been identified, which are encoded outside the *LEE* region, typically on cryptic prophages, but utilize the *LEE* type three secretion machinery to be translocated into host cells. Though not exhaustive, the following highlights some of these effectors.

Cycle inhibiting factor (cif) has an irreversible cytopathic effect on cells. It can assemble stress fibers and as its name indicates, inhibit eukaryotic cell cycle at the G_2/M phase transitions. EspJ is located on the cryptic prophage CP933U. This protein is not required for the formation of AE lesions, but plays a role in host survival and transmission as mutants in this gene influenced the clearance of the pathogen from the host intestinal tract (173). Recently, this protein was implicated in inhibition of macrophage phagocytosis independently of EspF (172). EspFu/Tccp is a proline-rich protein encoded downstream of EspJ on

CP933U (Figure 1.6). Interestingly, this protein shares remarkable similarity to the *LEE* encoded effector EspF (24% identity) and is able to complement a mutation in EspF. However, as described above, it has been shown to play a unique role in attaching and effacing lesion formation by connecting Tir and N-WASP (28, 93) . NleA has been shown to be essential for bacterial virulence (101, 190). This protein interferes with host cell intracellular transport by inhibiting Cop II –dependent protein secretion form the endoplasmic reticulum. CopII is involved in shaping intracellular protein transport vesicles (101, 144) .

A recent study used bioinformatics to identify putative translocated effector proteins in the Sakai strain of EHEC. This study revealed that the repertoire of non-LEE encoded effectors is much greater than previously suspected. Indeed, the authors of this study were able to experimentally confirm that 39 proteins were effectors based on proteomics and translocation assays (281). Investigation and characterization of these proteins is currently underway.

Regulation of the LEE Region

Regulation of the *LEE* region is extremely complex. Figure 1.9 shows a model of LEE regulation. While most identified regulators are shown, the model is not exhaustive. Numerous regulators both specific to EHEC and present in EHEC and the laboratory strain K-12 modulate expression of this pathogenicity island. Histone-like nucleoid structuring protein (H-NS) is able to repress the

transcription of *LEE* 2, 3, and 5 through direct interaction with these promoters (24, 102, 178, 230, 253, 287). Encoded in *LEE 1* is the *LEE* encoded regulator or Ler, which is able to overcome H-NS repression and activate transcription of *LEE* 2, 3, 4, and 5 (24, 71, 86, 102, 178, 230, 253). One study has shown that in EPEC Ler is able to autorepress *LEE* 1 for an additional layer of regulation (16). Integration host factor (IHF) is another activator of *LEE* expression through *LEE1* (86). Hha represses *LEE4* through environmental regulation (241). The stationary phase sigma factor (RpoS) activates both *LEE3* and *LEE1* (254). GrlR and GrlA, ORF10 and ORF11 of the *LEE* region are both specific to EHEC, and simultaneously repress and activate *LEE*1 respectively (59). These genes are both activated by Ler to complete a feedback loop (10) (71) (228). ClpX is a protease that degrades damaged or incomplete proteins and interacts with both RpoS and GrlR, thereby also regulating the *LEE* region (117). DsrA is an 85 nucleotide non-coding RNA that responds to environmental signals and stimulates the *LEE* region through RpoS (155). The nuceloid associated protein, Fis also modulates LEE expression; however, little is known about its method of regulation (96). EtrA and EivF are encoded on a second type three secretion system in EHEC that is non-functional and negatively regulate the *LEE* region (313). Three Pch proteins, A, B, and C, which have homologues in EPEC activate *LEE*1 expression (118). Finally, the DNA adenine methyltransferase (Dam) protein plays a role in LEE regulation at the post-transcriptional level. This protein is important for

normal cellular processes like chromosome replication and stimulation of the SOS response (165), but has also been shown to be involved pathogenesis of many bacterial species through control of type three secretion (90). *Dam* mutants in EHEC express increased levels of the *LEE* encoded proteins intimin and Tir (29).

The *LEE* region has also been shown to be modulated by the environmental signals, auto-inducer 3 (AI-3), epinephrine, and norepinephrine (257). These signals are modulated through several signaling proteins known as the Quorum *E. coli* Sensing Regulators A-G, which are present in both EHEC and K-12. QseA is a LysR-type regulator that has been shown to activate transcription of *ler* through direct interaction (242, 252). QseD is another LysR-type regulator, which is shown to have effects on both the *LEE* region and flagella and motility (Benjamin Habdas, unpublished data). The EHEC quorum sensing regulators and the effects of AI-3, epinephrine, and norepinephrine will be discussed in further detail in subsequent sections.

Prokaryotic signaling systems have also been shown to modulate the *LEE* region. The RcsCDB phosphorelay system, which is involved in biosynthesis and export of colanic acid, has been shown to modulate the *LEE* region through both positive and negative regulation (280) (33).

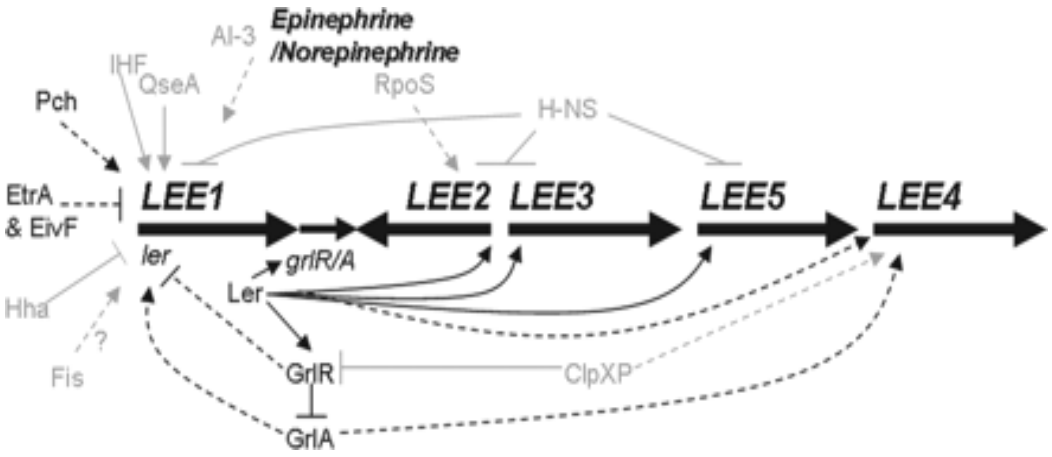


Figure 1.9 – Model of regulation of the LEE region. Numerous genes are involved in the regulation of the LEE region. Proteins that appear in grey are present in both K-12 E. coli and EHEC and factors that appear in black are specific to EHEC. A solid line represents an interaction that is direct with the target promoter and has been shown biochemically. Dashed lines show interactions that are either indirect or have not been shown to bind directly to a target biochemically. Figure from Walters, et al. 2006 (294).

Importantly, very little is known about the regulation of non-LEE encoded effector proteins. An initial study investigating environmental regulation of effectors showed that transcription of both EspJ and EspFu/TccP alters when temperature, pH, osmolarity, or O_2 pressure changed (91). In addition, a recent study examined the regulation of NleH, showing that it is post-transcriptionally regulated in a manner linked to expression of the LEE region (89).

pO157 Plasmid

All EHEC O157:H7 strains contain the highly conserved O157 plasmid. This plasmid, approximately 100 kb, encodes an enterolysin, potential adhesion factors, a catalase-peroxidase, a type II secretion system, a serine protease (EspP), and StcE (15, 22, 156, 237, 272). The role of the enterolysin in pathogenesis is unknown; however, the lysis of erythrocytes could release heme leading to enhanced growth of EHEC (15). StcE cleaves the C1 esterase inhibitor. C1 is part of the complement pathway and StcE's action could be involved in the tissue damage, edema, and thrombosis seen as a result of EHEC infection (156). The potential adhesion factor encoded on this plasmid is ToxB (272). Conflicting reports have been published about the role of this plasmid in pathogenesis and adherence. Using cultured epithelial cells, with some serotypes, the plasmid enhanced adhesion, while in others loss of the plasmid made no difference (104). In animal models, the plasmid has been tested with the K-12 strain and a report showed that K-12 containing pO157 was able to adhere to rabbit intestinal cells while K-12 alone was not (64). No role has been seen for this plasmid in development of diarrhea or intestinal histopathology (162, 285).

Shiga Toxin

Shiga toxin (Stx) is the major virulence factor of EHEC and differentiates it from other strains of attaching and effacing pathogenic *E. coli*. Originally

linked to outbreaks of enterocolitis and HUS by Karmali, *et al*.(134), this toxin is responsible for the mortality of EHEC and leads to the complications that cause HUS (239, 269, 277). Stx is divided into two toxins, Stx1 and Stx2, and EHEC may express one or both of them. Stx1 is highly conserved and is exactly the same in EHEC and *Shigella dysenteriae*. Stx2 is more variable and suggested to be more important in disease than Stx1 (202, 238) Takeda 1995(18, 87, 99)). Stx is an A-B toxin, in which the A subunit is proteolytically cleaved into two subunits connected by a disulfide bond. One subunit is enzymatically active, while the other connects the A subunit to the five identical B subunits that serve to bind the toxin to its receptor, globotriaosylceramide (Gb3), located on the surface of eukaryotic cells (163). The holotoxin is endocytosed through coated pits, transported to the golgi apparatus, to the endoplasmic reticulum (231), and then the A subunit is transferred to the cytoplasm, its site of activity. Subunit A is an N-glycosidase and acts on the 60S ribosomal subunit inhibiting protein synthesis (74). This ultimately leads to cell death in cells that contain the Gb3 receptor, including renal endothelial cells and intestinal epithelial cells. It is thought that Stx is translocated from the intestine enters the bloodstream becoming systemic. Stx in addition to LPS induces the body's inflammatory response to EHEC, which causes tissue disruption (227, 278, 300). Glomerular endothelial cells are a target of Stx. As these cells are damaged, the glomerular

filtration rate may be slowed leading to the acute renal failure that is seen in HUS patients (201) (167).

The genes for Stx are encoded on a lambdoid bacteriophage and this phage is capable of lytic growth and production of infectious particles, indicating its capability of continued evolution (201). This location of the genes encoding potent toxins makes the use of antibiotics very controversial and extremely risky in treatment of EHEC infection. The bacterial SOS response induces bacteriophages to enter their lytic cycle. In response to DNA damage, the bacterial RecA protease cleaves the CI phage repressor, inducing phage-encoded genes, production of phage particles, and bacterial cell lysis. Consequently, this allows the expression and release of the Stx toxin into the host (146, 197). Numerous antibiotic compounds including quinolones, trimethoprim, and metronidazole have been shown to induce the bacterial SOS response and in addition have been shown through reporter assays to induce Stx synthesis. Although reports about whether the use of antibiotics to treat EHEC infection increases the risk of developing HUS are mixed, the knowledge that these compounds induce Stx expression indicates that antimicrobial therapies should be avoided (145, 146). This reiterates the importance of discovering new methods of treatment for EHEC infection that will not induce an SOS response in the bacteria.

Immune Response

Although very little is known about the immune response to EHEC infection, many factors, such as the greater susceptibility of the young and elderly to HUS, indicate that it is important. Most EHEC immunological studies have involved cultured epithelial cells or sera collected from patients. Thus far, no protective immunity has been demonstrated (21); however, when sera from patients infected with EHEC has been examined, antibodies against Tir, Intimin, EspA and EspB have been detected indicating that the body mounts an immunological response to these proteins (122, 135, 161). It has also been shown that antibodies to LPS, hemolysin, and Stx are generated upon infection (236) (189). The role of the LEE region and attaching and effacing lesion formation in immune response has been debated. Although attachment of bacteria to epithelial cells is required for a polymorphonuclear leukocytes (PMN) infiltration, it has been shown that cells infected with LEE negative strains exhibit an even stronger cytokine response than wild type cells (227).

A strong pro-inflammatory response to EHEC infection has been seen with the recruitment of PMNs to the intestine. In addition, elevated levels of numerous cytokines such as: IL-8, Gro-α, β, and γ, ENA-78, and TNF-α are seen, which aid in the recruitment of PMNs (69, 135, 227, 232, 278). It has been shown that PMN infiltration is dependent on attachment of the bacteria to the epithelial cells (233). The increased concentration of cytokines is related to Stx

and treatment of cultured epithelial cells directly with Stx can induce these changes (278). In addition, the production of flagellin has been shown to elicit a robust cytokine response (227). It is thought that the influx of inflammatory cells into the intestine after infection leads to much of the tissue damage seen in the histopathlogy of EHEC infection. When cells are pretreated with anti-CD18, PMN infiltration and tissue damage is blocked (69). It has been shown that PMNs can bind to Stx. Since EHEC is a non-invasive bacterium, it has been suggested that PMN infiltration plays a large role in allowing Stx to reach its targets through the breakdown of the epithelial barrier.

Animal Models

Thus far a good animal model that replicates the pathogenesis of EHEC in humans has yet to be developed. However, numerous animals have been tested in infection models to make observations about infection with pathogenic *E. coli*. Several studies have used Streptomycin (Sm) treated mice in order to assess the virulence of different strains of EHEC. The mice show susceptibility to Shiga toxin; however, they do not exhibit AE lesion histology nor do they develop diarrhea (192, 193, 289, 290). A ferret model has also been investigated. This model also utilized Sm treatment of animals before inoculation to enhance intestinal colonization. Although these animals develop histological damage to the glomeruli and thrombocytopenia as seen in HUS in response to Stx, similar to

the mouse model, none of the animals developed colitis. When ferrets were infected with intimin mutants, they depicted less weight loss than animals infected with wild type indicating that attaching and effacing lesions may play some role in this animal model (307). Infant rabbits have been used as a model of infection and have been successful in showing the importance of *Stx*, *eae* and *tir* in colonization and pathogenesis *in vivo.* The infant rabbit model is advantageous over the mouse and ferret models in that no Sm pretreatment is necessary for colonization and that the initial features of EHEC infection such as diarrhea and attachment and effacement are seen. However, this model's major drawback is a lack of the Stx receptor, Gb3. The infant rabbits do not develop HUS and the lack of Gb3 may be the reason (207, 225).

Larger animals, such as the gnotobiotic piglet have been tested with EHEC infection and have been shown to mirror human infection. In this model, the piglets showed development of diarrhea, effacement of microvilli, epithelial damage, and the influx of inflammatory cells after EHEC colonized the large intestine (78, 284). Cattle are EHEC's natural reservoir and EHEC can successfully colonize these animals without causing disease. Calves less than three weeks old, however, have been shown to be susceptible to illness from EHEC, and have been used as a model of infection. These calves were inoculated with EHEC within 36 hours after birth and developed severe diarrhea and enterocolitis with the typical attaching and effacing lesion (55, 56, 171). The use

of nonhuman primates as models of infection has also been investigated. Studies have shown that injection of Shiga toxin into baboons elicits characteristic responses to HUS, including thrombosis in the renal flomerular and endothelial cell damage. When infected with EHEC, macaques exhibited all the signs of early human infection with EHEC including: diarrhea, attaching and effacing lesion formation, epithelial cell damage, and PMN recruitment to the intestine (129, 273). While infection of each of these three larger animals closely mirror human infection, the gnotobiotic pig, calf, and nonhuman primate models all prove difficult for large experiments as care for the animals is both costly and requires special facilities.

Rabbit-specific strains of EHEC, such as HInvEC and RDEC or rabbit EPEC (rEPEC), have been used to mimic the infection of humans with EHEC. These strains are able to induce diarrhea in rabbits as well as mucosal ulcers, bacterial invasion of the lamina propria, and PMN infiltration to the lamina propria typical of the attaching and effacing lesion and similar to what is seen in human infection with EHEC. This model was developed in 1977 and is currently used as the closest animal model to human infection with EHEC (30, 31, 316).

Vaccine Development

Currently, there is no vaccine for protection against infection with EHEC. Efforts to develop a vaccine have been hampered due to the lack of a good animal

model as discussed above. However, due to the continued occurrence of outbreaks and continual resurgence of routes of transmission aside from contaminated beef (produce, person-to-person, animal contact), there is a strong drive to develop a working vaccine. The current strategies involve either developing vaccines for humans or developing large scale vaccines for cattle herds in order to target EHEC's main reservoir from the start. Shiga toxin is a key candidate for human protection and several studies have tested toxoid vaccines in many animals. In order to avoid the negative effects that Shiga toxin elicits on cells, numerous forms of the toxin have been used in vaccine animal trials, including a subunit vaccine as well as a vaccine containing Shiga toxin mutated in its active site (E167Q) (1) (20). In addition, vaccine strains from other organisms such as *Vibrio cholerae* have been engineered to express Shiga toxin (25). These constructions have been used to inoculate rabbits and pigs and have had success being able to induce neutralizing antibodies and partial protection (1, 20, 25). Shiga toxin derived vaccines, however, will only protect against HUS and will not prevent colonization and attachment of EHEC to the intestinal lining. For this reason, the ideal vaccine might combine Shiga toxin derivatives as well as components involved in attachment and effacement (194).

Several of the factors involved in type three secretion and AE lesion formation have been characterized for their ability to produce an antigenic response in humans and have been investigated as potential vaccines for primarily

cattle, but also humans. One report indicated that sera from human patients showed the most robust response to Tir from the group of Tir, Intimin, EspA, and EspB tested. Each protein, however, has been used in various vaccination efforts with mixed results (65, 122, 161). Similar to the heterologous expression above, intimin has been expressed in the *Vibrio cholerae* vaccine strain (25). Several type three secreted proteins including EspA and Tir were used together to vaccinate cattle and these cattle showed a distinct decrease in shedding of bacteria in the feces (213). This study was followed by another examining how cross-reactive these vaccines might be in order to confer immunity of cattle to other serotypes of EHEC and found that the protection was quite sero-specific (7).

Collectively, these studies indicate that the prospect of a vaccine for EHEC could be quite near. However, the prevention of EHEC currently relies on improving the conditions of food supplies and sources.

Bacteria to bacteria signaling, Quorum Sensing

For many decades, the dogma stated that bacteria were simple single-celled organisms with no higher means of communication or way of acting in a multicellular fashion. In 1970, an exciting discovery drastically shifted the paradigm of how bacteria behave and made the the adaptation of both pathogenic and commensal microorganisms to certain niches more understandable. In this

study by Nealson, *et al*., the regulation of bioluminescence in *Vibrio fischeri* was described. This was the first report of a phenomenon known as quorum sensing (QS), in which bacteria could respond to chemical hormone-like compounds known as autoinducers. When the autoinducer reaches a critical threshold, bacteria are able to detect this signal and respond by altering their gene expression (195, 196). Subsequent studies to the one by Nealson, *et al*. showed that QS is widespread mechanism of gene regulation in bacteria.

Three main types of quorum sensing exist. The first, described by Nealson, is the LuxR/I system. In this system, a protein, LuxI produces acyl homoserine lactones (AHLs), while LuxR is activated by these autoinducers and increases luciferase operon transcription (75, 76). The synthase LuxI produces AHLs, which are composed of a conserved homoserine lactone ring connected through an amide bond to a variable length acyl chain. The number of carbons on the acyl chain can vary from 4 to 18, giving the AHL specificity. LuxI type proteins utilize S-adenosyl-methione to synthesize the homoserine lactone ring, while the acyl chains come from lipid metabolism (126, 187, 209). LuxR-like proteins are transcription factors that affect transcription of downstream genes after binding AHLs. They usually require AHL binding for stability and if not bound, are targeted for degradation (314, 317, 318). Typically, LuxR proteins recognize only one AHL and are involved in primarily intraspecies communication.

An exception to this rule is the SdiA gene. SdiA is a luxR homologue present in both *E. coli* and *Salmonella*. However, no LuxI homologue is present in either species. It has been shown that SdiA senses AHLs from other organisms (180, 265, 295). Although no role for SdiA is clear, its overexpression has been shown to repress the type three secretion genes intimin and EspD and adhesion (128).

LuxR/LuxI signaling is widespread and used by numerous different bacterial species to regulate phenotypes. Some examples are the production of antibiotics in *Erwinia*, motility in *Yersinia pseudotuberculosis*, and pathogenesis and biofilm formation in *Pseudomonas aeruginosa* (52, 54, 208).

As well as being widespread among prokaryotes, the LuxR/LuxI QS system has been implicated in inter-kingdom signaling. For example, the marine macroalga *Delisea pulchra* blocks interaction of the plant pathogen *Serratia liquifaciens* by producing a halogenated furanone that acts as a competitive inhibitor of the bacterium's AHL-based QS system (219). In addition, many reports have indicated that bacterial AHLs modulate gene expression in mammalian organisms indicating that response to AHLs may play a role in the beneficial or pathogenic outcomes of eukaryote-prokaryote interactions. For example, 3OC12-HSL from *P. aeruginosa* influences the production of several cytokines by immune cells (223, 246-248, 271, 276). In addition, Chun, *et al*. have reported that human airway epithelia inactivate 3OC12-HSL (37). Follow-

up studies showed that *P. aeruginosa* 3OC12-HSL can both enter mammalian cells and activate chimeric transcriptional factors based on its cognate LasR transcriptional activator (301). These studies set a precedent for prokaryotic-eukaryotic cell to cell signaling.

The second type of QS system is present in gram positive organisms. This system utilizes small peptides that act through two-component signaling systems. The small peptides are products of oligonucleotides that are cleaved or further modified in the cytoplasm before being exported from the bacterium by transporters. These peptides are recognized by sensor kinases once they reach threshold concentrations. This type of signaling is usually very specific and the receptors involved only recognize their cognate peptide (200). Some examples are toxin production from *Staphylococcus aureus* and cytolysin production by *Enterococcus faecalis (45, 103, 200)*.

The third QS system that has been described is the LuxS/AI-2 system. This system is also thought to be widespread as it is found in over 55 bacterial species, both Gram negative and Gram positive and for this reason is implicated as a universal signal for interspecies communication (182, 184, 308). The LuxS/AI-2 system was originally identified as part of the *Vibrio harveyi* bioluminescence regulatory cascade (235). LuxS is an enzyme involved in the metabolism of S-adenosyl methionine (SAM). It is able to convert S-ribosylhomocysteine into homocysteine and 4,5-dihydroxy-2,3-pentanedione

(DPD). DPD is a highly unstable compound and reacts with water to cyclize and form several furanones, including the proposed precursor for AI-2 (235, 257, 304). The structure of AI-2 has been determined by co-crystallizing the compound with its receptor LuxP in *V. harveyi* and is a furanosyl borate diester (34).

As shown in figure 1.10, *Vibrio harveyi* contains two hybrid sensor kinases, LuxN and LuxQ that sense AI-1 and AI-2, respectively. At low cell densities, without signal, both act as kinases and phosphorylate the intermediates, LuxO and LuxU (12-14, 84, 85). Phosphorylated LuxU is able to activate the transcription of several σ^{54} dependent small RNAs. These RNAs destabilize the message of LuxR, so that transcription of the luciferase operon (*luxCDABEGH)* does not occur (158, 159). At high cell densities, when LuxQ and LuxN are able to communicate with the cognate autoinducers, they act as phosphatases, and the dephosphorylated system is able to activate bioluminescence. Homologues of this system are limited to *Vibrio* species.

Both *E. coli* and *Salmonella* species have been shown to harbor the *luxS* gene and have AI-2 activity measured by the *V. harveyi* bioluminescence assay (234, 308). As shown in figure 1.10, in *Salmonella* and *E. coli*, AI-2 regulates the ABC transporter Lsr (LuxS regulated), which is responsible for AI-2 uptake (268). This has been shown to be a distinct form of AI-2, 2R, 4S-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (183). Once taken up by the cell, this AI-2 is

phosphorylated and interacts with LsrR. LsrR is transcription factor involved in repressing expression of the *lsr* operon. Binding of AI-2 derepresses this system leading to increased expression of the *lsr* operon and increased uptake of AI-2 (267). The genes of the *lsr* operon are the only genes in *Salmonella* and *E. coli* demonstrated to be directly regulated by AI-2 (268, 309).

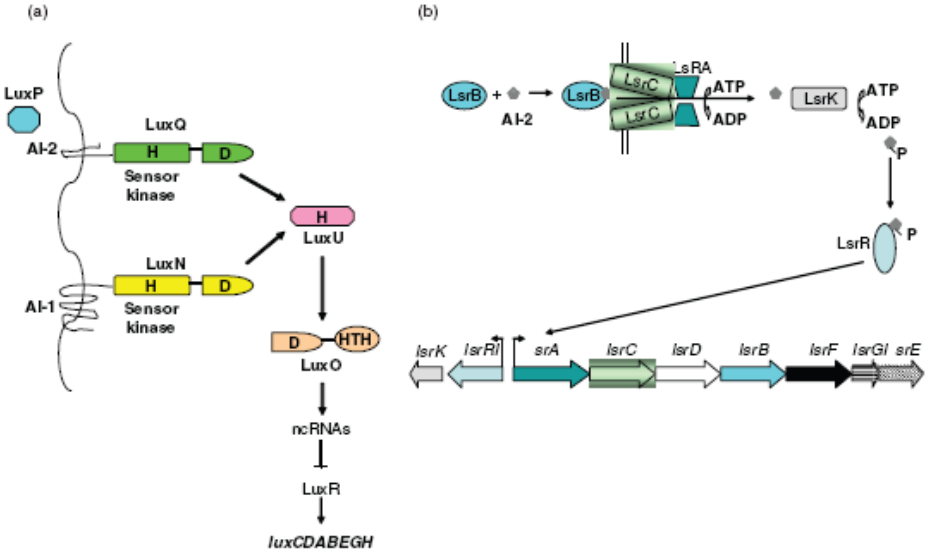


Figure 1.10 - Depiction of the LuxS/AI-2 quorum sensing system. A. *Vibrio harveyi* uses two sensor kinases, LuxN and LuxQ to recognize AI-1 and AI-2 respectively. LuxQ recognizes AI-2 complexed with its periplasmic receptor LuxP. Upon sensing these signals, these kinases become phosphatases and this complex phosphorelay system (through the intermediates LuxU and LuxO) is dephosphorylated. Dephosphorylated LuxO no longer activates transcription of small RNAs. Consequently, LuxR mRNA is no longer degraded, and LuxR is able to activate transcription of the luciferase operon. B. In *Salmonella* and *Escherichia coli* the AI-2 receptor is the LsrB periplasmic protein. Upon binding LsrB, AI-2 is transported inside the cell via the Lsr ABC transport system. AI-2 is then phosphorylated by LsrK, and presumed to interact with LsrR. LsrR is a transcriptional repressor of the *lsr* operon. Upon complexing of Lsr to AI-2, LsrR no longer represses *lsr* transcription. Figure from Reading, *et al*. 2005 (220).

Because LuxS in involved in metabolism of SAM as well as AI-2 production, its mutation will affect the expression of genes involved in QS and genes affected by the disruption of the SAM metabolic pathway. Recently, a fourth bacterial communication system has been discovered involving the eukaryotic hormones epinephrine and norepinephrine and a compound known as autoinducer -3 or AI-3 whose production is affected by the mutation of the *luxS* gene (257). This system is described in further detail below.

EHEC's Cell to Cell Signaling System

The AI-3, epinephrine, norepinephrine signaling system in EHEC was discovered serendipitously based on the fact that the *luxS* mutation affects not only genes affected by the loss of AI-2 production, but also genes affected by the disruption of the metabolism of SAM. Many initial studies examining QS's role in virulence in EHEC involved comparing WT EHEC to a *luxS* EHEC. In 1999 and 2001, based on such studies, Sperandio *et al*. reported that the genes involved in type three secretion, AE lesion formation, and Stx were controlled by QS (254, 255). In 2003, Sperandio *et al*. noticed that while there was a defect in the production of the proteins involved in type three secretion in the *luxS* mutant strain, this strain could still form AE lesions. This indicated that another compound was involved in type three secretion regulation that was present in

assays using HeLa cells. This compound was eventually narrowed down to epinephrine, which was present in the 10% fetal bovine serum (FBS) that is used for culturing HeLa cells. Indeed, purified epinephrine and norepinephrine were able to complement the *luxS* mutant and restore expression of proteins involved in type three secretion as well as flagellation (257).

Hormones are utilized by eukarotes for cell-to-cell signaling. There are three main groups of these chemical messengers: polypeptide hormones, steroid hormones, and hormones derived from tyrosine. The last group includes the hormones epinephrine and norepinephrine (109). Norepinephrine has previously been shown to induce bacterial growth and be taken up by bacteria (147, 168). Epinephrine and norepinephrine are both present in the gastrointestinal (GI) tract (68). Norepinephrine is synthesized within the adrenergic neurons present in the enteric nervous system (ENS) (88). Epinephrine is synthesized in the central nervous system (CNS) and the adrenal medulla and is able to act in a systemic manner after being introduced into the bloodstream, eventually reaching the intestine (217). Both hormones modulate smooth muscle contraction, submucosal blood flow, and chloride and potassium secretion in the intestine (111). The ability to monitor the level of these hormones might allow bacteria to gauge the metabolic state of the host. There are nine known human adrenergic receptors, partitioned into three subclasses: α-1, α-2, and β. The structure of the human β-2 adrenergic receptor was recently reported and predicted that the ligand-binding

sites for both epinephrine and norepinephrine are broadly similar (83). This indicates that these catecholamines are recognized by the same receptor. The α and β adrenergic antagonists, phentolamine (PE) and propranolol (PO) respectively have been shown to block response to catecholamines in the colon (111). PO and PE were also able to block epinephrine's rescue of the *luxS* mutant for type three secretion and transcription of the *LEE* genes. In addition, PO was able to block the *luxS* mutant from forming AE lesions on He-La cells after treatment with epinephrine, showing that these hormones are specific activators of virulence genes in EHEC (256).

In order to see the specific effects of AI-2 on virulence genes, in the same study in 2003, Sperandio *et al.*, purified AI-2, which was not commercially available at the time. Purified AI-2, however, was unable to activate *LEE1* nor could it complement the *luxS* mutant and restore type three secretion, although it was able to activate luminesceince in *V. harveyi*. AI-2 is extremely polar and does not bind to C-18 sepharose packed columns. In contrast, catecholamines, such as epinephrine and norepinephrine are able to bind these columns and can only be eluted with organic solvents. During the purification of AI-2, another fraction eluted from C-18 columns with MeOH that was able to activate *LEE1*, but not luminescence in the *V. harveyi* assay. This fraction was also able to restore type three secretion to the *luxS* mutant as well as activate transcription of genes involved in regulation of flagella and motility. These results indicated the

existence of another autoinducer, which has been named Autoinducer 3 or AI-3. Electrospray mass spectrometry has shown that the AI-3 fraction showed a major peak at 213.1 Da, in contrast to AI-2 (192.9), epinephrine (183.2), and norepinephrine (169.2). Initial structural studies of this compound indicate it that is an aromatic aminated compound. Considering EHEC has such a low infectious dose (50-100 cfus) that leads to outbreaks, it is likely that EHEC senses AI-3 from commensal flora in the intestine (256). AI-2 and AI-3 activity has been observed in spent supernatants from pathogenic organisms such as: *Shigella* and *Salmonella* and commensal organisms such as *E. coli*, *Klebsiella pneumonia*, and *Enterobacter cloacae*. In addition, AI-2 and AI-3 activity has been shown in spent supernatants from normal flora cultured from human stools. This confirms that AI-3 is present in the intestine and that it may be an interspecies QS signal (257, 293).

Since epinephrine, norepinephrine, and AI-3 can all substitute for each other in order to activate LEE gene transcription and type three secretion, AE lesions on cells, and flagellation, it is thought that cross-talk among these systems exists and that they use the same transduction pathway. Given that all three compounds seem to be derived from the same source, tyrosine, it is also possible that they share a common receptor. EHEC could take advantage of this, responding to both bacterial quorum sensing signals as well as eukaryotic signals

in order to most efficiently modulate virulence gene transcription throughout infection at different sites in the GI.

In 2006, Walters *et al*. confirmed that while AI-3 is not produced in a *luxS* mutant strain, its synthesis is not dependent on *luxS*. Walters *et al*. showed that when *luxS* is mutated, the cell only has one method of producing homocysteine. As this pathway is exclusively used, metabolism as a whole in the cell is altered, leading to a decrease in AI-3 production. Phenotypes that were dependent on AI-3 and altered in the *luxS* mutant, could be restored with aspartate and expression of an aromatic amino acid transporter or a tyrosine specific amino acid reporter. In addition, through the use of phenotype arrays, Walters *et al*. showed that the *luxS* mutation causes many metabolic deficiencies, while AI-3 does not seem to have a large effect on metabolism. Most importantly, Walters *et al.* showed that expressing the S-adenosylhomocysteine hyrdrolase from *Pseudomonas aeruginosa* so that homocysteine is synthesized directly from S-adneosylhomocysteine (SAH) was able to restore the defect in AI-3 production, but not AI-2 production.

In order to tease out the specifc role of the signaling molecules involved in EHEC virulence, a follow-up array to the one described in Sperandio *et al*. 2001 was performed in which the individual roles of AI-3, AI-2, and epinephrine were investigated. These results confirmed that the *luxS* mutant affects primarily metabolic genes in both pathogenic and non-pathogenic *E. coli* and that the

addition of pure AI-2 is unable to completely restore the expression profile of the *luxS* mutant. AI-3 and epinephrine addition to culture media of the *luxS* mutant increased expression of the *LEE* region. Importantly, when epinephrine was added to the *luxS* mutant, the most differential gene expression was seen and more was seen in pathogenic EHEC than commensal *E. coli* indicating that epinephrine may be a global virulence regulator (139).

During the initial studies in EHEC comparing WT EHEC to a *luxS* mutant, 10 % of the backbone genome shared between *E. coli* K-12 and EHEC was differentially expressed (255). This led to the identification of several regulators involved in QS and the epinephrine/norepinephrine/AI-3 system. Seven of these regulators, named Quorum Sensing *E. coli* Regulators A-G are currently being characterized. QseA is a LysR type regulator. It has been shown to autorepress itself by binding to its own promoter. In addition, QseA activates *ler* (the master regulator of the *LEE* region) by binding directly to its promoter (242, 251) (Kendall, unpublished data). A QseA mutant is defective in attaching and effacing lesion formation, but has no defect in flagellation (251). QseA is activated by epinephrine in a *luxS* background and both PO and PE can block this response (256). Recent data has shown that QseA also plays a role in regulation of Shiga toxin as well as numerous fimbrial genes (Kendall, unpublished data).

QseB and QseC are a two-component system, with QseC encoding a sensor kinase and QseB encoding a response regulator. This two-component

system is involved primarily in flagella and motility regulation, but is also thought to be the initial signal transducer that begins the cell-to-cell signaling virulence cascade in EHEC. QseC can respond to epinephrine, norepinephrine, and AI-3 in order to autophosphorylate and in turn activate QseB by phosphorylation. QseB activates its own transcription as well as the transcription of *flhDC* the master regulator of flagella and motility (39, 41, 42, 259). Recent data has shown that QseBC also plays a role in regulation of the *LEE* genes, iron uptake, Shiga toxin, and other two-component systems, including QseEF discussed below (112). QseC autophosphorylation in response to AI-3, epinephrine and norepinephrine can only be blocked by PE. However AE lesion formation can be blocked by both PE and PO. This indicates that more than one two-compon ent signaling system is involved in sensing of these signals (39, 257).

QseD is another LysR type regulator. This protein may provide one of the links between the AE lesion formation and flagella and motility pathways in EHEC. It has been shown to activate the flagella regulon and repress transcription of the *LEE* genes (unpublished data, Faith Sharp and Benjamin Habdas). QseE and QseF are a second two-component system that initial studies have shown is involved in the regulation of AE lesion formation as discussed in the following document (221). A signaling model for EHEC virulence is shown in Figure 1.11.

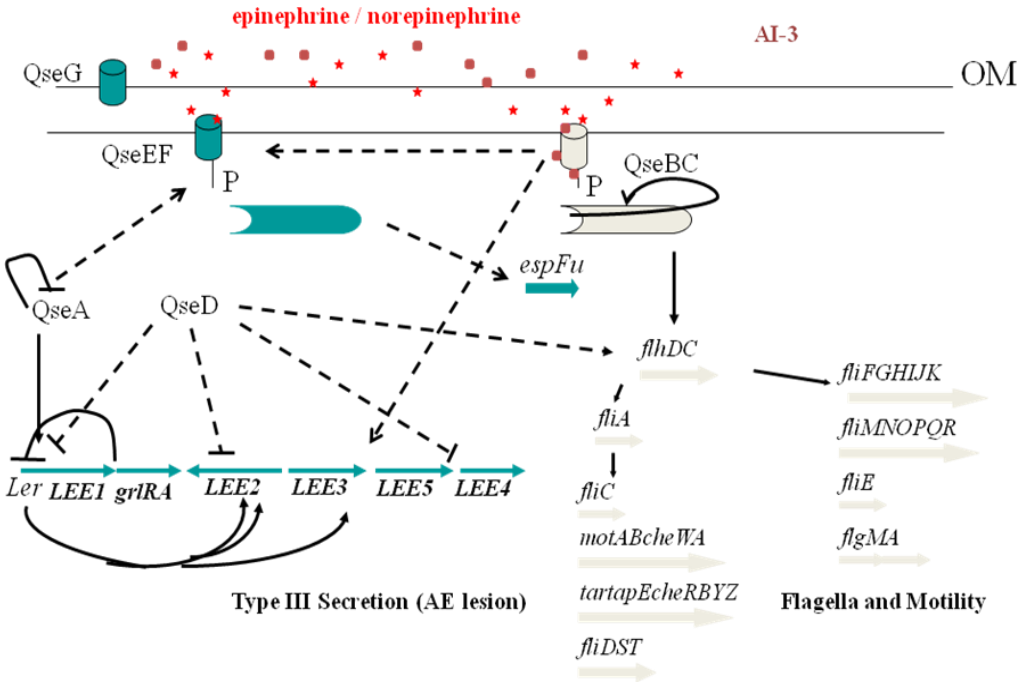


Figure 1. 11 – Virulence signaling model for EHEC. AI-3 and epinephrine/norepinephrine are thought to be recognized by the same outer membrane receptor(s). Once inside the cell, either through active transport of diffusion, these signals are recognized by QseC and partially by QseE. In response to the signals, both sensors autophosphorylate and phosphorylated their cognate response regulators, which modulate virulence. QseBC is involved in regulation of flagella and motility and possibility attaching and effacing lesion formation, while QseEF is involved in attaching and effacing lesion formation. QseG is located in the outer membrane, but its role in signaling has not been defined. Dashed lines show interactions that are either indirect of have not been confirmed by biochemistry.

The Alternative σ Factor, σ^{54}

σ^{54} (RpoN) is an alternative σ factor of RNA polymerase that has many unique properties. This factor has no similarity to any other prokaryotic σ factors,

indicating it has a different origin (166). Also interesting is that this factor can interact with promoter DNA without the entire RNA polymerase, unlike other σ factors (23). σ^{54} recognizes promoter DNA that is found at approximately -12 and -24 from the transcriptional start site in contrast to the -10 and -35 sequences that the typical σ^{70} factor recognizes (188). The sequence that RpoN recognizes, YTGGCACGrNNNTTGCW, is fairly well conserved and easy to recognize in DNA sequences (11, 262). When the EHEC EDL933 genome is scanned for this consensus sequence using PromScan (http://www.promscan.uklinux.net/), approximately 20 genes are recognized.

The most distinguishing characteristic of σ^{54} is its requirement for an upstream activator protein or enhancer binding protein (EBP). When σ^{54} binds promoter regions, it forms a closed promoter complex. Binding of an upstream regulator is necessary for transition to a transcriptionally open complex through an energy-dependent process, which the EBP catalyzes (154). EBPs must contain a DNA binding domain to bind upstream of the promoter and an additional domain that interacts with σ^{54} upon DNA looping. This looped structure was demonstrated by Su *et al*. when they showed the activator protein NtrC bound to its target *glnA* (264). All activator proteins contain this σ^{54} - interaction domain as well some additional domains, such as a DNA-binding domain. The σ^{54} interaction domain contains a sequence that is invariant in almost all activator

proteins, GAFTGA, and is most likely the structural feature that directly interacts with the σ^{54}. This region is amino acids 215-220 in NtrC from *Escherichia coli*, one of the most well-characterized enhancer binding proteins (263). Genes that are transcribed by σ^{54} – dependent promoters are often involved in processes that are highly environmentally regulated, like nitrogen fixation and regulation of virulence. Correspondingly, many enhancer-binding proteins are the regulator component of two-component signaling systems (263).

Two-Component Signaling Systems

With environmental signaling playing such an important role in the adaptation to niches by bacteria, the importance of a system to recognize and transduce signals that indicate the external or internal environment is paramount. Two-component system signaling is a widely conserved mechanism by which many organisms do this. Although two-component systems are present in fungi and plants as well as bacteria, they have not been identified in animals making them excellent drug targets (260).

The most classical two-component systems in gram negative bacteria are composed of an integral inner membrane histidine sensor kinase protein and a cytosolic response regulator protein. The periplasmic region of the sensor kinase is able to sense external signals and catalyze an autophosphorylation reaction. Once phosphorylated, the sensor kinase is able to transfer the phosphoryl group to

a conserved aspartate residue on the cytosolic response regulator. This enables the regulator to act on downstream genes most often activating transcription (110). Although most two-component systems have been identified as two protein systems, there is also an abundance of phosphorelay systems identified in the genomes of numerous bacteria. Some of these are composed of four proteins, two containing conserved histidine domains and two containing conserved aspartate domains. Others are composed of a hybrid protein containing both histidine and aspartate domains as well as an additional regulator protein containing an aspartate domain. The latter system requires an intermediary protein that contains a histidine-containing phosphotransfer (HPT) domain (315). In addition to phosphorelay systems, examples of two-component systems that rely on additional membrane or cytosolic proteins have been reported, especially when the additional protein plays a role in connecting two different two-component systems. For example, the *Salmonella enterica* two-component systems, PhoQ/PhoP and PmrB/PmrA are connected by the cytosolic protein, PmrD. This allows resistance to antimicrobials in response to different signals (136). In addition, the CpxA/CpxR two-component system involved in sensing "touch" and adjusting gene expression after bacteria adhesion in *E. coli* is negatively regulated by a periplasmic small protein, CpxP (77, 205). A small protein, B1500 was recently identified that connects the EvgA/EvgS system to the

PhoQ/PhoP system, both involved in drug resistance (67). Finally, the outer membrane protein RcsF is involved in the signaling pathway of RcsCDB (33).

As well as adapting to environmental niches, many two-component systems are intimately involved in virulence. In *Streptococcus pneumonia* eight of the known 13 systems that it contains are involved in virulence in a mouse model (279). Other examples of two-component systems' roles in virulence are: persistence in infection by *Mycobacterium tuberculosis* (312), invasion and antimicrobial resistance in *Salmonella typhimurium* (3, 8), and adhesion by *E. coli* (205). In addition, the RcsB/RcsC/RcsD system has been implicated in positive regulation of the *LEE* region (280)

In the *E. coli* K-12 genome, there are 29 histidine kinases, 32 response regulators, and one HPT domain identified (185). Among these systems, much cross talk exists without compromise of specificity (157, 310). This adds another layer of complexity to environmental signaling in *E. coli* as the systems take advantage of cross-talk, but also block out unwanted communication.

Quorum Sensing *E. coli* Regulators E, F, and G

The quorum sensing *E. coli* regulators E, F, and G contain the components of a traditional two-component system. These proteins are conserved among numerous enteric organisms including species of *Shigella, Salmonella, Yersinia,*

and *Klebsiella*, and commensal *E. coli*. The genes encoding these proteins are co-transcribed in an operon with *glnB***.** As seen in Figure 1.12, *qseE, qseF, qseG, glnB* contain little or no space between the stop codon of one gene and the translational start codon of the next, indicating they may be co-transcribed in one operon. The gene upstream of *qseE* is 513 base pairs upstream, indicating that it is probably not co-transcribed with the remaining genes. Downstream of *glnB* is *hmpA*, which lies in the opposite orientation. QseE is a putative sensor kinase containing two transmembrane domains, a signal histidine kinase domain and an ATPase domain. Likewise, QseF is a putative two-component response regulator protein containing a response regulator domain with a key aspartate residue, a σ^{54} interaction domain including the GAFTGA motif, and a helix-turn-helix DNA-binding domain (Figure 1.12). The helix-turn-helix domain was reported by Liu *et al*., but has not been confirmed (164). QseE and QseF are juxtaposed around QseG. QseG is predicted to be an outer membrane protein and shares homology with many alpha-helical proteins. It contains a secretion sequence with a cleavage site. The presence of a gene encoding a membrane protein with no predicted catalytic sites or domains between open reading frames (ORFs) encoding a sensor kinase and a reponse regulator is very unusual and makes QseG's function intriguing. *glnB* encodes the PII protein, which is a negative regulator of the NtrC/NtrB two-component system. This two-component system is involved in nitrogen regulation. The most efficient source of nitrogen for

Escherichia coli is ammonia. However, in ammonia-limited conditions, the cell must utilize alternative sources of nitrogen, which are converted to glutamine through the action of glutamine synthetase (encoded by *glnA*). This process is extremely costly for cells. The proteins NtrB, NtrC, and PII are involved in regulating this process in order to conserve energy. *ntrC, ntrB,* and *glnA* are encoded together in one auto-regulatory operon driven by phosphorylated NtrC through a σ^{54} promoter upstream of *glnA*. In nitrogen-limited cells, NtrC is phosphorylated by NtrB. In nitrogen-replete cells, PII interacts with NtrB so that NtrC remains unphosphorylated and glutamine synthetase is not transcribed. PII is uridylated to PII-UMP in nitrogen-replete cells, therefore unable to interact with NtrB (164, 170, 199) . QseF shows more similarity with NtrC than any other EHEC response regulator. The presence of *glnB* downstream of *qseEFG* presents the possibility that *qseEFG* may have a role in nitrogen signaling.

In 2005, Yamamoto, *et al.* confirmed that QseEF behave as a two-component system by showing that the cytoplasmic portion of QseE autophosphorylates and transfers the phosphate to QseF *in vitro* (310).

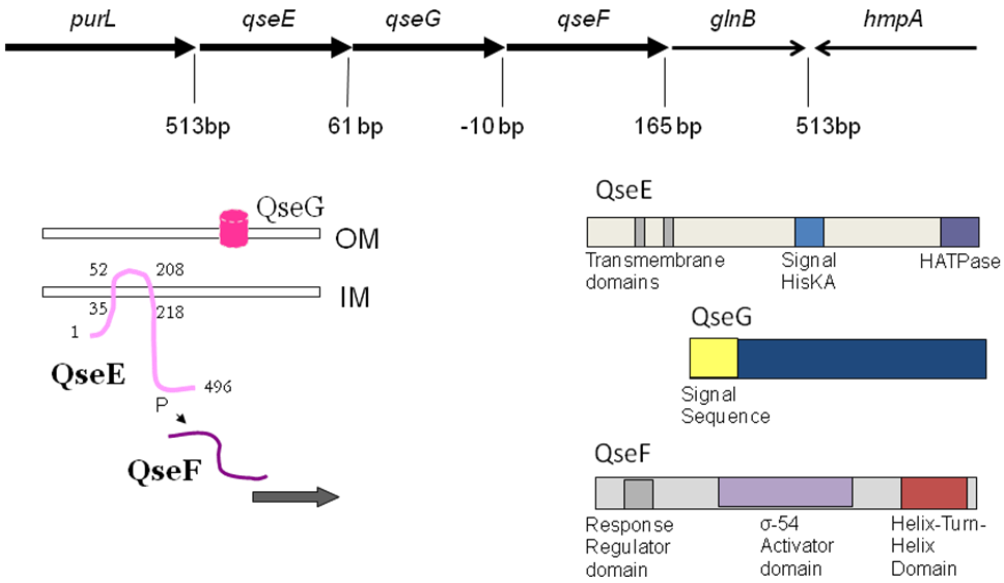


Figure 1.12 - The QseEFG system. *qseEFGglnB* are encoded together. There are 513 basepairs between *qseE* and *purL* indicating that *purL* is probably not a part of this operon. In addition, *hmpA* is oriented opposite to *glnB*, indicating that it is not a part of this operon. *qseE* encodes a sensor kinase that contains two transmembrane domains, a signal histidine kinase residue, and an ATPase domain. It is predicted to localize to the inner membrane. *qseF* encodes a response regulator that contains a conserved aspartate residue in a response regulator domain, a σ^{54} activator domain, and a putative helix-turn-helix domain for DNA-binding. *qseG* encodes a putative lipoprotein and contains a secretion signal sequence. It is predicted to localize either to the inner or outer membrane.

CHAPTER TWO

OVERALL PURPOSE OF RESEARCH

Enterohemorrhagic *E. coli* continues to be a major health concern responsible for cases of hemorrhagic colitis and life-threatening hemolytic uremic syndrome (HUS) worldwide. Despite the serious nature of this disease, no treatment is currently available as described in Chapter 1. One of the hallmarks of EHEC infection is the formation of attaching and effacing lesions (AE), which destroy the microvilli and result in tight adherence of the bacteria and actin reformation into pedestal-like structures that raise the bacteria above the surface of the epithelium.

In 1999, Sperandio *et al.* reported that genes involved in AE lesion formation (the *LEE* region) were under the control of quorum sensing based on studies involving a *luxS* mutant strain. LuxS is required for the synthesis of auto-inducer 2 (AI-2) and a *luxS* mutant is also unable under certain circumstances to produce autoinducer 3 (AI-3), the autoinducer involved in EHEC virulence (254). This study was followed with a microarray analysis, in 2001, comparing a wild type EHEC strain to an isogenic *luxS* mutant, which showed that numerous genes were potentially under the control of quorum sensing. Among the differentially regulated genes were many putative regulators (255). One of these regulators, QseC, was shown to be involved in flagellation and motility (259) . However,

many additional regulators, including QseE were identified in the study indicating a complex signaling cascade.

The purpose of this study was an in depth characterization of a new type of signaling system involving three-proteins. We sought to address what roles QseE, QseF, and QseG play in pathogenesis of EHEC and to characterize their mechanism in this role through detailed genetic and biochemical analysis. Since no treatment currently exists for EHEC infection, new drug targets are highly desirable. Blocking virulence signaling could be a highly effective method of treatment considering it would render the bacteria harmless without killing them or inducing an SOS response. In depth understanding of the signaling systems involved in EHEC pathogenesis will pave the way to these novel therapeutics that target cell to cell signaling.

CHAPTER THREE

MATERIALS AND METHODS

Strains and Plasmids. All bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were grown in aerobic conditions in low-glucose Dulbecco's modified Eagle medium (DMEM) at 37º for microarray studies, RT-PCR, and virulence assays. For purifications, strains were grown in Luria-Bertani broth (LB). All overnight cultures were grown in Luria-Bertani broth at 37 º unless otherwise noted. Antibiotics for culture growth were added at the following concentrations: ampicillin, 100 µg/ml, chloramphenicol, 30 µg/ml, kanamycin, 50 µg/ml, and tetracycline, 25 µg/ml.

Table 1: Strains and plasmids used in this study

Plasmid or strain	Relevant genotype	Reference or source
Strains		
86-24	Stx2+ EHEC strain (serotype O157:H7)	Griffen et al. 1998
NR01	86-24 *qseE* non-polar mutant	Reading, et al. 2007
NR02	86-24 *qseF* non-polar mutant	Reading, et al. 2007
NR03	86-24 *qseG* non-polar mutant	This study
NR04	NR01 complemented with plasmid pNR01	Reading, et al., 2007
NR05	NR03 complemented with plasmid pNR03	This Study
NR06	NR02 complemented with plasmid pNR02	Reading, et al, 2007

KRL7	86-24 *qseE* polar mutant	Reading, et al., 2007
NR26	pK187 in NR02	Reading, et al., 2007
NR27	pKC471 in NR02	Reading, et al., 2007
NR28	pKH35-4 in BL21 DE3 cells	Yamamoto, *et al.,* 2005
NR30	pNR15 in NR02	Reading , et al., 2007
NR31	pET16 in NR02	Reading, et al., 2007
NR32	p635 in NR02	Reading, et al., 2007
NR33	pNR30 in BL21 DE3 cells	This Study
NR34	pcya in 8624	This Study
NR35	pcya in qseE-	This Study
NR36	pcya in qseG-	This Study
NR37	pcya-tir in 8624	This Study
NR38	pcya-tir in qseE-	This Study
NR39	pcya-tir in qseG-	This Study
NR40	pcya in qseE+	This Study
NR41	pcya in qseG+	This Study
NR42	pcya-tir in qseE+	This Study
NR43	pcya-tir in qseG+	This Study
NR44	pDE2GFP in 8624	This Study
NR45	pDE2GFP in qseG-	This Study
NR46	pDE2GFP in qseG+	This Study

Plasmids

pBadMycHis	C-terminal Myc-His-tag cloning vector	Invitrogen
pNR01	*qseE* in pBadMycHis	Reading, et al., 2007

pNR02	*qseF* in pBadMycHis	Reading, et al., 2007
pNR03	*qseG* in pBadMycHis	This study
pKD3	pANTSγ derivative containing FRT-flanked chloramphenicol resistance	Datsenko and Wanner, 2000
pKM201	λ red recombinase expression plasmid	Murphy, 1998
pCP20	TS replication and thermal induction of FLP synthesis.	Cherepanox, 1995
pRS551	*lacZ* reporter gene fusion vector	Simons, *et al*. 1987
pNR10	*espFu* in pRS551	Reading, et al., 2007
pNR30	pET21 + *qseE*	This Study
pK187	low copy number KanR vector	Campellone *et al*. 2002
pKC471	*espFu*-myc in pK187	Leong *et al*. 2004
pKH35-4	*yfhA* in pET21a(+)	Yamamoto, *et al*., 2005
pNR15	*qseF* in pACYC177	Reading, et al. 2007
pACYC177	Cloning vector	New England Biolabs
pVS262	*espFu* in TOPO	Reading, et al., 2007
Topo	Commercial Blunt-End Cloning Vector	Invitrogen
p635	*espFu* in pET16	A kind gift from John M. Leong
pET16	Expression Vector	Novagen
pET21	Expression Vector	Novagen
pCya	*cyaA* of *Bordetella pertussis* cloned into pACYC177	A kind gift from James Kaper; Crawford, et al., 2002
pCya-Tir	*tir-cyaA* fusion cloned into pACYC177	A kind gift from James Kaper; Crawford, et al., 2002
pDE2GFP	*gfp* vector driven by the *lac* promoter	Clonetech

Recombinant DNA Techniques. PCR, restriction digestions, plasmid preparations, transformations, gel electrophoresis, and ligations were all completed using standard methods (229). Primers used in real-time PCR and cloning are listed in Table 2. Plasmid pNR30 was made by amplifying *qseE* from 86-24 genomicDNA utilizing Invitrogen *Pfx* polymerase with primers: qseEpet21F and qseEpet21R. The PCR product was cloned into the BamHI and NotI sites of Pet21 (Novagen). Plasmid pNR01 was constructed by amplifying the *qseE* gene from the EHEC strain 86-24 using *Pfx* DNA polymerase (Invitrogen) using primer YfhKFBAD and YfhKRBAD and cloning the resulting PCR product into the *EcoRI-KpnI* cloning site of vector pBADMycHisA (Invitrogen). Plasmid pNR02 was constructed by amplifying the *qseF* gene from the EHEC strain 86-24 using *Pfx* DNA polymerase (Invitrogen) using primers QseFFBAD and QseFRBAD and cloning the resulting PCR product into the *KpnI-HindIII* cloning site of vector pBADMycHisA (Invitrogen).

Plasmid pNR03 was created by amplifying *qseG* from 86-24 overnight culture and cloning the PCR product into the EcoRI and KpnI sites of the pBadMycHis (Invitrogen) vector.

TABLE 2. Oligonucleotides used in this study.

Primer Name	Sequence	Description
yfhKP1	GGCAAAGCCTGAATGCGCCTTAGCGACC AGGCGGCGCTGGTCAACCGCACCACGCT TATCGATGCCCGGCGCAGCGAAGCAATG ACCAACGCGGCGCTGGATGTAGGCTGGA GCTGCTTC	Construction of the *qseE* Isogenic Mutant
yfhKP2	TTGCCCGCTCTCGTCGACCAGATACAGTT CCCCTTGCATACGGCGAATACAATCCCTG GCAATGCTTAATCCCAGACCGCTGCCCTT CACCGCCCCTTTTATATGAATATCCTCCT TA	Construction of the *qseE* Isogenic Mutant
yfhARedP1	GAAACTGCTTGGCCTGCGCCTGACCAGC GAAGGCTACAGCGTGGTCACGGGTGTAG GCTGGAGCTGCTTC	Construction of the *qseF* Isogenic Mutant
yfhARedP2	AATTCTGTCCGGTTGCGCCCCGCCATTCT CGCCGCGTGGGTGACGTTGCCCATATGA ATATCCTCCTTA	Construction of the *qseF* Isogenic Mutant
QseFFBAD	GGTACCGTGAAAAGCCCGCGCCATCCA	Construction of pNR02
QseFRBAD	AAGCTTATCGTTTGCATCCAGCTCGTGT	Construction of pNR02
YfhKFBAD	GGTACCTATCTGAACTTCCCCTCGGTT	Construction of pNR01
YfhKRBAD	GAATTCCCTTTCGTGTTTTTCGACGACGG	Construction of pNR01
EspFuF	CGCGGATCCCTGTCGGCTCTCTTCTAGAT	*espFu* activity assay, EMSA, RT-PCR
EspFuR	CGC GGA TCC ATA TTG CGG TTG ACG GTT GG	*espFu* activity assay
EspFuSEQ	ATA TTG CGG TTG ACG GTT GG	Primer Extension, Sequencing Ladder
QseE RTR	CGC GCC ATG ATC TTC GA	Real-Time Analysis
QseE RTF	CCC TTC ACC GCC CCT TT	Real-Time Analysis
EspFuCInv	TGC GGC GAT GTA TAA ATG AC	Primer Extension
EspFuBInv	ACA GCC ATT CCT CCT GTG T	EMSA
EspFuCInv	TGC GGC GAT GTA TAA ATG AC	EMSA
EspFuRN	GTTCATTTTGTACTGGCGGC	RT-PCR
EspFuRT2	GCATCCTATTTATTGCTCACGTTA	RT-PCR

EspFuProbeR	CGAGCGCTTAGATGTATTAATGCC	RT-PCR
EspFuProbeF	ATGATTAACAATGTTTCTTCACTTTTTCC	RT-PCR
QseF RT R	CGT AAG CTG CTG CAA ATT ACC A	Real-Time Analysis
QseF RT F	CGC CCC GCC ATT CTC	Real-Time Analysis
YfhG-F-Bad	GGT ACC CAA CGA TTA TTG CCC CGA CG	Construction of pNR03
YfhG-R-Bad	GAA TTC CCG ACC TCA TGG GTG GAT GGC GCG	Construction of pNR03
yfhG Red P1	ATC CTC ACG TAC GAC ATG TAC GCT CCG GTT TCT CCG CGC TGT CCA TGT CCG TGT AGG CTG GAG CTG CTT C	Construction of the *qseG* isogenic mutant
yfhG Red P2	TCC AGT TGC TGC TGT AGA ACG TGA TGT TGC TGG CGC AAT GTA TCC AGC TCC ATA TGA ATA TCC TCC TTA	Construction of the *qseG* isogenic mutant
QsePet21 F-final	CGA ATG ACG CAC GGA TCC GAG CCT GCC GT	Construction of pNR30
QseERpET21	GGG ATA GGC TGT GCG GCC GCT TTC GTG TTT	Construction of pNR30
Z0463F	CGG TGC CAG CGG GTA TT	Real-Time Analysis
Z0463R	CGC ACC GCC TGT ACT AAC TCT	Real-Time Analysis
YfeRBF	GGG CAG CAG TTG GCT TTG	Real-Time Analysis
YfeRBR	TCG CGC AAC GTG CTG TTA	Real-Time Analysis
YchFAF	TTC GCT GCT TTG AAA ATG ACA	Real-Time Analysis
YchFAR	CAA TAT CGT CAG CCG GGT TAA	Real-Time Analysis
YeiEF	TGC AAG CGG TGC TGG AT	Real-Time Analysis
YeiER	TGT GGC ACG GTC CTT CAA	Real-Time Analysis
RcsCF	GCA GGA GAT GGC ACA AGC A	Real-Time Analysis
RcsCR	TGA CGG TGG CAA GGA ACA T	Real-Time Analysis
RcsBF	TCT CTC GCC AAA AGA GAG TGA AG	Real-Time Analysis
RcsBR	CGA TCT CGG TCA CCA GGA A	Real-Time Analysis

RpoZ RTF	TGC AGG TAG GCG GAA AGG	Real-Time Analysis
rpoZRTR	GCG CAG CGC GAT TAC AGT	Real-Time Analysis
livKA RTF	CGA TAA GCT GGT TGG CGT AGA	Real-Time Analysis
livKA RTR	GAC CGC AAC GGC TTG TTT	Real-Time Analysis
PhoP RTF	CCG CTG GCG TAG CAA TG	Real-Time Analysis
PhoP RTR	AGC TTT CAC GGG CGG TTA A	Real-Time Analysis
PhoQ RTF	GCC GCC TGG TGG AGT TTA C	Real-Time Analysis
PhoQ RTR	TCT TCC AGT TCG CGG ACT TC	Real-Time Analysis
FepE RTF	GGC GAT TAT TGT GAT CCT TTC C	Real-Time Analysis
FepE RTR	CGC AAT AAC ACG CTA CCA CAAG	Real-Time Analysis
EntB RTF	GAT GCG CTG GCC GAT TT	Real-Time Analysis
EntB RTR	AAC GTC CGG CCA CAT ATT TC	Real-Time Analysis
PrpB RTF	AGC GTG CGC AGG CCT AT	Real-Time Analysis
PrpB RTR	AAT CGC CTC CGG GAA CA	Real-Time Analysis
TonB RTF	CCC CTG GCC GAC GTT ACT	Real-Time Analysis
TonB RTR	AGA GCA GAC CCG CCA CAA C	Real-Time Analysis
eae RTF	GCT GGC CCT TGG TTT GAT CA	Real-Time Analysis
eae RTR	GCG GAG ATG ACT TCA GCA CTT	Real-Time Analysis
espA RTF	TCA GAA TCG CAG CCT GAA AA	Real-Time Analysis
espA RTR	CGA AGG ATG AGG TGG TTA AGC T	Real-Time Analysis
QseG RTF	TGC GCA GGC ATT AAA CGA T	Real-Time Analysis
QseG RTR	CTG CGG GCG ATG GAT TGT GCT	Real-Time Analysis

ler RTF	CGACCAGGTCTGCCC	Real-Time Analysis
ler RTR	GCGCGGAACTCATC	Real-Time Analysis
glnBRTF	CACTGGCTGAAGTCGGCATT	Real-Time Analysis
glnBRTR	TGGCGGCCAAAACCTTT	Real-Time Analysis

Isogenic Mutant Construction. The *qseE* polar mutant (KRL7) was constructed by replacing *qseE* with the *tet* gene amplified from plasmid pACYC184 using suicide vector pCVD442, as previously described (62). Construction of the nonpolar isogenic *qseE* (NR01), *qseF* (NR02), and *qseG* (NR03) mutants was constructed utilizing λ-Red assisted recombination as previously described (51). Briefly, a chloramphenicol resistance cassette was amplified from pKD3 using primers specific for the cassette and containing 50-80 base pair overhangs homologous to either the *qseE, qseF,* or *qseG* genes using primers: yfhGRed F, yfhGRed R, yfhKP1, yfhKP2, yfhARedP1, and yfhARedP2. 86-24 cells containing the λ red recombinase expression plasmid, pKM201 were made electro-competent using ice cold water washes and the above PCR product was electroporated into the competent cells. The cells were incubated shaking at 37º for three hours, then plated on media containing 30 µg/ml chloramphenicol and grown overnight at 37º. Colonies that grew were patched onto ampicillin and chloramphenicol plates and ampicillin sensitive and chlormaphenicol resistant

colonies were selected. Absence of *qseE*, *qseF*, or *qseG* was confirmed by PCR. To create the non-polar mutants, NR01, NR02, and NR03, the choramphenicol cassette was resolved using pCP20, which encodes a resolvase(35). pCP20 was electroporated into the mutant strains and resulting colonies were patched onto chloramphenicol. Chloramphenicol sensitive colonies were selected. NR01, NR02, and NR03 were complemented with plasmids pNR01, pNR02, and pNR03 to create strains NR04, NR06, and NR05 respectively.

SDS-PAGE and Immunoblotting. To isolate protein from whole-cell lysates, strains NR01, NR02, NR03, NR04, NR05, and NR06 were grown in DMEM to an optical density at 600nm (OD_{600}) of 1.0. 3 mls of culture was centrifuged at full speed (13,000 rpm for 5 minutes) and pellets were resuspended in lysis buffer (50mM Tris-HCL, pH 7.5, 50 mM NaCl, 5% glycerol, 1 mM dithiothreitol, and 30 mM phenylmethylsulfonyl fluoride) and treated with lysozyme at a final concentration of 300 µg/ml for 4 hours at 4º. Subsequently, preparations were treated with DNaseI for 45 minutes at 4º. Cell debris were pelleted (13,000 rpm for 10 minutes) and supernatants containing whole cell protein were removed. Sodium-dodecyl sulfate –polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were completed as previously described (229). Protein concentration from whole-cell lysates was determined using the Bradford assay (229). Preparations were probed by western blot analysis using polyclonal

antisera against: EspA, EspB, Tir, or intimin (a gift from James Kaper). Enhanced chemiluminescence (ECL) (Bio-Rad) was used to detect protein.

Isolation of Secreted Proteins. Secreted proteins were isolated from NR01, NR02, NR03, NR04, and NR05 using the previously described method by Jarvis, et al. (121). Briefly, NR03 and NR05 were grown to an OD_{600} of 1.0. Cultures were centrifuged for 15 minutes at 10,000 rpm. The protease inhibitors EDTA, PMSF, and aprotinin were added to the resulting supernatants and protein was precipitated using tri-chloroacetic acid. Total protein was collected by high-speed centrifugation. Protein was resuspended in phosphate-buffered saline (PBS) and total samples were subjected to SDS-PAGE and immunoblotting with antisera against EspA, EspB, and Tir (a kind gift from James Kaper), and visualized using ECL (Bio-Rad).

Reporter Gene Assays. Plasmid pNR10 was generated by amplifying the regulatory region of *espFu* with primers EspFuF and EspFuR, and cloning the resulting fragments into the *Bam HI* restriction sites of plasmid pRS551 (243). Because of plasmid compatibility issues, to perform these assays, the *qseF* mutant was complemented by cloning the *qseF* gene into the *SmaI* restriction site of pACYC177, generating plasmid pNR15. Bacteria containing the *lacZ* fusions were grown overnight at 37^{o}C in LB containing the appropriate selective

antibiotic. Cultures were diluted 1:100 and grown in DMEM to an $O.D._{600}$ 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 (181).

Flourescent Actin Staining. Flourescent actin staining (FAS) was performed as previously described by Knutton, et al. (148). Briefly, HeLa cells (ATTC) were grown to confluency on 18mm coverslips in 12-well plates at 37º + 5% CO_2. 86-24, NR01, NR02, NR03, NR04, NR05, and NR06 were grown in LB overnight and diluted 1:100 in low-glucose red DMEM containing 10% fetal bovine serum and no antibiotics. Diluted cultures were used to infect HeLa cells. Cells were grown at 37º with 5% CO_2 for three hours, fresh media was added to wells, and cells were grown for another three hours. After six hours, coverslips were washed 3 times with 1X PBS, permeablized for 10 minutes with 0.2% Triton-X-100 (check), and treated with fluorescein isothiocyanate (FITC)-phalloidin to visualize actin. Propidium iodide was added to stain bacteria, and coverslips were treated with RNaseA for 10 minutes. Coverslips were visualized by immunofluorescence with a Zeiss Axiovert microscope. The entire field of at least six coverslips for each strain was analyzed and images of AE lesions were taken.

EspA Immuno-fluorescent Filament Staining. Immunoflourescence was performed as previously described (150). HeLa cells were seeded on 18mm coverslips in 12-well plates and allowed to grow to confluency at 37º and 5% CO_2. After overnight growth, 86-24, NR03 and NR05 containing pDE2GFP (NR44, NR45, and NR46) and induced with 0.5 molar isopropyl-beta-D-thiogalactopyranoside (IPTG) were diluted 1:100 in low-glucose red DMEM +10% fetal bovine serum (FBS) and used to infect the confluent He-La cells. Cells were incubated at 37º + 5% CO_2 for either 3 or 6 hours. At designated time points, coverslips were washed 6 times in 1X PBS and fixed with 2% for 30 minutes, washed 6 times with 1X PBS, and treated with polyclonal antisera against EspA (a kind gift from James Kaper) at a concentration of 1:80 in DMEM + 10% FBS for 1 hour. Subsequently, coverslips were washed 3 times for 10 minutes in 1X PBS and treated with tetramethyl rhodamine iso-thiocyanate (TRITC) labeled secondary antibody against rabbit IgG (Sigma) at a concentration of 1:3000 in DMEM containing 10% FBS for one hour. Coverslips were washed 3 times for 10 minutes and mounted on slides. Coverslips were visualized by immunofluorescence with a Zeiss Axiovert microscope. The entire field of at least six coverslips for each strain and images of AE lesions were taken.

Assay for Tir Translocation. Tir translocation assays were performed as previously described by Crawford, et al. (48). Briefly, HeLa cells (ATTC) were

grown to confluency in 12-well plates at 37% with 5% CO_2. Overnight cultures of 8624, NR03, and NR05 containing either pcya or pcya-tir (NR34, NR37, NR36, NR39, NR41, NR43) were diluted 1:10 in LB plus the appropriate antibiotic and grown statically for 1.5 hours or until each strain reached an OD_{600} of exactly 0.3. HeLa cells were washed 2 times with warm 1X PBS and were infected with bacteria at a multiplicity of infection of 100:1 (EHEC:HeLa cell) in warm low-glucose red DMEM containing 10% FBS. Cells were incubated at 37% with 5% CO_2 for 1.5 hours. Cells were washed 2 times in 1X PBS. 50 mM hydrochloric acid was added to each cell monolayer and cells were scraped out of wells and placed in microcentrifuge tubes. Samples were boiled for 5 minutes, 0.5N NaOH was added to neutralize, a 20 µl aliquate was taken, and protein concentration was determined by Bradford assay (BioRad). To extract cAMP, ice-cold 100% ethanol was added to sample to a final concentration of 60%. Samples were dried under vacuum. Total cAMP levels were determined using a cAMP enzyme immunoassay (EIA) kit (Amersham) according to manufacturer's instructions. Values from strains containing pcya were subtracted from strains containing pcya-tir for final values and translocation is expressed as percentage with WT EHEC (86-24) set at 100%.

Purification of QseF. QseF was purified after being expressed from plasmid pKH35-4 (310). *E. coli* BL21(DE3) containing plasmid pKH35-4 was grown at

37^{o}C in LB to an O.D.$_{600}$ of 0.7, at which point IPTG was added to a final volume of 0.5mM and allowed to induce for three hours. His-tagged QseF protein was then purified under native conditions using a nickel column according to manufacturer's instructions (Qiagen).

Electrophoretic Mobility Shift Assays (EMSA). In order to study the binding of QseF to the *espFu* promoter, EMSAs were performed using purified QseF-His and PCR amplified DNA probes. Probes were end-labeled with [γ-^{32}P]-ATP (NEB) using T4 polynucleotide kinase using standard procedures (229), and gel purified using the Qiagen PCR purification kit. EMSAs were performed by adding increasing amounts of purified QseF protein (0 to 3μg) to end-labeled probe (10ng) in binding buffer (500μg ml^{-1} BSA (NEB), 50ng μl^{-1} poly-dIdC, 60mM HEPES pH 7.5, 5mM EDTA, 3mM DTT, 300mM KCl, 25mM $MgCl_2$) with or without 0.1M acetyl phosphate for 20 minutes at 4^{o}C. A 5% ficol solution was added to the mixtures immediately before loading. Reactions were then electrophoresed on a 6% polyacrylamide gel, dried, and exposed to KODAK X-OMAT film.

RNA Purification and Primer Extension Analysis. RNA purification was performed according to manufacturer's instructions using the Trizol reagent (Invitrogen). RNA was isolated from strain 86-24 grown in DMEM aerobically at

37^{o}C to an O.D.$_{600}$ of 1.0. Primer extension analysis was then performed as described previously (178). Briefly, EspFuSEQ, approximately 40 basepairs downstream of the ATG start site and EspFuCInv, approximately 100 basepairs upstream of the ATG start site (Table 2) were end-labeled using [γ-^{32}P]-ATP. A total of 35ug of RNA, was incubated with the end-labeled primer and reverse-transcribed using the SuperScript^{TM} First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's instructions. A sequencing ladder was generated using the Sequenase Version 2.0 DNA Sequencing Kit (USB) according to manufacturer's instructions. The sequencing ladder was generated using primer EspFuSEQ and the plasmid pVS262. Plasmid pVS262 was created by amplifying the *espFu* regulatory region and cloning the product into blunt end TOPO (Invitrogen). These experiments were repeated three times, with two different primers to ensure the correct mapping of this promoter.

Purification of QseE-His and Reconstitution into Liposomes. 500 mls of NR33 cells were grown in LB medium with the appropriate antibiotic at 37 º until reaching a density of OD_{600} 0.3**,** at which point, cells were induced with 0.5M IPTG. Cells were grown for 4 more hours at 37º, then harvested by centrifugation at 10,000 rpm for 15 minutes. Inclusion bodies were isolated by re-suspending the cells in 10 mls of BPERII Bacterial Protein Extraction Reagent (Pierce) and allowing the resuspension to shake for 10 minutes. Cells were centrifuged at

15,000 rpm for 15 minutes and the pellet was re-suspended in 10 mls of BPERII. Lysozyme to a final concentration of 200 µg/ml and 50 µl of His-tag protease inhibitor cocktail (Sigma) were added and cells were incubated at room temperature for 10 minutes. 50 mls of diluted BPERII (1:20) was added to sample and then cells were centrifuged for 15 minutes at 15,000 rpm. Cells were washed again with 50 mls of diluted BPERII and spun. Cells were washed a total of 3 times and each time the supernatant was saved for analysis. The final pellet contained purified inclusion bodies composed of mostly QseE-His. Liposomes were reconstituted as previously described (119). Briefly, 100 mg of *E. coli* phospholipids contained at 20 mg/ml in chloroform (Avanti Polar Lipids) were evaporated over several days and then re-suspended in 5 mls of 20 mM potassium phosphate buffer containing 80 mg of N-octyl-β-D-glucopyranoside. This product was dialyzed overnight at 4º against 1-liter of potassium phosphate buffer. The final liposome mixture was frozen and thawed 3X in liquid nitrogen. 20 mg (1 ml) of the liposomes were destabilized by the addition of 5.22 mg of dodecylmaltoside. Approximately 1 ml of the purified inclusion bodies (3 µg/µl) was combined with the destabilized lipids and the mixture was gently stirred at room temperature for 10 minutes. 52 mg of fresh Biobeads (BioRad) were added to the loaded liposomes and allowed to incubate overnight at 4ºC. The supernatant was incubated for 1 hour with fresh Biobeads at room temperature, then the resulting loaded liposomes were frozen in liquid nitrogen and stored at -

80ºC. The remainder of the unloaded dialized liposomes were stored at -80ºC until used.

Phosphorylation of QseE-His in Liposomes. 20 µl of the QseE-His loaded liposomes were adjusted to 10 mM $MgCl_2$, 1mM DTT, and various concentrations of signal molecules. Liposomes were frozen and thawed three times in liquid nitrogen, and kept light protected at room temperature for one hour allowing the loading of signals and the reformation of the liposomes. [γ-^{32}P]-ATP (.5 µl) (110 TBq/mmol) was added to each reaction and tubes were incubated for 5 minutes. After 5 minutes, 20 µl of SDS-loading dye containing 20% SDS was added to each reaction, samples were subject to SDS-PAGE analysis without boiling, and phosphorylation was visualized using a PhosphorImager. Bands were quantified using IMAGEQUANT version 5.0 software (Amersham Pharmacia).

Identification of Proteins by Mass Spectrometry. Kinase assays were completed as detailed above, but [γ-^{32}P]-ATP was replaced with unlabeled ATP. Samples were subjected to SDS-PAGE as described above and gels were stained with Biosafe Comassie Stain (BioRad). Bands corresponding to the size of bands from autoradiography detailed above were excised and submitted to the UT Southwestern Protein Chemistry Core Facility. Briefly, in-gel bands were subject to trypsin digestion and reversed-phase non-high-performance liquid

chromatography/ion trap mass spectrometry. Resulting data sets were used to search against the EHEC EDL933 genome.

RNA Extraction. Cultures of 8624, NR01, NR02, and NR03 were grown aerobically in LB medium at 37ºC overnight and then were diluted 1:100 in low-glucose red DMEM and allowed to grow at 37ºC until they reached OD_{600} 1.0. RNA was extracted from three replicates of each strain using a RiboPure bacterial RNA isolation kit (Ambion) according to manufacture's instructions.

Microarrays. The GeneChip *E. coli* Genome 2.0 array by the Affymetrix system contains 10,000 probe sets towards genes from four different 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.affix). These GeneChips were used in order to compare to the transcriptome of 86-24 to strains NR01, NR02, and NR03. Processing of extracted RNA, cDNA labeling, hybridization, and slide-scanning procedures were performed according to manufacture instructions found in *Affymetrix Gene Expression Technical Manual* (http://www.affymetrix.com/support/technical/manual/expression_manual.affx).

Microarray Data Analysis. The data from scanning a single replicate of the Affymetrix GeneChip *E. coli* Genome 2.0 array for each strain was gathered using GCOS v 1.4 as per the manufacturer's instructions. Normalization of data was conducted using Robust Multiarray analysis (19, 116) at the RMAExpress website (http://www.rmaexpress.bmbolstad.com). Output data were analyzed for different gene expression resulting from the removal of *qseE*, *qseF*, or *qseG*.

Real Time RT-PCR. Primers used in real-time RT-PCR analysis were all designed using Primer Express v 1.5 (Applied Biosystems) and are listed in Table 2. Real-time RT-PCR analysis was conducted using Applied Biosystems ABI 7500 sequence detection system using a one-step reaction. Each primer set was checked for amplification efficiency by standard curves resulting from using varying concentrations of RNA template. To ensure template specificity, products were heated to 95ºC for 15 seconds, cooled to 60ºC, and heated to 95ºC while fluorescence was monitored. To analyze gene expression in NR01, NR02, and NR03 compared to 86-24 *E. coli*, relative quantification analysis was used. Parameters for cDNA generation and amplification were as follows: 1 cycle at 48ºC for 30 minutes, 1 cycle at 95ºC for 10 minutes, and 40 cycles at 95ºC for 15 seconds and 60ºC for 1 minute. The RNA polymerase subunit Z, *rpoZ*, was used as an endogenous control. In each reaction of 20 µl, 10 µl 2X SYBR master mix,

0.1 µl Multiscribe reverse transcriptase (Applied Biosystems), and 0.1 µl of RNase inhibitor (Applied Biosystems) were added.

Detection, Quantification, and Statistical Analysis. Applied Biosystems ABI Sequence Detection 1.3 software was used for initial collection of data. Values were normalized to *rpoZ* and analyzed using the comparative critical threshold (C_T) value as previously described (6). Expression is shown in graphs as *n*-fold change in expression level compared to wild type levels at late exponential growth. Error bars represent the standard deviations of the $\Delta\Delta C_T$ value (6). The Student's T-test was performed to assess statistical significance. A P-value of <0.05 was considered significant.

Membrane Preparation and Sucrose Density Gradient Centrifugation. Membrane separation methodology was adapted from several previously published methods for isolation of outer membranes from gram-negative bacteria (114, 198, 204). 500 mls of 86-24 and NR05 were grown in LB and the appropriate antibiotic at 37ºC until they reached OD_{600} 0.2. Cells were induced by the addition of 10 mls of 20% arabinose and harvested once they reached OD_{600} 1.0 by centrifuging at 10,000 rpm for 15 minutes. Cells were resuspended in 10 mls of 0.75M sucrose in 5mM Tris, pH 7.5. While stirring on ice, 40 mls of 10 mM EDTA-Tetrasodium in 5mM Tris, ph 7.5 was added slowly and cells were

incubated on ice stirring for 30 minutes. Lysozyme was added slowly to a final concentration of 200 µg/ml and cells were incubated stirring at room temperature for 30 minutes. Cells were osmotically lysed by the slow addition of 4.5 volumes or 240 mls of ice cold dH_2O. Cells were incubated on ice for 30 minutes while swirling. Debris was collected by centrifuging cells at 10,000 rpm for 30 minutes. To collect total membranes, supernatants from the previous spin were divided into 8 tubes and ultracentrifuged for 2 hours at 200,000 X g at 4ºC. Total membrane pellets were resuspended each in 1 ml of resuspension solution (25% sucrose in 5 mM Tris, 30mM $MgCl_2$, 1 EDTA-free Mini-Complete (BioRad), 15 µl Benzonase (Invitrogen), and 13 µl of His-tag protease inhibitor cocktail (Sigma). Tubes were incubated, shaking at room temperature for 30 minutes to degrade DNA. Linear sucrose gradients were poured in 14 by 99 mm Beckman ultra-centrifuge tubes by layering 1.8 mls each of 55%, 50%, 45%, 40%, 35%, and 30% sucrose and crude membrane preparations were layered on top of the 30% sucrose. Sucrose gradients were ultracentrifuged overnight for 17 hours in a SW-40 Beckman rotor at 256,000 X g at 4ºC. Approximately 20 fractions of 500 µl each were collected from each sucrose gradient by puncturing the bottom of the tube with an 18 gauge needle and letting fractions leave tube by gravity flow. The refractive indexes of each fraction was determined using a refractometer (Fisher Scientific, Hampton, NH), and the density of each fraction in g/ml was calculated based on the refractive index (215). Fractions were diluted in SDS-loading dye,

subjected to SDS-PAGE, and immunoblotted as described above in order to detect QseG.

CHAPTER FOUR

A NOVEL TWO-COMPONENT SIGNALING SYSTEM THAT ACTIVATES TRANSCRIPTION OF AN ENTEROGEMORRHAGIC *ESCHERICHIA COLI* EFFECTOR INVOLVED IN REMODELING OF HOST ACTIN

Introduction

Sperandio *et al.* have shown that many of the genes involved in EHEC virulence, including the *LEE* region and the flagella and motility genes are under the control of cell-to-cell signaling (254) (255). The two-component system QseBC is well characterized and known to respond to epinephrine, norepinephrine, and AI-3 in order to regulate the flagella and motility genes (39, 42, 259). Recent data also indicates that QseC is the sensor kinase that initiates the signaling cascade which leads to activation of the *LEE* region and AE lesion formation (David Hughes, unpublished data).

The existence of more than one two-component system involved in the regulation of EHEC cell-to-cell signaling is likely considering the precise timing involved in switching from a motile state to an attaching state when EHEC is colonizing the intestine. Indeed, numerous regulators thought to be under the control of quorum sensing were identified in a recent microarray study conducted by Sperandio, *et al.* (255) In this chapter, we report the identification of an additional two-component regulatory system, YfhK and YfhA, herein re-named,

quorum sensing regulators E and F (QseEF). QseE is putative sensor kinase while QseF is a putative response regulator. QseEF are part of the AI-3/epinephrine/norepinephrine signaling cascade, and activate transcription of *espFu* to drive actin polymerization during AE lesion formation.

Results

QseEF constitute a novel putative two-component system required for pedestal formation.

The gene *yfhK* encodes a 55 KDa putative sensor kinase protein predicted to lie in the inner membrane. This protein contains two transmembrane domains, a signal histidine kinase domain, and an ATPase domain. The gene *yfhA* encodes a 49 kDa putative response regulator and contains a response regulator domain, a helix-turn-helix DNA binding domain, and a σ^{54} activation domain (Fig. 4.1).

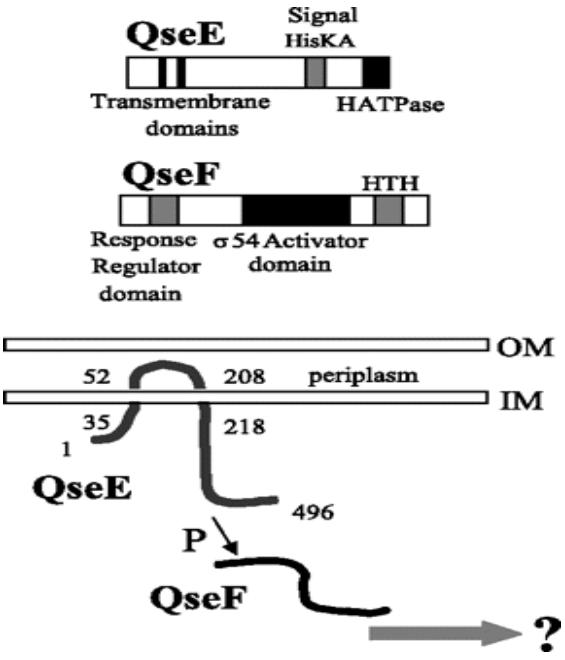


FIG. 4.1. Schematic of QseE and QseF. QseEF is a predicted two-component signaling system that is a part of the EHEC cell-to-cell signaling cascade. Shown is a schematic of QseE and QseF and their respective domains and the expected localization of QseE and QseF within the bacterial cell and proposed signaling mechanism.

These genes were originally identified in a microarray study comparing differential gene expression between a wild-type (WT) EHEC strain and that for an isogenic EHEC *luxS* knockout strain. Under defined environmental conditions, the *luxS* mutant does not produce the AI-3 bacterial signal (292). Transcription of *yfhK* was up-regulated two-fold in the *luxS* mutant in late exponential growth compared to WT (255). To confirm the array data, we performed real-time reverse transcriptase (RT)-PCR analysis using cDNA synthesized from RNA extracted from WT and an isogenic *luxS* mutant during mid-exponential (OD_{600}0.5) and

late-exponential growth (OD_{600}1.0). Transcription of *yfhK* was mildly decreased 0.5 fold in the *luxS* mutant during mid-exponential growth ($p \leq 0.0075$), whereas it was up-regulated two-fold in the *luxS* mutant during late exponential growth ($p \leq 0.0051$) (Fig. 4.2). In the same microarray study, transcription of *qseBC* and *qseA* were altered in the *luxS* mutant compared to WT (251, 258), leading to the subsequent description of these regulators as being part of the AI-3/epinephrine/norepinephrine signaling cascade in EHEC (251, 257, 259). These results suggested that *yfhK* and *yfhA* could also be part of the AI-3/epinephrine/norepinephrine signaling cascade. QseA, a LysR family regulator (255) is activated by the AI-3/epinephrine/norepinephrine and subsequently activates the LEE region (252, 256). Transcription of *yfhK* in a *qseA* mutant during mid-exponential growth is increased two-fold ($p \leq 0.0028$) and decreased 0.5 fold at late exponential growth ($p \leq .0047$) (Fig. 4.2A), providing additional evidence that *yfhK and yfhA* are part of this signaling pathway. Finally, transcription of *yfhK* and *yfhA* are upregulated by epinephrine in WT EHEC, while it is downregulated in a *qseC* mutant either in the presence or absence of epinephrine (Fig. 4.2B) QseC is a sensor for AI-3, epinephrine and norepinephrine, and an EHEC *qseC* mutant is unable to sense these three signals (39). Hence we renamed these genes quorum sensing regulators E and F (*qseEF*), in which QseE is a putative sensor kinase and QseF is a putative response regulator.

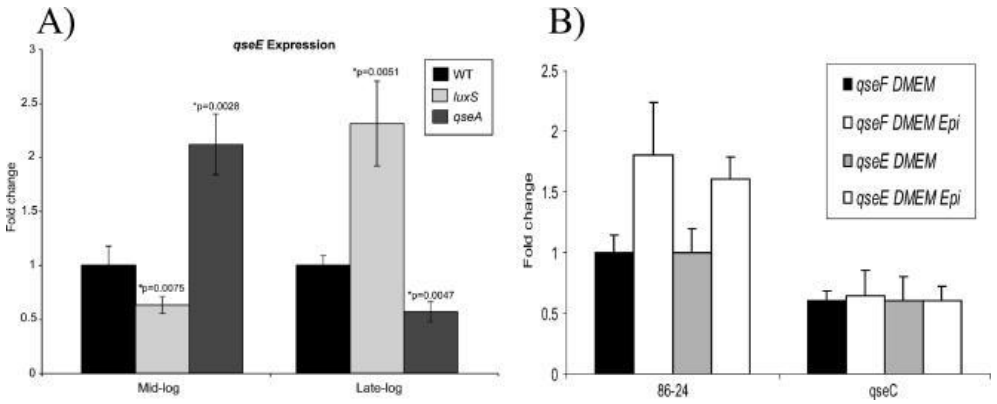


FIG. 4.2. (A) Real-time analysis of *qseE* expression in a *luxS* mutant and a *qseA* mutant compared to WT. *qseE* expression is down-regulated at mid-exponential growth and up-regulated at late-exponential growth in the *luxS* mutant. Conversely, *qseE* expression is up-regulated at mid-exponential phase and down-regulated at late-exponential phase in the *qseA* mutant. (B) Real-time analysis of *qseF* and *qseE* expression in a *qseC* mutant compared to WT in the presence and absence of epinephrine (*Epi*). *qseF* and *qseE* expression is increased in the presence of epinephrine and decreased in the *qseC* mutant with or without epinephrine.

To test QseEF's role in this pathway, a polar mutation in *qseE* was constructed utilizing a tetracycline cassette. We then tested whether any of the phenotypes regulated by the AI-3/epinephrine/norepinephrine signaling cascade were altered, namely AE lesion (pedestal) formation and flagellation and motility. We used fluorescent-actin staining (FAS) (148) to visualize pedestal formation in the WT and mutant strains. Actin was stained using FITC-labeled phalloidin, and He-La cell nuclei and bacteria were stained with propidium iodide. Pedestal formation was visualized as brilliant patches of stained actin (green) localized

underneath a red bacterium. Wild type EHEC formed pedestals; however, the *qseE* mutant was abrogated for pedestal formation (Fig. 4.4A). There were no differences in flagella expression as seen by western blot and no differences in motility between WT and the *qseE* mutant (data not shown), indicating that QseEF is involved in the regulation of the genes required for pedestal formation but not motility.

The *qseE* and *qseF* genes are encoded in a cluster of several genes, including *yfhG*, an uncharacterized gene, and *glnB*, which encodes the PII protein involved in nitrogen regulation (Fig. 4.3). To determine if these genes are transcribed in one operon, we designed primers flanking *qseE* to *qseF* and *qseF* to *glnB* and performed RT-PCR. RT-PCR was performed using cDNA synthesized from RNA isolated from the WT strain. RT-PCR indicated that transcriptional read-through occurred across the intergenic region between *qseE* and *qseF* and *qseF* and *glnB* (Fig. 4.3), suggesting that these four genes are transcriptionally linked.

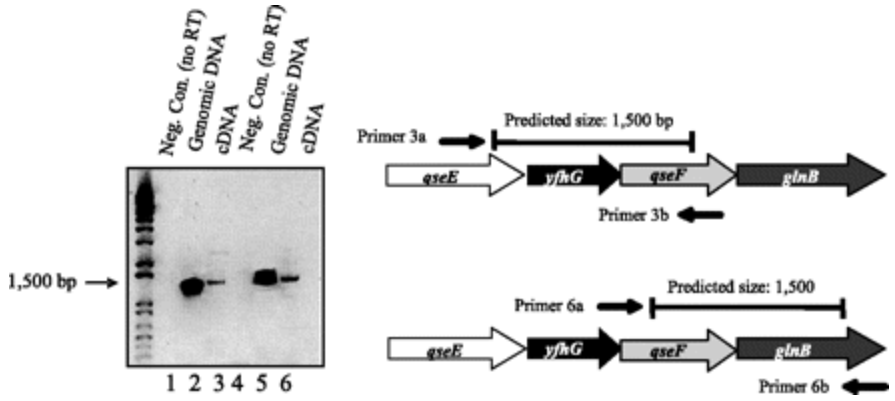


FIG. 4.3. RT-PCR analysis showing that *qseE*, *yfhG*, *qseF*, and *glnB* are co-transcribed. In lanes 1 to 3, primers flanking *qseE* to *qseF* show no product when no RT is added (lane 1) and show product when either a genomic DNA control or cDNA is used (lanes 2 and 3); in lanes 4 to 6, primers flanking *qseF* to *glnB* show no product when no RT is added (lane 4) and show product when either the genomic DNA control or cDNA is used (lanes 5 and 6). Neg. Con., negative control.

Because *qseE, yfhG, qseF*, and *glnB* are co-transcribed, we constructed non-polar mutations in *qseE* and *qseF*. Each mutant was tested for its ability to form pedestals on HeLa cells using the FAS assay. The non-polar *qseE* mutant was still able to form pedestals; however, the *qseF* mutant was abrogated for pedestal formation. Pedestal formation was restored in the *qseF* mutant upon complementation with the QseF gene on a plasmid (Fig. 4.4A).

QseE and QseF are predicted to be a cognate two-component system. This is supported by the fact that the genes encoding QseEF are co-transcribed and that QseE phosphorylates QseF (310). However, QseF is also phosphorylated by multiple non-cognate sensor kinases (QseE, UhpB, BaeS, EnvZ, RstB) (Fig.

4.4B) (310), which may account for the contrasting phenotypes of the *qseE* and *qseF* mutants.

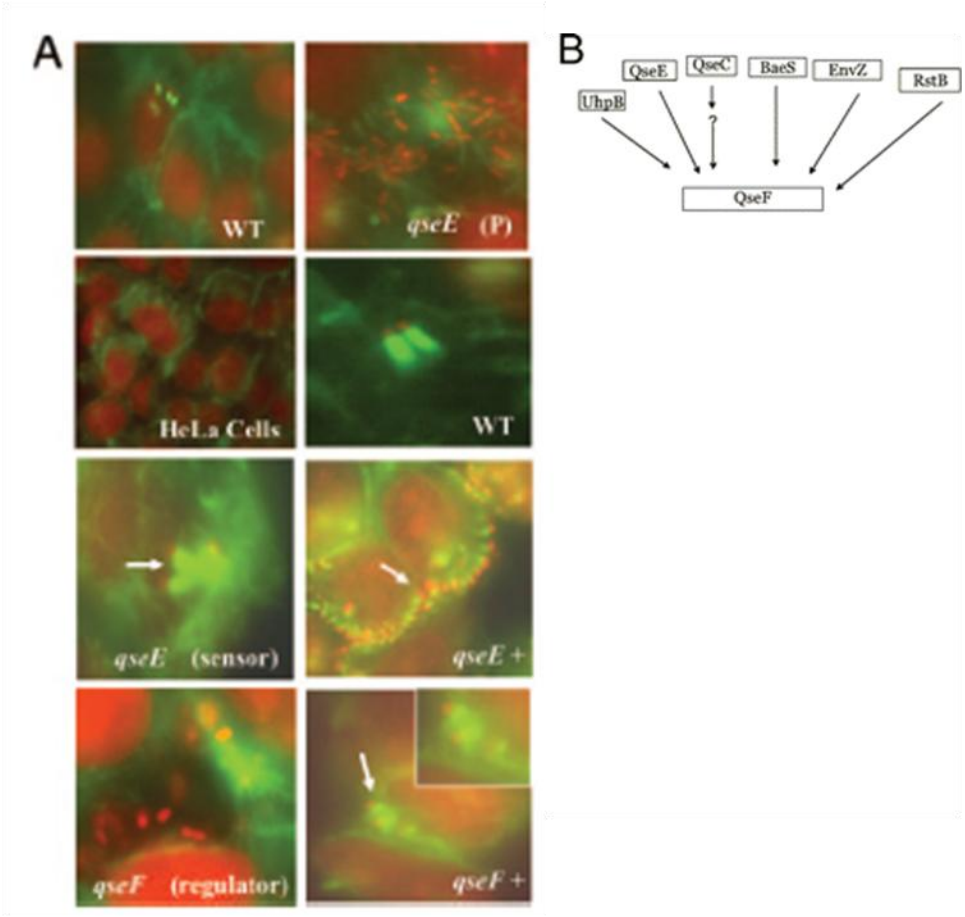


FIG. 4.4. Detection of AE lesion formation using the FAS test on HeLa cells. (A) Comparison of WT and a *qseE* polar (P) mutant. Green shows the HeLa cell actin cytoskeleton, and red shows the bacteria and cell nuclei. WT EHEC forms pedestals (top left); the *qseE* polar mutant does not (top right). Nonpolar mutant strains were tested on HeLa cells by use of a FAS assay. Nonpolar mutations reveal that only the *qseF* mutant is unable to form AE lesions. The *qseE* mutant formed AE lesions, while the *qseF* mutant could not (left bottom two panels). Each mutant strain was complemented. Cells were viewed at a magnification of

x640. (B) QseF is cross-phosphorylated by several *E. coli* noncognate sensors. It is not known if QseC is able to phosphorylate QseF.

QseF activates expression of *espFu* to induce pedestal formation.

Several factors are required for pedestal formation in EHEC, including components of the TTSS, intimin, and the effector protein Tir (130) (Fig. 4.5A). We suspected that one of the genes encoding proteins that comprise different portions of this system might be activated by QseF. We tested the expression of EspA, EspB, Tir, and Intimin by western blot of whole cell lysates and found no defect in production of any of these proteins in a *qseF* mutant (Fig. 4.5B). In addition, we tested secretion of the TTSS proteins Tir (data not shown), EspA, and EspB and again no defect was seen in secretion of these proteins (Fig. 4.5B).

EspFu was recently identified as a non-LEE encoded effector protein necessary for pedestal formation in EHEC (28, 93), possibly serving as a Nck-like protein to recruit N-WASP and Arp-2/3 to Tir. We tested whether QseF activated *espFu* transcription using an *espFu::lacZ* transcriptional reporter fusion. In the *qseF* mutant, transcription of *espFu* was abolished. Transcription of *espFu* in this mutant was restored to WT levels upon complementation with a functional copy of *qseF* (Fig. 4.5C). Transcription of *espFu* was only mildly decreased in the *qseE* mutant (twofold) (Fig. 4.5C). This mild decrease in the *qseE* mutant, in contrast to the striking decrease in the *qseF* mutant, can be attributed to the fact that QseF can be phosphorylated by multiple kinases (Fig. 4.4B). To further investigate

whether the lack of *espFu* expression was the reason for the defect on pedestal formation in the *qseF* mutant, we performed FAS assays using the *qseF* mutant containing a plasmid expressing *espFu*. Under these conditions, we were able to reconstitute pedestal formation (Fig. 4.5D).

A recent report suggested that *espFu* is co-transcribed in an operon with *espJ*. The authors of this study created *gfp* transcriptional fusions with the upstream regions of these genes and found low levels of expression of *espFu::gfp*. However, no primer extensions, Northern blots, or RT-PCR analysis were performed in these studies (91). Given that there are 324 bp in between the *espJ* and *espFu* genes (AE005419), it is possible that *espFu* is a stand-alone gene. To examine the operon structure of *espFu* and upstream genes (*espJ* and Z3069), we

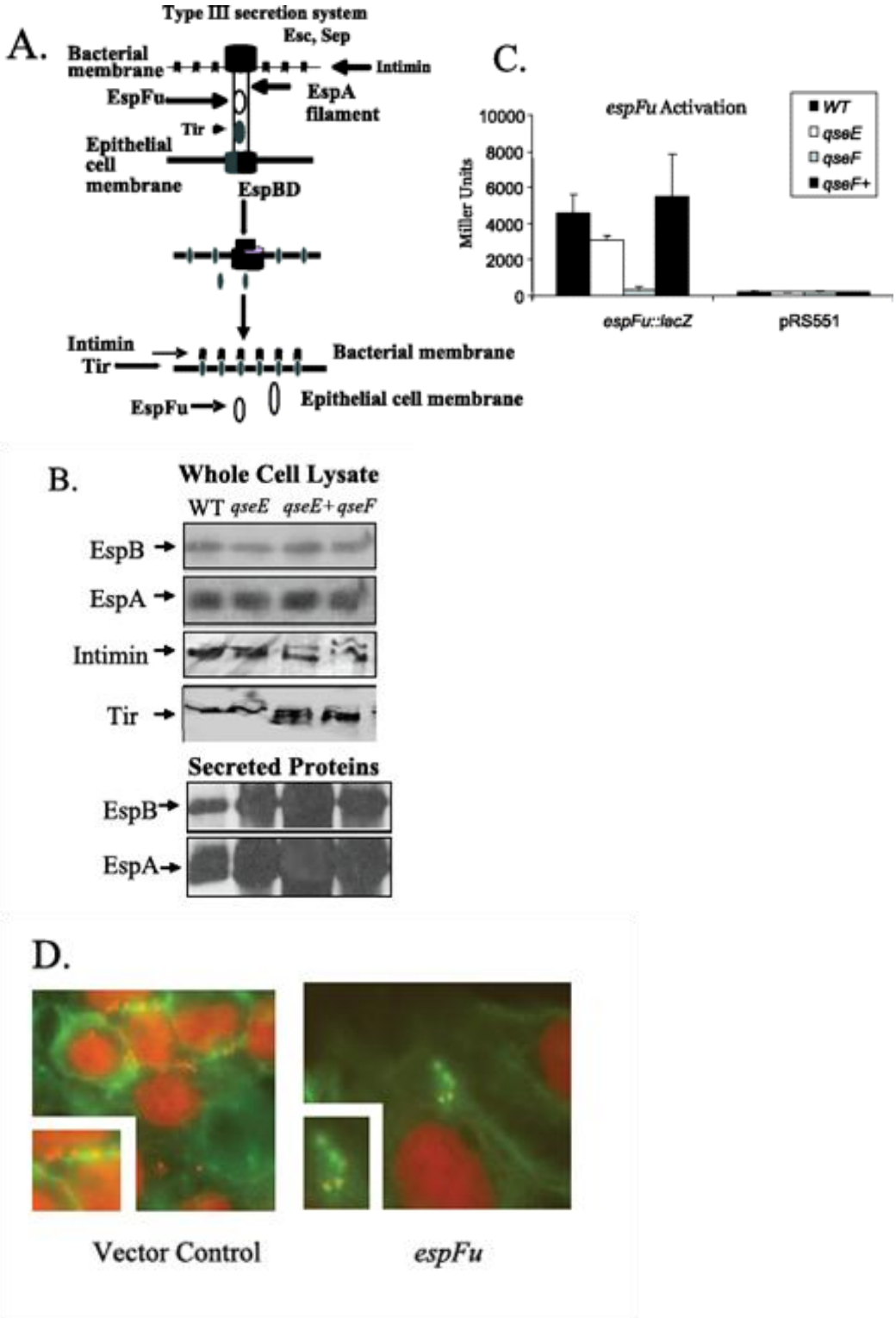

A.
Type III secretion system
Esc, Sep
Bacterial membrane
Intimin
EspFu
EspA filament
Tir
Epithelial cell membrane
EspBD
Intimin
Tir
Bacterial membrane
EspFu
Epithelial cell membrane
C.
espFu Activation
WT
qseE
qseF
qseF+
Miller Units
10000
8000
6000
4000
2000
0
espFu::lacZ
pRS551
B.
Whole Cell Lysate
WT qseE qseE+qseF
EspB
EspA
Intimin
Tir
Secreted Proteins
EspB
EspA
D.
Vector Control
espFu

FIG. 4.5. QseF regulates transcription of *espFu*. (A) Schematic of the TTSS machinery in EHEC and injection of effectors, Tir and EspFu, into host cells, enabling tight adherence to the host cell and AE lesion formation. (B) Western blots were performed on whole-cell lysates and secreted protein preparations from mutant and WT cultures grown to an OD_{600} of 1.0 in DMEM with antibodies specific to EspA and B, intimin, and Tir. The mutant *qseE* strain is complemented with *qseE* in pBADMycHisA. Production and secretion, where applicable, were not deficient in mutant strains. (C) ß-Galactosidase assay using an *espFu*::*lacZ* transcriptional reporter construct in the WT strain, the *qseF* mutant, and the *qseF* mutant containing *qseF* in pACYC177. *espFu* activation in the mutant is at background levels (third bar). (D) FAS assay on the *qseF* mutant strain containing an empty vector (left) or *espFu* under an inducible promoter (right). In the presence of *espFu*, pedestal formation is restored. Cells were viewed at a magnification of x640.

used RT-PCR. We detected transcription of *espFu* using an internal primer set to this gene (Fig. 4.6). No amplification was observed with primers flanking *espFu* and either of the two upstream genes (Fig. 4.6). These data indicate that *espFu* and *espJ* are not transcriptionally linked. The contrasting data obtained by our group to that of Garmendia *et al*. may be due to the low sensitivity of *gfp* reporters. To identify the *espFu* promoter, we performed primer extensions. These primer extension studies were performed three times using two different primers to ensure the correct mapping of the transcriptional start site of *espFu*. The

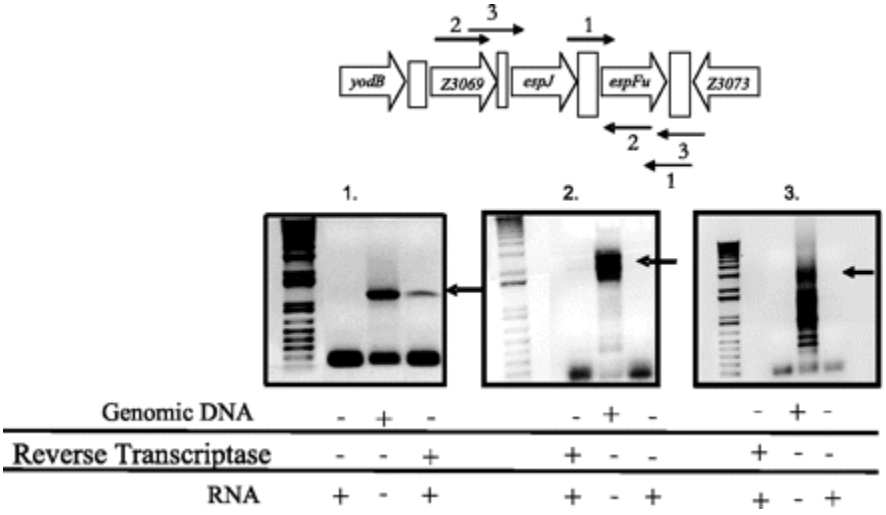


FIG. 4.6. The *espFu* operon. Reverse transcription analysis was performed to evaluate the operon structure of *espFu* by use of *espFu* internal primers (1., EspFuProbeR and EspFuProbeF), primers flanking *espFu* and the two upstream genes *espJ* and *Z3069* (2., EspFuRN and EspFuRT2), and primers flanking *espFu* and one gene upstream, *espJ* (3., EspFuF and EspFuRN). Only the primers to amplify *espFu* alone from WT cDNA showed a product, indicating that *espFu* is a stand-alone gene. For each primer set, PCR was performed using genomic DNA as a positive control and RNA with no reverse transcriptase added as a negative control.

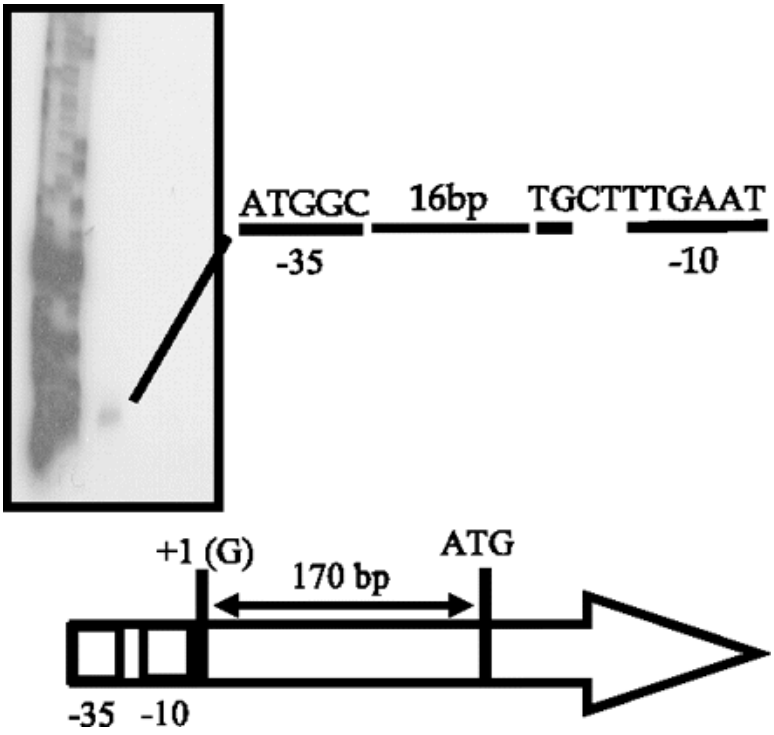


FIG. 4.7. Mapping of the *espFu* promoter. The promoter of *espFu* was mapped using primer extension with primers downstream and upstream of the ATG start site. The promoter region of *espFu* contains an extended σ^{70} consensus sequence corresponding to –10 and –35 from the +1 transcriptional start site that was mapped.

espFu gene contains a conserved extended σ^{70} consensus sequence (Fig. 4.7). The combined RT-PCR and primer extension data led us to conclude that *espFu* is encoded by itself and driven by its own promoter. In-silico analysis indicates that QseF contains a σ^{54}-activator domain (www.promscan.uklinux.net/; Fig 4.1). Because *espFu* contains a σ^{70} rather than the alternative σ^{54} promoter, it is likely that QseF indirectly activates transcription of this gene, possibly through an additional regulatory protein. Consistent with this hypothesis, we were unable to

observe direct binding of QseF to the *espFu* regulatory region using electrophoretic mobility shift assays EMSAs, even after addition of acetyl phosphate as a phosphate source for activation of QseF (Fig. 4.8). This suggests that QseF does not directly bind to the promoter region of *espFu* and that QseF regulation of *espFu* is indirect, involving an intermediary factor transcribed in a σ^{54}–dependent fashion.

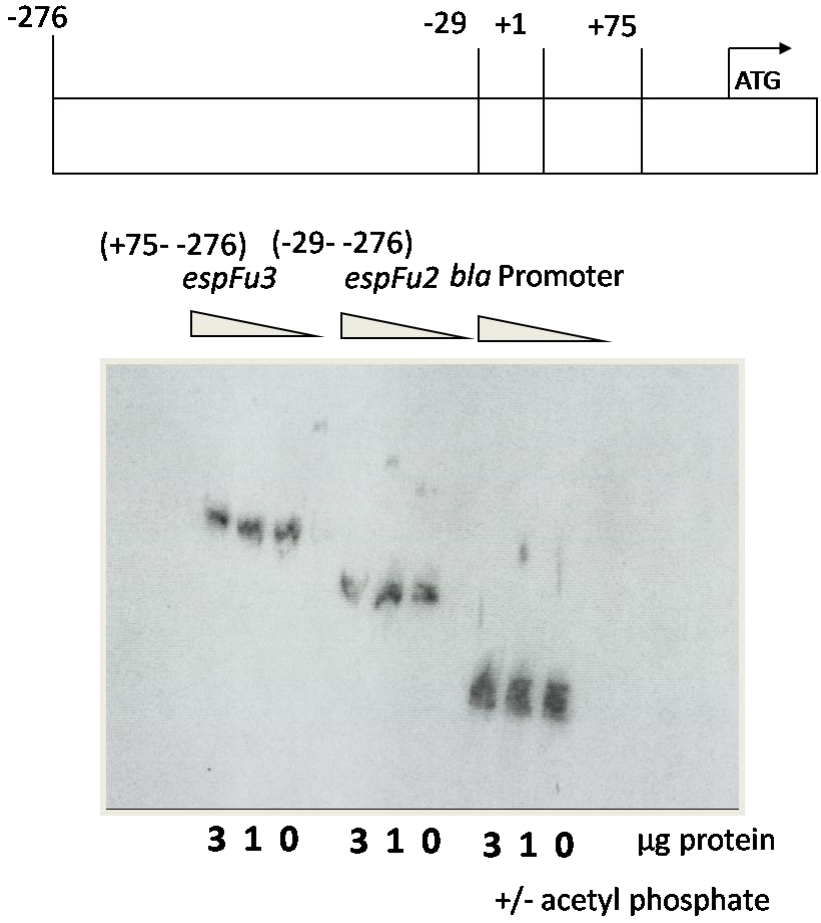


FIG. 4.8. Electromobility shift assay. DNA probes from the *espFu* regulatory region were amplified using primers EspFuF/EspFuBInv for probe #2 and EspFuF/EspFuCInv for probe #3. No concentration of purified QseF was able to

shift the mobility of the radiolabeled probes in the presence or absence of a phosphate source.

Discussion

The cell-to-cell signaling system utilized by EHEC to control virulence is highly complex, involving many levels of gene regulation. Although we know that multiple EHEC virulence genes are activated in response to the host epinephrine/norepinephrine and the bacterial signal AI-3, numerous aspects of this regulation are still unresolved, including how the signals are sensed and transduced by the bacterial cell as well as the global hierarchy of signaling that leads to gene activation. With respect to this signaling, EHEC virulence can be divided into two main pathways: (i) flagellation and motility and (ii) the formation of AE lesions (Fig. 4.9). The two-component system QseBC activates the flagella regulon in response to AI-3 and epinephrine/norepinephrine. QseC's autophosphorylation in response to these signals can be blocked only by α-adrenergic antagonists (39). AE lesion formation, however, can also be blocked by a β-adrenergic antagonist (256). This suggests that more than one sensor kinase responds to AI-3 and epinephrine/norepinephrine. QseE could function as an additional sensor, transducing environmental signals in the direction of AE lesion formation rather than flagella regulation.

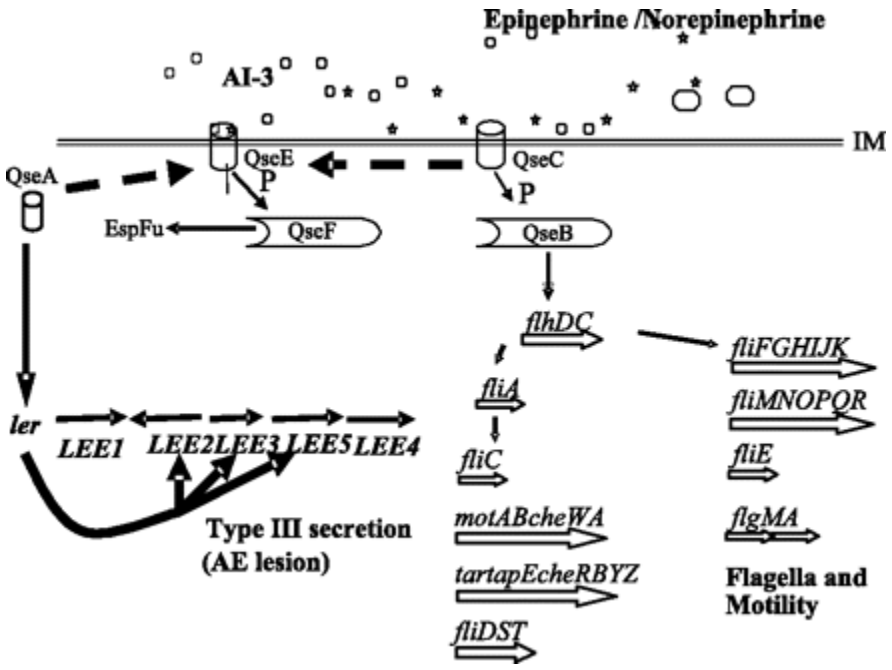


FIG. 4.9. Schematic of AI-3, epinephrine, and norepinephrine signaling in EHEC. AI-3, epinephrine, and norepinephrine are sensed by sensor kinases in the EHEC membrane. QseBC regulate flagella and motility, while QseEF may be a second two-component system that regulates AE lesion formation in conjunction with other EHEC regulators such as QseA.

In this chapter, we describe QseEF, a two-component system regulated by cell-to-cell signaling in EHEC. These proteins regulate pedestal formation through indirect activation of *espFu*. The response regulator QseF does not bind directly to the σ^{70} promoter region of *espFu*. Instead, it is likely that QseF regulates *espFu* through an unidentified σ^{54} – dependent intermediate. Based on our data, QseEF do not appear to regulate the LEE genes. These data reinforce

the complexity of EHEC virulence signaling and indicate that another two-component system, along with intermediates such as QseA, is involved in AE lesion formation by EHEC (Fig. 4.9).

The timing of flagella production and formation of AE lesions is crucial for successful EHEC colonization of the human intestine, thus making the kinetics of activation of these genes equally important. Interaction of environmental signals with multiple sensors would allow a precise timing mechanism enabling successive rather than simultaneous production of these systems, and ultimately more efficiency. We suspect that upon initial colonization of the intestine, EHEC receives signals from the host and commensal flora allowing the bacteria to activate the flagella genes and swim across the mucus layer of the intestine. Once in proximity to the epithelium of the intestine, the TTSS genes are activated for AE lesion formation. At this point, the flagella genes must be down-regulated. It is further intriguing that there is yet an additional level of fine tuning through QseEF regulation of *espFu* transcription, but not LEE expression. Determining how these two systems are connected and how they use cell-to-cell signaling to orchestrate regulation in response to the environment will not only give us further insight into cell-to-cell signaling, but also to the biology of the intestine and interaction between pathogenic and commensal organisms.

Only a small number of signals for two-component systems are currently known. This along with the difficulty in purifying membrane proteins prompted

Yamamoto *et al.* (310) to use only the cytoplasmic region of the sensor kinases in their study of all two-component systems in *E. coli*. Although QseE has been shown to phosphorylate QseF (310), the physiological signal that activates QseE is still unknown. We have attempted to purify QseE and insert it into liposomes in order to test possible chemical and environmental signals to which it responds. However, despite attempting to express QseE in several different vector contexts, this protein remains insoluble and difficult to purify in large quantities. In addition, we have attempted to examine potential interaction between the two two-component systems, QseBC and QseEF, by conducting cross-phosphorylation experiments in which we assess whether phosphorylated QseC can phosphorylate QseF. However, the similar sizes of QseF (49 kDa) and QseC (56 kDa) and similar isoelectric points (QseE = 5.6, QseC = 6.0) have made separation of these proteins after phosphor-transfer assays challenging. Alternative methods to resolve these problems are under investigation.

Two-component signaling systems are found primarily in prokaryotes and have not yet been identified in animals and humans making them an ideal target for drug inhibitors and therapeutics (299). This is important in an age where antibiotic resistance is becoming increasingly prevalent. In particular, in EHEC infection, no current treatment exists (131), and antibiotics can worsen the infection and lead to hemolytic uremic syndrome (HUS). Blocking EHEC's signaling mechanisms for expressing virulence genes, could potentially render

this bacteria harmless. Elucidating the EHEC hierarchy of virulence expression is crucial in order to utilize this pathway in treatment.

CHAPTER FIVE

THE OUTER MEMBRANE PROTEIN QSEG IS THE THIRD MEMBER OF A THREE COMPONENT SIGNALING SYSTEM IMPORTANT FOR EHEC PATHOGENESIS

Introduction

The events that must occur in order for EHEC to form pedestals and be a successful pathogen are well orchestrated and require precise timing of expression of numerous virulence genes. Indeed, most pathogens must develop mechanisms to exist in certain niches and to recognize when they have reached this niche. This is particularly relevant in an environment such as the large intestine, in which an organism encounters not only chemicals and nutrients from the host, but also large numbers of commensal flora. The large intestine contains approximately 10^8 bacteria per ml of intestinal content and hundreds of different species of bacteria (17). Given the nature of this environment, it is logical that there exists a signaling system which connects EHEC with the commensal flora in the intestine and with the host itself as EHEC does utilizing AI-3, norepinephrine, and epinephrine (39, 220, 257).

Regulation of the genes involved in the formation of AE lesions through these signals is highly complex and involves many intermediary signaling

proteins such as the LysR-type regulators QseA (242, 251) and QseD (Benjamin Habdas, unpublished data). In addition to QseBC as an initial two-component signaling system (38) in the EHEC virulence gene signaling cascade, we recently reported the initial characterization of another two-component system, QseEF, which is also involved in the regulation of AE lesion formation. QseEF are a part of EHEC's cell to cell signaling cascade and play a role in AE lesion formation through the indirect regulation of the effector protein EspFu (221). It has been shown that QseE and QseF are a cognate pair and that QseE is capable of autophosphorylating and transferring a phosphate to QseF. However, QseE's cognate signal that activates autophosphorylation is still unkown. In addition, QseF is cross-phosphorylated by at least four non-cognate sensors: BaeS, EnvZ, RstB, and UhpB (310). *qseEF* have a very unusual operonic structure in that they are juxtaposed to a small gene (800 bp), *yfhG*. The YfhG protein contains a predicted secretion signal sequence and is predicted by *in silico* analysis to be a membrane protein. However, it lacks significant homology to other proteins and does not contain any predicted active sites or domains. This distinguishes it from other proteins that are involved in two-component phospho-relay signaling (315). An increasing number of reports have implicated membrane proteins as key players in allowing two-component systems to recognize their cognate signals and communicate or coordinate with other two-component systems (33, 67, 77), indicating that these systems are even more complex than originally thought.

None of these reports, however, have described a membrane protein encoded within an operon encoding a two-component system.

The aim of this study was to elucidate the role of *yfhG* in QseEF function and to further characterize the entire QseEFG system and its role in responding to environmental signals and regulating attaching and effacing lesion formation.

Results

The *qseEFG* operon

QseG is a 237 amino acid protein that is encoded between the genes that encode the sensor kinase QseE and the response regulator QseF and separates on an SDS-PAGE gel at approximately 27kDa. The first 25 amino acids of QseG are predicted to be a signal sequence and the protein is predicted to contain lipid attachment sites (http://expasy.org/tools/scanprosite/). In addition, QseG shows homology to putative membrane proteins as well as putative alpha-helix proteins (http://expasy.org/tools/blast/). However, no additional domains or active sites were found in QseG based on *in silico* analysis. The sensor kinase, QseE lies in the inner membrane and the response regulator, QseF, lies in the cytoplasm (Fig. 5.1A).

In order to definitively determine the localization of QseG in EHEC cells and to have a better understanding of how QseG might interact with QseE and QseF, we isolated crude membrane fractions and separated these fractions over a

density sucrose gradient. We determined the density of each fraction based on its refractive index. As seen in figure 5.1B, 18 fractions ranging from 1.092 to 1.2073 g/ml were isolated. An anti-Imp antibody (a generous gift from Thomas Silhavy) was used to check the validity of our fractions. This antibody recognizes an inner membrane protein of approximately 55 kDa and the outer membrane protein, OmpA, at approximately 19 kDa. As seen in Figure 5.1B, when fractions were blotted with this antibody, OmpA appeared primarily in fractions 1 and 2 at 1.2076-1.2046 g/ml while Imp appeared primarily in fractions 7-10 at 1.1493-1.1311 g/ml. These weights correspond well with the densities expected for the inner and outer membranes from previously reported membrane fractionations (198, 204). When we probed the fractions using both an anti-His antibody and an anti-QseG antibody, in both instances, QseG appeared in the first two to three fractions (1.2073-1.18 g/ml), indicating that QseG localizes to the outer membrane.

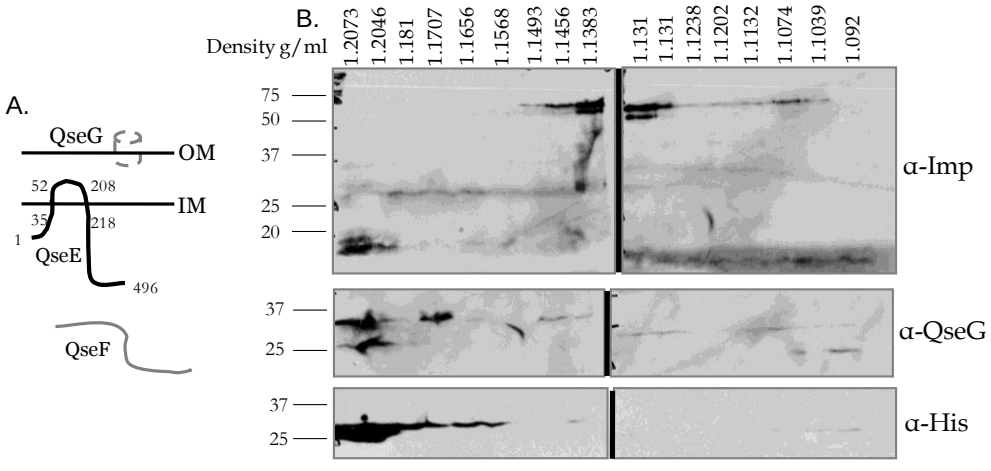


Figure 5.1 – QseEFG constitute a three-component system that contains: QseE, an inner membrane sensor kinase, QseF, a cytosolic response regulator, and, QseG, an uncharacterized protein predicted be a lipoprotein. A. Depiction of the localization of QseE, QseF, and QseG. B. Membrane fractions separated over a density dependent sucrose gradient. QseG localizes to the first 4 fractions, similar to the outer membrane protein OmpA recognized by α- Imp.

We previously reported that *qseEGFglnB* are co-transcribed in one operon (221). Previous reports in the literature and *in silico* analysis reveal that the structure and regulation of this operon is quite complex with at least two promoter sites that have been mapped, one upstream of *glnB* and one upstream of *qseE*. Both of these promoters were mapped as typical σ^{70} promoters *(108, 164)*. On the other hand, based on *in silico* scans of the *Escherichia coli* EDL933 genome using a well conserved sequence (11), *qseG* is additionally predicted to have a σ^{54} promoter (http://www.promscan.uklinus.net/RpoN/promscan.outfile.53.html) (Fig. 5.2A). Finally, auto- regulation is often seen within two-component systems, where the regulator of the system drives the transcription of the operon (40) (263). Indeed, the targets of response regulators, particularly ones containing σ^{54} activator domains like QseF, are often located chromosomally close to their regulators (263). To this end, we wanted to investigate auto-regulation in the *qseEFGglnB* operon and to determine if the transcription of any of the genes is dependent on any other portion of the operon. In order to investigate the autoregulation of the *qseE* promoter, we fused the regulatory region of *qseE* to a promoterless *lacZ* and tested *qseE's* activation in the *qseE*

non-polar mutant. As seen in Figure 5.2 B, the transcription of *qseE* in the *qseE* mutant does not change, indicating that QseE is not required for its own transcription.

To test whether *qseG* or *qseF* modulated *qseE* transcription, real-time RT-PCR for the *qseE* transcript was performed in both the *qseF* and *qseG* mutants. Transcription of *qseE* was increased in the *qseG* mutant suggesting that QseG exerts a repressive role in the expression of *qseE* (Fig. 5.2 C). Conversely, expression of *qseE* was decreased in a *qseF* mutant, suggesting that QseF activates the expression of *qseE* (Fig. 5.2C). These data indicate that the promoter upstream of *qseE* is activated through QseF in a QseE-independent manner, which can be explained by the previous observation that QseF is a promiscuous reponse regulator that can be phosphorylated by four other sensor kinases in addition to QseE (310).

Due to the presence of two additional internal promoters in this operon, we wanted to further investigate the possibility that the transcription of any one of these genes could be autoregulated through either one of these promoters. Transcription of both *qseG* and *qseF* was unaltered in any of the three mutants, suggesting that transcription of these two genes is not subject to autoregulation. In contrast, transcription of *glnB* is increased in the *qseF* and *qseG* mutants. This suggests that these proteins may act through the σ^{70} promoter upstream of *glnB* to repress its transcription. These data highlight the complexity of the auto-

regulatory circuit that governs the expression of the *qseEGFglnB* regulon, suggesting that fine tuning the transcription of this operon is important for signaling (Fig. 5.2C).

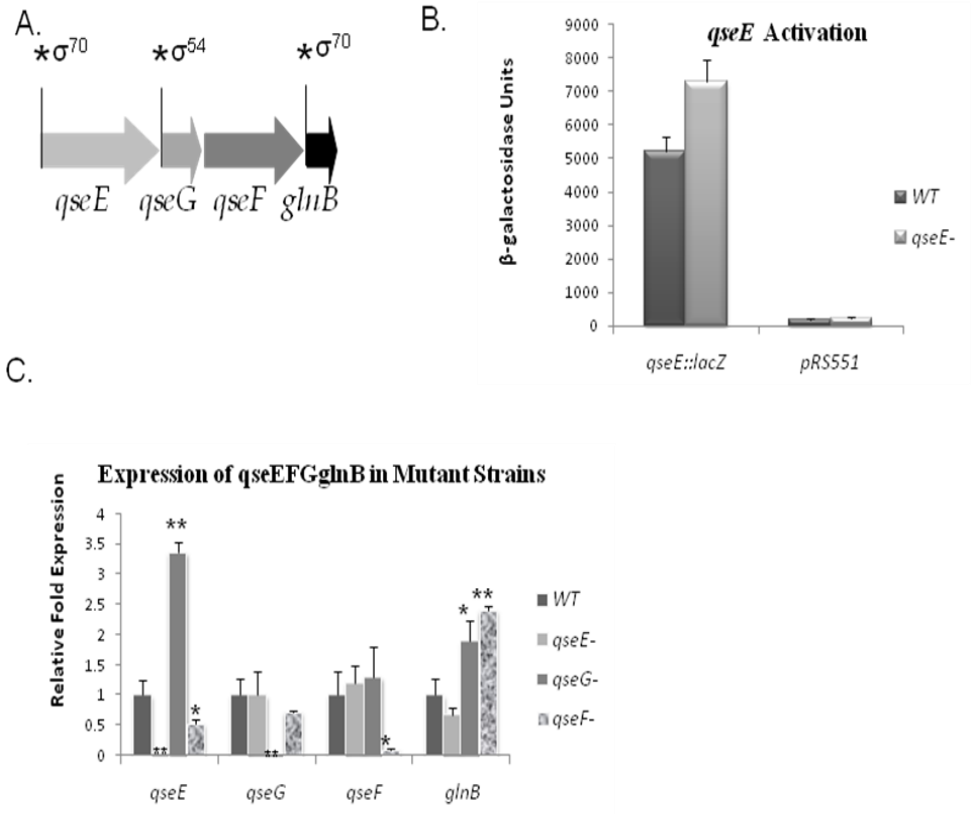


Figure 5.2 – The *qseEGFglnB* operon. A. The *qseEGFglnB* operon contains several promoters including two mapped σ^{70} promoters upstream of *qseE* and *glnB* as well as a predicted σ^{54} upstream of *qseG*. B. *qseE* activation using *qseE::lacZ* fusions in β-galactosidase assays. This construct is still activated in a Δ*qseE* background. C. Real-time RT-PCR analysis examining the expression of *qseE*, *qseG*, *qseF*, and *glnB* in WT versus *qseE*, *qseG*, and *qseF* mutant backgrounds. *qseG* expression is upregulated three-fold in the *qseE* mutant, while *qseF* expression is down-regulated. *glnB* expression is upregulated two-

fold in the *qseG* and *qseF* mutant backgrounds. * denotes a p-value of ≤ .05 and ** denotes a p-value of ≤ .005 using a student's T-test.

Microarray analysis of Δ*qseE*, Δ*qseF*, and Δ*qseG* vs. WT EHEC

In order to identify additional targets for the QseEGF three-component system, we used Affymetrix GeneChip *E. coli* Genome 2.0 arrays to compare the transcripts of WT EHEC to Δ*qseE*, Δ*qseF*, and Δ*qseG*. The results of these analyses revealed many patterns among the three genes. For example, across the three mutant strains, 445 similar genes increased expression and 130 similar genes decreased expression. Tables 3 and 4 summarize the results of these studies in EHEC (Table 3), and among different serotypes (Table 4). More similarity is seen between the effect of knocking out these genes, when Δ*qseE* and Δ*qseG* are compared.

Table 3. Conserved genes activated or repressed in response to mutation

	Mutation
	E. coli* 8624 Δ*qseE
Increased	1726
Marginally increased	173
Decreased	1331
Marginally decreased	429
No Change	6549
	E. coli* 8624 Δ*qseF
Increased	1101
Marginally increased	535
Decreased	304
Marginally decreased	472
No Change	7796
	E. coli* 8624 Δ*qseG
Increased	1092
Marginally increased	433
Decreased	569
Marginally decreased	545
No Change	7565
	In Δ*qseE*, Δ*qseF* and Δ*qseG*
Increased	445
Marginally increased	0
Decreased	130
Marginally decreased	0
No Change	5318
Differentially regulated	4314

Table 4. Pathovar specific distribution of genes

	MG1655 (n=4070)		EDL933 (n=1787)		Sakai (n=373)		CFT073 (n=2486)		Intergenic (n= 1297)	
8624 v ΔqseE										
Decrease	871	21.40	263	14.72	33	8.85	127	5.11	33	2.54
Marginal decrease	253	6.22	93	5.20	18	4.83	53	2.13	10	0.77
Increase	558	13.71	406	22.72	83	22.25	235	9.45	395	30.45
Marginal increase	74	1.82	48	2.69	5	1.34	17	0.68	28	2.16
No change	2314	56.86	977	54.67	234	62.73	2054	82.62	831	64.07
	4070		1787		373		2486		1297	
8624 v ΔqseF										
Decrease	183	4.50	69	3.86	9	2.41	33	1.33	10	0.77
Marginal decrease	280	6.88	114	6.38	24	6.43	37	1.49	14	1.08
Increase	304	7.47	207	11.58	50	13.40	213	8.57	285	21.97
Marginal increase	272	6.68	134	7.50	15	4.02	44	1.77	65	5.01
No change	3031	74.47	1263	70.68	275	73.73	2159	86.85	923	71.16
	4070		1787		373		2486		1297	
8624 v ΔqseG										
Decrease	332	8.16	158	8.84	23	6.17	38	1.53	14	1.08
Marginal decrease	328	8.06	140	7.83	25	6.70	27	1.09	17	1.31
Increase	300	7.37	250	13.99	49	13.14	176	7.08	287	22.13
Marginal increase	217	5.33	88	4.92	15	4.02	47	1.89	62	4.78
No change	2893	71.08	1151	64.41	261	69.97	2198	88.42	917	70.70
	4070		1787		373		2486		1297	

The total number of genes assigned to the specific genomes included is 10,013.
There are an additional 96 features that are used as controls and 99 features that are associated with phage and plasmids and thus not directly linked to a genome project.
Total number of features on the array is 10208.

Several notable areas of regulation were identified through this screen, including many metabolic genes and numerous outer membrane proteins and transport machines. Genes involved in both iron and nitrogen regulation, particularly, were regulated by this system (Figs. 5.3A and5. 4A).

Given the abundance of metabolic genes that appeared to be differentially regulated in the array, we would expect to see a difference in the growth ability between WT and each of the mutants. To follow up on the our microarray results showing that the genes involved in iron and nitrogen regulation were differentially regulated in the Δ*qseE*, Δ*qseF*, and Δ*qseG* mutants, we conducted a series of growth experiments. Based on the presence of *glnB* in the operon, it is

not surprising that nitrogen metabolism gene regulation was altered in the mutant strains. *glnB* encodes the α-ketoglutarate sensor PII. This protein negatively regulates the two-component system NtrB/NtrC, which activate glutamine synthetase when the cell has low nitrogen levels (199). However, we found no defect in growth in any of the mutants compared with wild type grown in minimal media nor when glutamine was supplemented into the media (Fig. 5.3B).

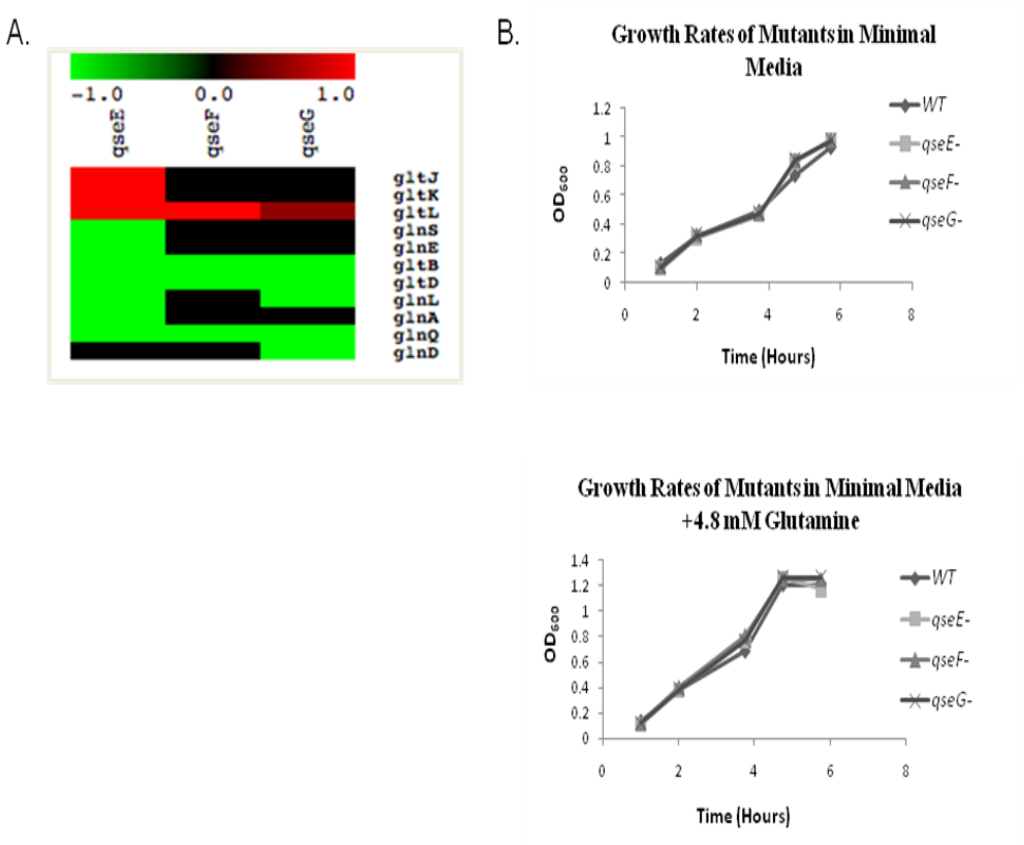


Figure 5.3 – *qseEGFglnB* affects nitrogen gene regulation. A. Heat map showing genes involved in nitrogen regulation. The mutant strains are listed across the top and genes probed listed on the right side. Green indicates down-

regulation of the indicated gene compared with WT, red indicates up-regulation, and black indicates no change. These genes are primarily downregulated in *qseE, qseG,* and *qseF*. C. Growth curves showing the growth of each mutant in minimal media and with glutamine titrated in. In both cases, there is no difference in growth between WT EHEC and the *qseE, qseG,* and *qseF* mutant strains.

We additionally tested the ability of each mutant to grow on plates containing arginine as the sole nitrogen source. Again, each one of the mutant strains was able to grow on this minimal media (data not shown). These data indicate that although this three component system modulates expression of genes involved in nitrogen metabolism, the Δ*qseE*, Δ*qseF*, and Δ*qseG* mutants are not defective for nitrogen utilization. This suggests that QseEFG does not overcome the primary nitrogen signaling system, NtrB and NtrC.

Our gene array studies suggested that QseEFG modulate expression of several iron uptake systems. To confirm the array studies, we performed real-time RT-PCR. Our real-time experiments confirmed that transcription of *entB* is decreased in the *qseG* mutant, while it is unaltered in the *qseE* and *qseF* mutants. Expression of *fepE* is decreased in each of the mutants, while expression of *tonB* is unaltered (Fig. 5.4A). EntB is involved in the synthesis of the enterobactin siderophore (106), while FepE is involved in enterobactin synthesis and transport (206) and has been implicated in modification of LPS in *Salmonella* (191).

In order to investigate differential iron uptake and utilization in each of the mutant strains, we performed several iron utilization and uptake assays. As

shown in Fig. 5.4B, when each of the mutants were grown on Luria Broth plates containing the iron chelator 2,2 di-pyridyl (DPD) or hemin for recovery over 48 hours, there is no difference between the effect of these compounds on any of the mutants compared with WT. The growth of all strains was inhibited by

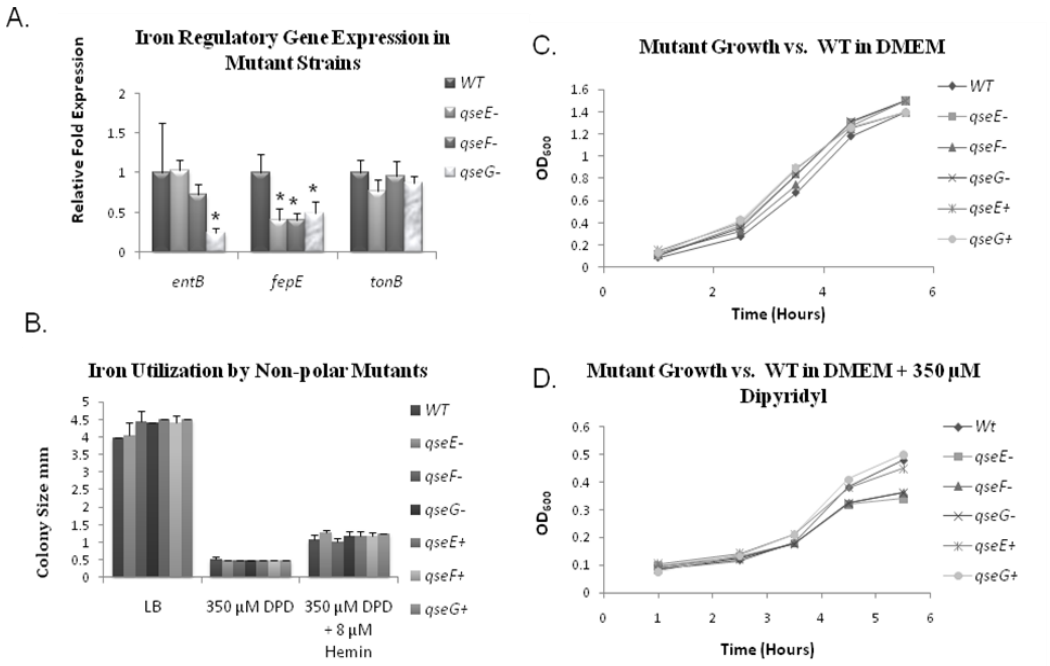


Figure 5.4 – A. Real-time RT-PCR showing the regulation of iron ultilization genes in the *qseE, qseG,* and *qseF* mutant backgrounds. Significant down-regulation is seen in *fepE* and *entB* expression. B. Colony growth of mutant and complement strains on media containing either 350 µM DPD or 350 µM DPD + 8 µM hemin. Each strain was equally affected by DPD addition and able to recover equal to WT when hemin was added. C. Growth of mutant strains versus WT EHEC in DMEM. No growth defect is seen in any of the mutant strains. D. Growth of mutant strains versus WT EHEC in DMEM containing 350 µM DPD. The *qseE* and *qseG* mutants grow slightly slower than wild-type and this is complemented by the addition of the gene expressed on a plasmid to the WT strain. * denotes a p-value of $\leq .05$ using a student's T-test.

the addition of 350 µM DPD and all strains were able to equally recover with the addition of 8µM Hemin. When the strains were grown in minimal media (Fig. 5.4C), no difference in growth is seen. When the strains were grown in minimal media containing DPD, the *qseE* and *qseG* mutants grew slightly slower in the presence of 350 µM DPD (Fig. 5.4D). This slight defect in growth is complemented when a copy of any one of the genes is expressed in the mutant strains (Fig. 5.4D). These data indicate that while numerous iron uptake and utilization genes are differentially regulated in the mutants, the effect on iron utilization or growth is minimal. However, there does seem to be a small defect in mutant growth under iron-limiting conditions in minimal media, which is the media utilized in the transcriptional studies.

Cross-talk has been shown to exist between two-component systems at the protein level by non-cognate phosphorylation (310). Our gene array studies indicated that numerous two-component signaling systems may also be regulated by QseEFG at the transcriptional level. To validate this finding, we performed real-time RT-PCR and found that *phoP* expression is down-regulated in all three mutant strains, while *phoQ* expression is down-regulated in both the *qseF* and the *qseG* mutants. PhoPQ is a two-component system known to be involved in sensing of antimicrobial peptides (8). In addition, the uncharacterized response regulator Z0463 is upregulated in all three mutants. Finally, the RscBC system,

which has been shown to modulate expression of the *LEE* region (280) showed regulation. *rscB* is upregulated in *qseE, qseF,* and *qseG* mutants, while *rscC* is upregulated in the *qseG* mutant (Fig. 5.5). These data indicate that QseEFG may regulate numerous two-component systems transcriptionally and provides a link between QseEFG and the *LEE* region.

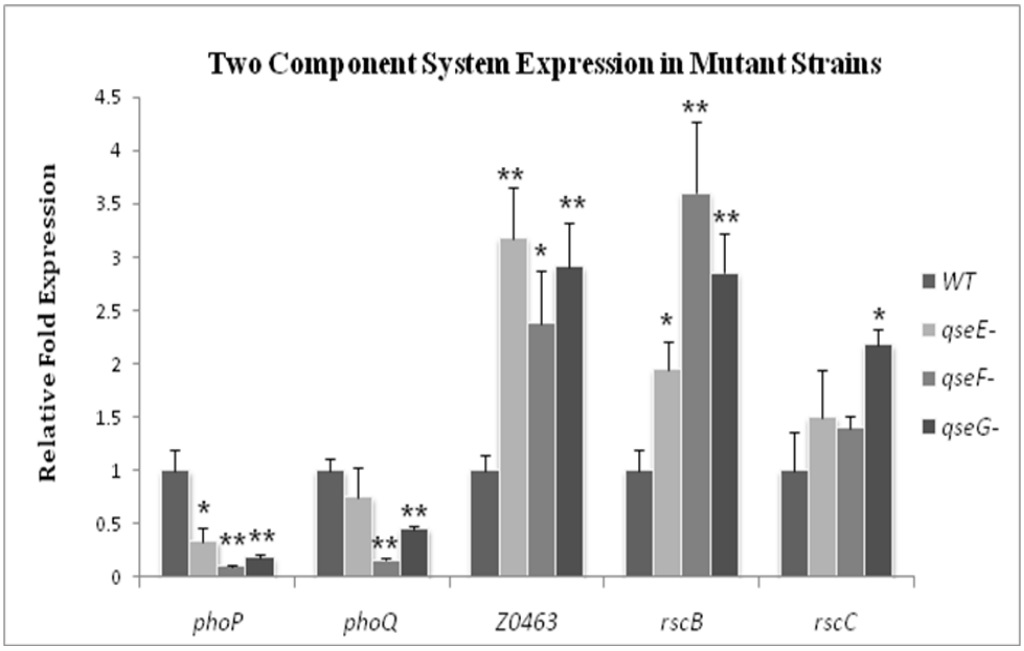


Figure 5.5 – QseEFG cross-talks with additional signaling systems. Real-time RT-PCR analysis was used to investigate transcriptional regulation that QseEFG exerts on other two-comonent systems. Overall, expression of *phoP* and *phoQ* were down-regulated in *qseE, qseF,* and *qseG* mutants, while *Z0463*, *rscB,* and *rscC* were up-regulated in the mutant strains. * denotes a p-value of $\leq .05$ and ** denotes a p-value of $\leq .005$ using a student's T-test.

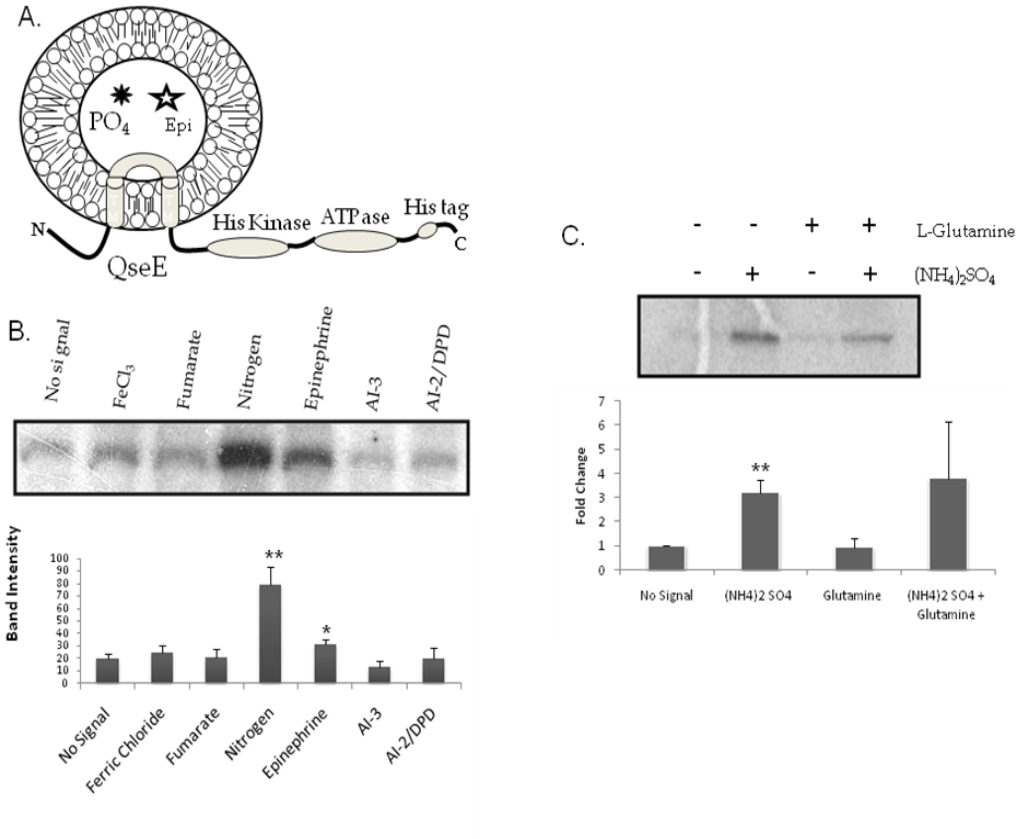
QseE is activated by epinephrine, phosphate, and sulfate sources.

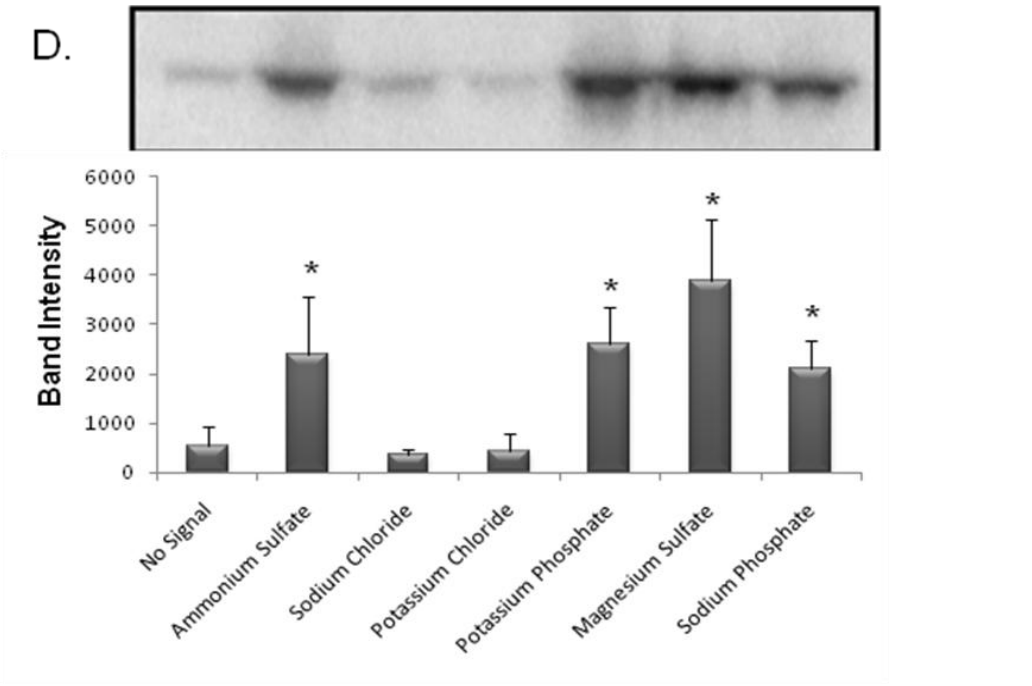
QseE is a predicted sensor kinase and its cytoplasmic domain has been shown to autophosphorylate and transfer that phosphate to its cognate response regulator QseF (310). In order to conduct a high-throughput study examining all the two-component signaling systems in *E. coli* without using cognate signals, many of which are unknown, Yamamoto, *et al.* used only the cytoplasmic domains of the sensor kinases. Previous reports have shown that intact sensor kinases often will not function unless in the physiological presence of membranes (119, 120). In order to facilitate this environment in *in vitro* assays, liposomes have been utilized. This system not only allows for the sensor kinase to be oriented in a membrane, the kinase is inserted into the liposome in an inside out orientation. This orientation, as shown in Fig.5.6A, allows different chemicals to be pre-loaded into the liposome and tested for their ability to enhance the kinase's ability to phosphorylate (39, 120). QseE has been shown to be a part of EHEC's virulence cell-to-cell signaling cascade. Genetically, it has been shown to be regulated by epinephrine through QseC and QseA, other members of the cascade, and the presence or absence of the *luxS* gene (221). In order to investigate which physiological signals the QseEFG system may respond to directly, we used the liposome system. QseE was inserted into liposomes. We confirmed the insertion of QseE into the liposomes by western blot with an anti-C-terminal His antibody (data not shown). In order to test QseE's responsiveness to numerous signals, we

used the QseE/liposome preparations in *in vitro* autophosphorylation assays in the presence or absence of numerous signal molecules. Based on QseE's putative role in the EHEC's AI-3/epinephrine/norepinephrine signaling cascade, we used sources of 50 μM epinephrine as well as 50 μM AI-3. In addition, we treated the liposomes with 100 μM 4, 5-dihydroxy-2, 3-pentanedione (DPD), the precursor to autoinducer 2 as well as 20 mM fumarate, the known signal for DcuS (120). Finally, based on our microarray studies, we used both sources of iron (150 μM $FeCl_3$) and nitrogen, to which QseE might also respond. As shown in Fig. 5.6B, QseE exhibits a low level of basal phosphorylation when no compounds are added. However, QseE shows a robust response to nitrogen sources (15 mM glutamine and 15 mM $(NH_4)_2SO_4$). In addition, QseE significantly increases phosphorylation when 50 μM epinephrine is loaded into the liposome, but shows no response to AI-3, AI-2, or iron. We confirmed the phosphorylated band from autoradiography as QseE by western blot using an anti-His antibody and by mass spectrometry. These results suggested that QseE senses nitrogen sources and epinephrine, but not quorum sensing molecules.

To further investigate QseE's enhanced phosphorylation in response to nitrogen sources, we pre-loaded QseE liposomes with glutamine or ammonium sulfate separately. This demonstrated that QseE was responding specifically to ammonium sulfate and not to glutamine (Fig. 5.6 C). Considering the presence of *glnB* in this operon, it would be logical for QseE to be a nitrogen sensor.

However, we wanted to distinguish whether QseE was responding to ammonium or its counter ion sulfate. To this end, we conducted the autophosphorylation assays after preloading the QseE liposomes with numerous sources of 15 mM phosphate or 15 mM sulfate (Fig. 5.6D). Here, QseE's autophosphorylation in each case is stimulated by the presence of either phosphate or sulfate sources, but not by the controls, 15 mM NaCl or 15 mM KCl. Although these experiments demonstrated that QseE autophosphorylation is stimulated by phosphate and sulfate sources, they did not rule out the possibility that QseE is a nitrogen sensor. In order to further elucidate QseE's role as a nitrogen sensor, we tested the ability of numerous ammonium compounds to activate QseE autophosphorylation when loaded into the QseE liposomes. As seen in Figure 5.6E, only the ammonium compounds containing a phosphate or sulphate counter ion were able to stimulate QseE autophosphorylation above background levels. These data indicate that QseE may be a phosphate, sulfate, and epinephrine sensor.


A.
PO4
Epi
N
QseE
His Kinase
ATPase
His tag
C
B.
No signal
FeCl3
Fumarate
Nitrogen
Epinephrine
AI-3
AI-2/DPD
Band Intensity
No Signal
Ferric Chloride
Fumarate
Nitrogen
Epinephrine
AI-3
AI-2/DPD
C.
L-Glutamine
(NH4)2SO4
Fold Change
No Signal
(NH4)2 SO4
Glutamine
(NH4)2 SO4 + Glutamine


D.
Band Intensity
6000
5000
4000
3000
2000
1000
0
*
*
*
*
No Signal
Ammonium Sulfate
Sodium Chloride
Potassium Chloride
Potassium Phosphate
Magnesium Sulfate
Sodium Phosphate

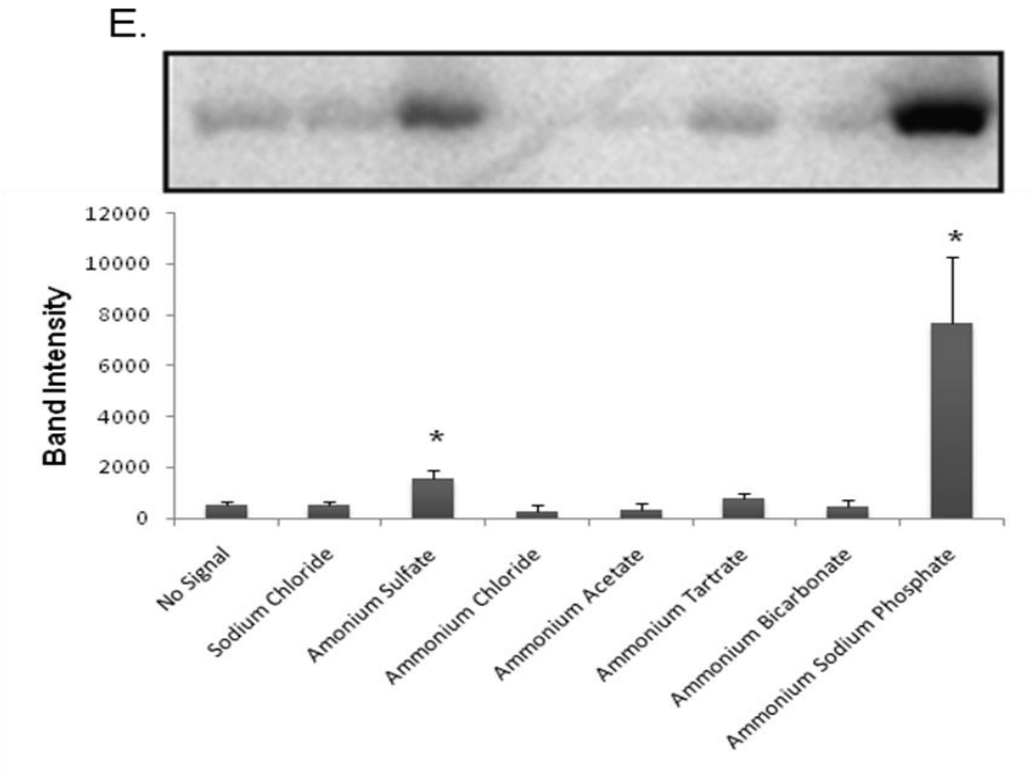

E.
Band Intensity
12000
10000
8000
6000
4000
2000
0
*
*
No Signal
Sodium Chloride
Amonium Sulfate
Ammonium Chloride
Ammonium Acetate
Ammonium Tartrate
Ammonium Bicarbonate
Ammonium Sodium Phosphate

Figure 5.6 – QseE autophosphorylation in response to various agonists. QseE was inserted into liposomes. Autophosphorylation assays were conducted in the presence and absence of various chemical agonists or signals. A. Schematic of QseE's orientation in the liposome. B. QseE phosphorylation increases in reponse to nitrogen sources and epinephrine, but not in reponse to AI-3 or iron. C. QseE's response to nitrogen occurs in response to ammonium sulfate, not glutamine. D. QseE increases autophosphorylation in response to phosphate and sulfate sources. E. QseE is not a nitrogen sensor. The only nitrogen sources that were able to activate QseE were those containing phosphate or sulfate counter ion groups, ammonium sodium phosphate and ammonium sulfate. * denotes a p-value of $\leq .05$ and ** denotes a p-value of $\leq .005$ using a student's T-test.

QseG is required for pedestal formation by EHEC.

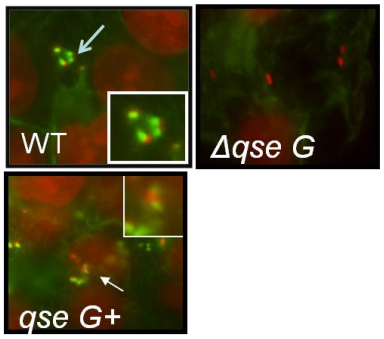
In a previous study, we showed that QseF is required for EHEC to make pedestals on epithelial cells due to its indirect regulation of *espFu* (221). In order to investigate whether QseG is involved in the same process, we tested the ability of the Δ*qseG* mutant strain to make pedestals using the fluorescence actin staining test (148). Here, actin was stained with FITC-labeled phalloidin and the bacteria and He-La cells nuclei were stained with propidium iodide and pedestals were visualized as brilliant patches of green underneath a red bacterium. As shown in figure 5.7A, the *qseG* mutant is unable to form pedestals. This defect in pedestal formation is restored upon complementing the mutant with *qseG* expressed from a pBadMycHis vector. As is seen with QseF, Δ*qseG* is still able to produce and secrete EspA ,EspB (Fig. 5.7B), and Tir (data not shown). This mutant is also still able to express intimin (data not shown). To investigate whether QseG activates *espFu* transcription in a similar fashion to QseF, we used an *espFu*::*lacZ*

fusion to test the activation of *espFu* in the *qseG* mutant. We found that in contrast to Δ*qseF*, there was no significant lack of *espFu* activation (Fig. 5.7 C). Since the Δ*qseG* mutant is able to produce and secrete each one of the components necessary for assembly of the type three secretion system and its effectors (Fig. 5.7B, C, and data not shown), we investigated whether the secretion apparatus was properly assembled and whether effectors, while secreted into the media, were properly translocated into epithelial cells.

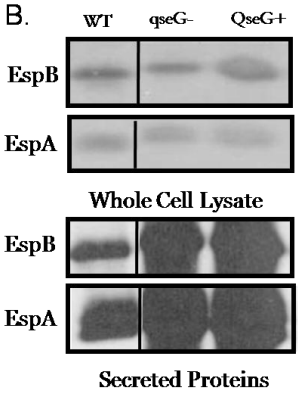
In order to visualize the type three secretion system in the Δ*qseG* strain, we utilized immunofluoresence. After infecting He-La cells with WT and Δ*qseG* EHEC expressing GFP, we stained the filament of the type three secretion system using an EspA primary antibody and a TRITC-conjugated secondary antibody. Preliminary data show that after three hours, as seen in Figure 5.7D, wild type GFP fluorescing cells are shown with the EspA filament protruding from the cell. In the Δ*qseG* mutant strain, however, although EspA filaments could be localized on He-La cells, none were seen associated with bacteria. This indicates that the Δ*qseG* mutant strain has a defect in the translocation of the type three secretion machinery. To further investigate this possibility, we utilized the *cyaA* gene reporter system developed by Sory and Cornelis (1994). This system utilizes a fusion of a type three secreted effector and the calmodulin-dependent adenylate cylcase domain (*cyaA*) of *B. pertussis* cytolysin. This gene relies on calmodulin for activation, which is present in eukaryotic cells, but not prokaryotic cells (306).

Therefore, cAMP from this system will only be produced if the fusion is translocated into host cells rather than simply secreted into the media. This system has been successfully used to measure translocation of effector molecules in numerous studies (48, 177, 179, 305). We used the *tir-cyaA* gene fusion (a kind gift from James Kaper) to monitor the translocation of Tir into host cells in WT EHEC versus Δ*qseG* EHEC. Wild type levels of cAMP were set at 100% and each other category was expressed as a percentage of wild type levels. As shown in Figure 5.7E, Δ*qseG* shows no translocation of Tir. Translocation of Tir is restored when *qseG* in pBadMycHis is introduced to Δ*qseG*. The complemented strain translocates Tir 3.5-fold higher than the WT strain, suggesting that overexpression of qseG enhances Tir translocation. These data provide evidence that Δ*qseG* has a defect in type three secretion system translocation. In order to further investigate the role of QseG in type three secretion, we performed real-time RT-PCR analysis of representative LEE genes in a *qseG* mutant background. As shown in Figure 5.7F, expression of *LEE1* (operon encoding the Ler master regulator of the *LEE* genes), *LEE4* (encodes the EspA filament), and *LEE 5* (encodes intimin and tir) is not altered in the *qseG* mutant. These results suggest that QseG does not transcriptionally regulate the *LEE* or *espFu* genes necessary for pedestal formation. Instead, QseG may aid in type three secretion system assembly and consequently translocation of effectors to mammalian cells.

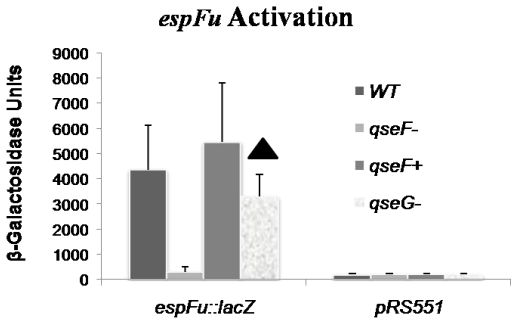
A.


WT
Δqse G
qse G+

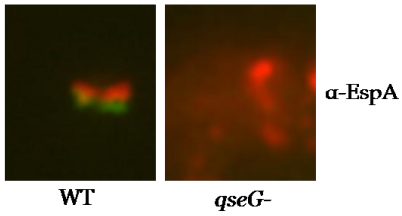
B.


WT
qseG-
QseG+
EspB
EspA
Whole Cell Lysate
EspB
EspA
Secreted Proteins

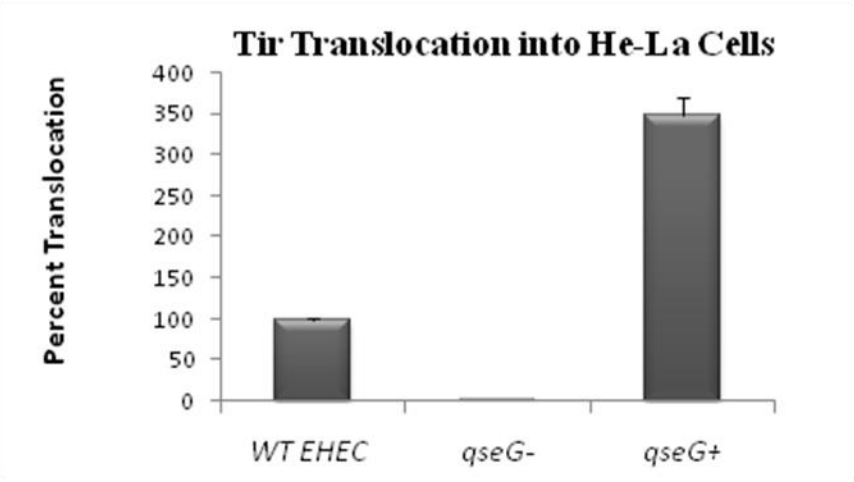
C.


espFu Activation
β-Galactosidase Units
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
WT
qseF-
qseF+
qseG-
espFu::lacZ
pRS551

D.


α-EspA
WT
qseG-

E.


Tir Translocation into He-La Cells
Percent Translocation
400
350
300
250
200
150
100
50
0
WT EHEC
qseG-
qseG+

F.

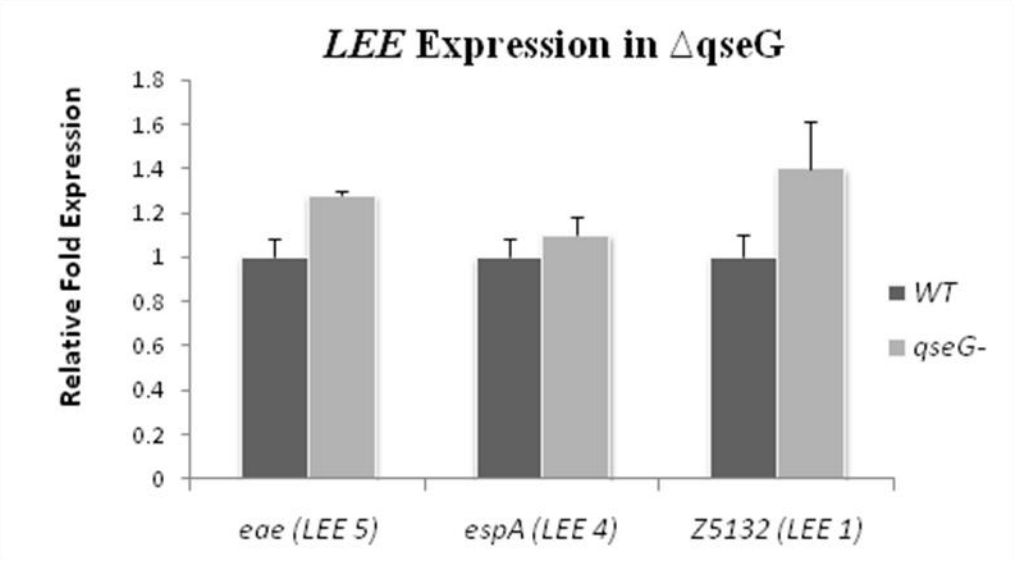

LEE Expression in ΔqseG
Relative Fold Expression
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
eae (LEE 5)
espA (LEE 4)
Z5132 (LEE 1)
WT
qseG-

Figure 5.7 – q*seG* is required for pedestal formation by EHEC. A. Detection of pedestal formation by FAS assay. Green shows the He-La cell actin and red shows the He-La cell nuclei and bacteria. WT EHEC is able to form pedestals while the *qseG* mutant is abrogated for pedestal formation. B. Western blots were performed on whole cell lysates and secreted protein preparations from WT, Δ*qseG*, and *qseG+* strains. Production and secretion where applicable of proteins involved in AE lesion formation were not defective in the mutant strain. C. *espFu* activation determined by a *espFu*::*lacZ* fusion. In a *qseG* mutant, *espFu* activation is unaltered. D. Detection of the EspA filament of the type three secretion apparatus using immune-fluorescence. Wild type shows EspA protruding from GFP expressing bacteria. The *qseG* mutant strain shows the presence of EspA, but not associated with bacteria. E. *tir*::*cyaA* fusions were used to assess translocation of Tir by Δ*qseG*. WT levels of translocation were set at 100%. The *qseG* mutant strain is unable to translocate Tir. This defect is corrected upon addition of *qseG* expressed in pBadMycHis into the mutant strain. F. Expression of the *LEE* genes using real-time RT-PCR. *LEE1*, *LEE4*, and *LEE5* are unaltered in the *qseG* mutant. Δ denotes a p-value of ≥ 0.05 using a student's t-test.

Discussion

Two-component signaling systems are major prokaryotic players in the recognition and transduction of environmental signals in order to most efficiently and effectively adapt to certain niches. These systems have been shown to be crucial in bacterial pathogenesis in both Gram positive and Gram negative organisms. In this study, we report a classical sensor kinase and response regulator system that involves a third protein, named QseG. The outer membrane protein, QseG, is the third member of the recently reported two-component system QseEF (221). QseG is shown to be important to form AE lesions, with a *qseG* mutant unable to form pedestals. However, unlike QseF,

QseG does not play a role in *espFu* expression. The inability of Δ*qseG* to form pedestals can be attributed to its inability to translocate the effector Tir and effectively assemble the EspA filament, which constitutes the translocon of the TTSS, and is required for the translocation of effector proteins to epithelial cells (150).

Our autoregulatory studies highlight the complexity of this operon by revealing several levels of autoregulation, both positive and negative. Through microarray analysis, we confirmed that many genes are equally affected transcriptionally compared to wild type by knocking out *qseE, qseF,* and *qseG* (Tables 3 and 4, Fig. 5.3 and 5.4). This is seen in complete sets of genes including many metabolism genes. However, within these data sets, independent regulation of some genes was also seen by each of the three proteins. When examining this regulation further, we were unable to detect any significant growth defect phenotype in numerous growth mediums in any of the mutants. However, due to the sweeping affects seen in transcription of the genes involved in iron regulation and nitrogen regulation, we cannot rule out a role for QseEFG in metabolism. Another possibility might be that these genes are involved in a global stress response rather than the metabolism of one specific nutrient.

While the genetics of many two-component systems have been characterized, very few physiological signals that sensor kinases recognize are known. We attempted to discover the signals that QseE responds to in order to

activate QseF and regulate AE lesion formation. It has been shown genetically that QseE and QseF are members of EHEC's epinephrine, norephinephrine, and AI-3 virulence signaling cascade as they are regulated by both QseA and QseBC (221). We have shown that QseE does increase autophosphorylation significantly in the presence of epinephrine, but not AI-3. This is logical considering QseEF play a role in pedestal formation, but not in flagellation or motility. EHEC is known to have a very low infectious dose, so it is hypothesized the AI-3 that EHEC responds to upon entering the intestine is produced by the normal flora, which resides in the intestinal lumen. In the lumen, it would be advantageous for EHEC to express flagella associated with motility, and not genes associated with AE lesion formation. Epinephrine is a systemic stress signal and norepinephrine is released by adrenergic neurons present in the enteric nervous system (88, 217). The presence of these compounds in the intestine suggests that they are in high concentration close to the epithelial layer, the same location where EHEC begins the process of attaching and effacing. Although there are many parallels between QseE and QseC, the sensor kinase that activates flagella and motility, QseE appears to be downstream in the signaling cascade from QseC (221) ; David Hughes unpublished data), and these two molecules most likely function coordinately.

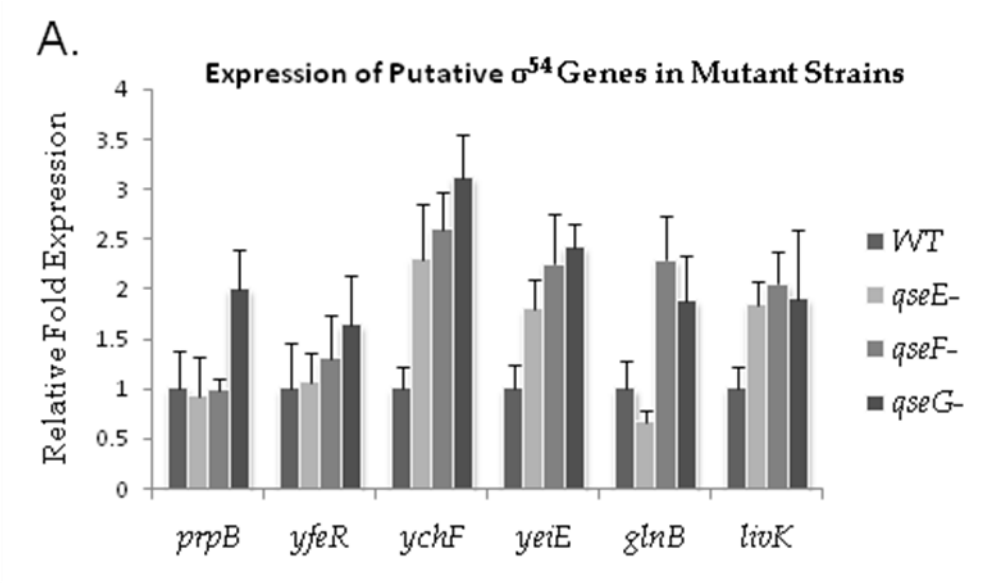
Most interestingly, QseE has a strong response to both phosphate and sulfate sources. Little is known about phosphate and sulfate levels in the intestine

and how EHEC's sensing of phosphate and sulfate might be involved in virulence. QseE's phosphate and sulfate sensing ability may play a role in allowing the bacterium to out-compete the overwhelmingly large population of microorganisms in the gastrointestinal tract in gaining nutrients, therefore, allowing EHEC to be more successful in localizing closely to the intestinal epithelium.

While we have shown that QseE responds to epinephrine, phosphate and sulfate sources, the search for cognate signals that activate QseE has not been exhausted. As mentioned before, several two-component systems are involved in cross-talk between systems, possibly enabling QseE to recognize several environmental cues. We have shown that the genes encoding numerous two-component systems are up and down regulated (Fig. 5.5) in the *qseE, qseF*, and *qseG* mutants. *rscB* and *rscC* are upregulated in the *qseE, qseF*, and *qseG* mutants. The two-component system encoded by these genes is involved in modulation of the *LEE* gene expression (280), possibly creating a link between QseEFG's regulation of *espFu* and the *LEE* region in AE lesion formation.

During this study, we also sought to identify intermediate factors that might link *espFu* activation to QseF. QseF contains a well conserved σ^{54} activator domain suggesting that its target is a gene containing a σ^{54} dependent promoter. To this end, we looked for regulation of genes predicted to contain σ^{54} dependent promoters and found that many σ^{54} dependent genes are under QseEFG control

(Fig. 5.8). However, their role in activation of *espFu* still remains to be determined. An alternative is that QseF regulates small RNAs that in turn act as transcriptional regulators.



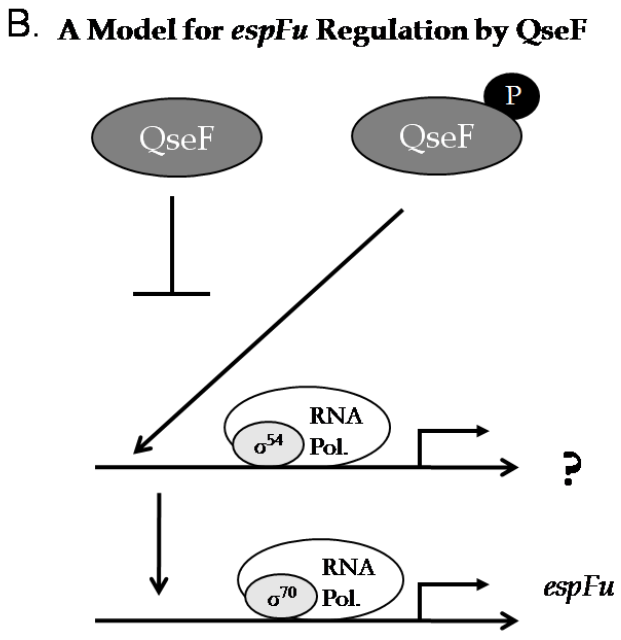


Figure 5.8 – Intermediate targets of QseF regulation. A. Real-time RT-PCR analysis of genes containing predicted σ^{54} RNA polymerase binding sites in the *qseE, qseF,* and *qseG* mutant strains. Up-regulation of *ychF, yeiE,* and *livK* is seen in all three mutant strains. In addition, *prpB* is two-fold up-regulated in the *qseG* mutant and *glnB* is upregulated in the *qseF* and *qseG* mutants. No lack of activation is seen among the predicted σ^{54} promoter containing genes in any of the mutant strains. B. A model for QseF regulation of *espFu*. QseF binds to the promoter region of a gene containing a σ^{54} dependent promoter, which in turn activates the σ^{70} promoter driven gene, *espFu*.

This study takes the first steps to characterize QseEFG, a new twist on well-conserved two-component signaling systems. The data presented indicate that the regulation and function of the QseEFG system is highly complex and much work is still needed to elucidate its mechanism within EHEC. Since QseEFG are all present in non-pathogenic strains of *E. coli* as well as pathogenic, it is likely that they have two distinct roles in non-pathogenic and pathogenic strains. It is possible that while QseEFG are involved in AE lesion formation in EHEC, their role in nonpathogenic *E. coli* involves metabolism or stress response. These roles could coincide if they involve sensing environmental cues in order to allow EHEC to inhabit the intestine. The ability to elucidate how commensal *E. coli* genes have been diverted to also play a role in regulation of virulence genes in EHEC will play an important role in the understanding of pathogenic *E. coli*. This is increasingly important as two-component systems are evaluated as drug targets.

CHAPTER SIX

CONCLUSIONS, PERSPECTIVES, AND FUTURE DIRECTIONS

Once EHEC successfully reaches the intestine, many events must simultaneously occur in order for the bacterium to reach the proximity of the intestinal epithelium and form attaching and effacing lesions to successfully colonize the host. Flagella and motility are crucial for the bacteria to swim across the mucus layer. Cell-to-cell and environmental signaling must play a key role, not only in prompting EHEC to produce flagella, but also in targeting the bacteria to the intestinal epithelium and in initiating the production of a type three secretion system once the bacterial cells are situated near the intestinal lining. All of this must be accomplished all the while competing for nutrients, space, and resources with approximately 10^8 bacterial cells and hundreds of bacterial species that inhabit the intestine. Genetic and biochemical work in the Sperandio laboratory has shown that EHEC activates many of its virulence genes in response to the signals AI-3 from the intestinal flora and epinephrine and norepinphrine, produced by the host, which are hormones present in the intestine (39, 257).

In prokaryotes, the most common systems of signal transduction employ histidine sensor kinases and their cognate response regulators. Thus far, in EHEC, the transduction of AI-3, epinephrine, and norepinephrine has been reported to occur primarily through the two-component system QseBC (39).

Although QseBC is a well-characterized system, the environmental signaling seen in the intestine and in EHEC pathogenesis is only recently starting to be understood. A study by Sperandio, *et al.* in 2001 identified 16 additional regulators that might respond to quorum sensing signals (255). The work in this thesis sought to characterize one such regulator, QseE, and its two counterpart proteins, QseF and QseG and their putative role in cell-to-cell signaling and EHEC pathogenesis.

By constructing isogenic mutations in each of these genes, we were able to show that they are involved in EHEC's ability to form AE lesions on intestinal epithelial cells. Through the use of microarray analysis, we showed that these genes have similar global effects on the transcriptome of EHEC, but that the same studies combined with genetic and biochemical studies indicate that QseE, QseF, and QseG can also each function independently. This is seen especially in the *qseF* and *qseG* mutants, neither of which is able to form pedestals on epithelial cells. However, Δ*qseF*'s defect in pedestal formation occurs because in this mutant the effector *espFu* is not expressed, while the defect in Δ*qseG* appears to involve the correct assembly of the type three secretion system. Δ*qseE* is able to form pedestals, but this may be due to redundancy. Moreover, QseE does seem to play some role in pedestal formation given that overexpression of *qseE* from a plasmid to complement Δ*qseE* results in an over-abundance of pedestal formation on epithelial cells (Figure 4.4).

We have shown in Chapter 4 that the gene that encodes the effector protein EspFu is activated by QseF and that activation is indirect. In Chapter 5, we began the search intermediates in this pathway. The main targets that were investigated were all transcription factors predicted to contain σ^{54} dependent promoter regions. The regulation of these genes was tested in the *qseE, qseF,* and *qseG* mutant strains using real-time RT-PCR. While many of these genes are regulated by QseEFG, their role in *espFu* activation must still be tested. An additional approach to real time RT-PCR can be used, which would identify regulators immediately upstream of *espFu*. Recently, a bank of mutant strains from K-12 *E.coli* (*E. coli* Keio library by Open Biosystems) was published that contains a knock-out for every non-essential gene in K-12 *E. coli*. Our existing *espFu*::*lacZ* fusion construct can be utilized to test the activation of *espFu* in the mutant background for each of the genes that contain a σ^{54} promoter consensus sequence. Using this two-pronged approach, identification of intermediary targets in the QseF-*espFu* pathway will be more likely. Moreover, CHIP (Chromatin Immunoprecipiation) assays could also be performed to further pull out targets directly bound by QseF.

The possibility exists that QseF may regulate expression of small RNAs rather than a transcription factor. Precedence for small RNA regulation of transcription through two-component regulation has been shown (159). The very large 5' untranslated region upstream of *espFu* supports the theory of small RNA

regulation. In the *qseF* mutant array analysis, numerous characterized small RNAs as well as intergenic regions, which could constitute non-characterized small RNAs, were differentially regulated. This further indicates that this pathway may involve RNA regulation. We have scanned the intergenic regions that were differentially regulated in the *qseF* mutant versus WT EHEC array for the σ^{54} promoter consensus sequence, unearthing more potential candidates for regulation by QseF. Finally, a mutant strain of the small RNA chaperone, *hfq* has recently been constructed. Activation of *espFu* in this mutant strain should be tested to further indicate whether small RNA regulation is involved in the QseF pathway.

Using liposomes containing QseE, we have tested the ability of many environmental signals to stimulate QseE's ability to autophosphorylate *in vitro*. This portion of the study aimed to identify the cognate signal, which activates QseE, and we were able to show that epinephrine, phosphate, and sulfate sources activate QseE and ultimately AI-3 and nitrogen sources do not. These results have been somewhat surprising. Given QseEFG's role in EHEC's signaling cascade, a response to epinephrine is not surprising, but the observation that QseE discriminates between epinephrine and AI-3 is exciting. QseE may respond to epinephrine and not AI-3 due to its role in AE lesion formation that occurs near the intestinal epithelium rather than in the lumen where AI-3 producing micro-organisms reside. QseE's response to phosphate and sulfate sources is intriguing,

however. In order to follow up the *in vitro* studies showing QseE's response to phosphate sources, genetic studies should be performed examining the response of EHEC to phosphate and or sulfate abundant and starved conditions in the *qseE, qseF,* and *qseG* mutants. These studies may provide some clues about the significance of phosphate and sulfate sensing by this system. Phosphate levels have been shown to be involved in the synthesis of antibiotics and other secondary metabolites, but the mechanism behind this effect is unknown (36, 250). In *E. coli*, the two-component system PhoP PhoR regulates alkaline phosphatase and phosphate transport genes under phosphate limitation and investigation as to whether synthesis of antibiotics and secondary metabolites is also controlled by PhoP PhoR is under investigation (113, 214, 250). Given that cross-talk and synergy exists among two-component systems, it is possible that QseE may be linked to these processes through PhoP PhoR.

Cross-phosphorylation of two-component systems has been reported in the literature. Although this cross-phosphorylation occurs with reasonable frequency, Yamamoto, *et al*. reported only 22 cross-phosphorylation reactions out of 692 combinations (310). While this study was able to investigate non-cognate signaling by the QseEF system, the authors were unable to purify QseB and QseC. Since both QseBC and QseEF are involved in EHEC pathogenesis, both play a role in AE lesion formation, and both respond to the signal epinephrine, understanding the hierarchy of signal transduction between these two systems

might further elucidate EHEC's strategy of corralling cell-to-cell signaling to its advantage in infection. Given that neither Δ*qseE* nor Δ*qseF* have a defect in motility or flagellation, it is unlikey that QseE phosphorylates QseB. In Yamamoto's study, while QseF was shown to be a promiscuous response regulator, which is phosphorylated by at least four other sensor kinases aside from its cognate sensor QseE, QseE only phosphorylated its cognate response regulator, QseF. Consequently, the hypothesis that QseC may cross-phosphorylate QseF remains to be tested.

Finally, as shown by real-time PCR analysis, in Fig. 5.5, QseEFG affects the regulation of many two-component systems indicating that QseEFG communicates with other two-component regulatory systems at the transcriptional level. Autoregulation is often seen in two-component signal transduction systems, indicating that QseEFG could have a role in activating or repressing the transcription of the genes that encode these two-component systems. However, to investigate the extent of the effect of QseEFG on these systems, the transcription of each two-component system's target genes should be tested to see if they exhibit a similar effect in gene expression.

The microarrays analyses comparing gene expression by the *qseE, qseF*, and *qseG* mutants indicated that large metabolic changes occur when any of these genes, particularly, *qseE* and *qseG* are deleted. Though our efforts to find a phenotype involved in iron or nitrogen regulation were unsuccessful, we saw no

defect in any of the strains in uptake of nitrogen, and only a mild defect on iron uptake. Given the level of redundancy that exists with these systems, many phenotypes involved in iron regulation are hard to assess, so the possibility of QseEFG's role in iron regulation cannot be eliminated. Since several sets of metabolic genes are differentially regulated, including transcriptional systems, the response seen may be a global stress response rather than a specific response to either iron or nitrogen. To this end, experiments such as acid resistance tests should be performed to assess the ability of each of these mutants to respond to stress.

The structure and composition of the *qseEFGglnB* operon is very unusal and intriguing. The nature of the autoregulation we discovered in this operon leads us to believe that the complexity of this operon has not been fully uncovered. For example, *qseG* is predicted to contain a σ^{54} dependent promoter, indicating that it might have different levels of regulation than the rest of the operon. Transcriptional fusions and truncations of this promoter region should be constructed in order to evaluate its activity and regulation. Additionally, this promoter is putative (based on promscan) and should be mapped definitively by primer extension. Another intriguing finding is that *qseE* expression is upregulated three-fold in the *qseG* mutant strain. This indicates that QseG has a negative regulatory effect on *qseE*. The molecular mechanisms underlying this regulation must still be determined.

Finally, the fact that *glnB* is co-transcribed with *qseE, qseF,* and *qseG* suggests that this three component system may have a role in nitrogen metabolism. Both our efforts and those of Lu, *et al*. , however, have failed to show a role for QseE, QseF, and QseG in nitrogen regulation (164). We observe an increase in *glnB* transcription in both the *qseF* or *qseG* mutants. However, the link between NtrC/B and QseEFG through the PII protein is still elusive and will have to be investigated. Recently, a *glnB* mutant has been constructed. Characterization of this mutant may provide further insight into *glnB*'s role in this operon. A study showed that *glnB* is not always essential for the cell growth under nitrogen limiting conditions and that a close homologue of *glnB, glnK* is responsible for this redundancy. Unlike, *glnB, glnK* contains a σ^{54} dependent promoter and depends on the NtrB/NtrC two-component system for activation (288). This raises the possibility that *glnB* has a second role. Recently, preliminary data showed that *espFu* expression is decreased in the *glnB* mutant, suggesting that *glnB* may also play a role in virulence (unpublished data, Cristiano Moreira). The PII protein encoded by *glnB* controls the phosphorylation state of the two-component system NtrB and NtrC, and consequently, their regulation of downstream genes. It is possible that in the presence of the PII protein, cross-talk exists between the NtrB/NtrC and the QseEFG signaling systems. This would implicate a role for PII in virulence as well as a role for QseEFG in nitrogen metabolism.

QseG's localization to the outer membrane is surprising. Although this protein contains a signal sequence and is predicted to be a lipoprotein, utilizing BLAST analysis, QseG is shown to have homology with numerous proteins predicted to be alpha helical. Although there are some examples of alpha-helical proteins that localize to the outer membrane (46, 61), this is not common. Structural studies should be undertaken to examine the nature of QseG including whether it is truly alpha helical and whether it may act as a transporter as seen in the case of another alpha-helical outer membrane protein, Wza (61).

There are two possibilities for QseG's functions in the membrane, which are not mutually exclusive. Given that Δ*qseG* is unable to translocate Tir, it is probable that in this mutant the type three secretion system is not properly formed. Indeed, preliminary studies indicate that the EspA filament in the *qseG* mutant is compromised. These characteristics of the *qseG* mutant indicate that as an outer membrane protein, it may play a role in the events that lead to or are involved in the formation of the type three secretion machinery and translocation of effector molecules into epithelial cells. Two proteins, SepD and SepL have been shown to be involved in the switch that occurs upon the completion of the type three secretion machinery to enable the translocation of effector molecules. It is possible that QseG also plays a role in this process, especially considering that the *qseG* mutant is still able to secrete proteins involved in type three secretion. To begin investigating this process, it will be necessary to identify

proteins that QseG interacts with. These studies can begin with interaction screens, such as a bacterial two-hybrid screens or a GST-pulldown. Another possibility in this paradigm is that QseG alters the topology or stability of the bacterial outer membrane. Based on the array results, compared to WT, in the *qseG* mutant, expression of numerous outer membrane proteins and transporters was altered. This indicates that the cell might be trying to compensate for the lack of *qseG* in the outer membrane to maintain proper function. More evidence for this line of reasoning lies in the fact that the protein OmpW, an *E. coli* outer membrane protein is upregulated in both the *qseE* and *qseG* mutants (see Figure 6.1).

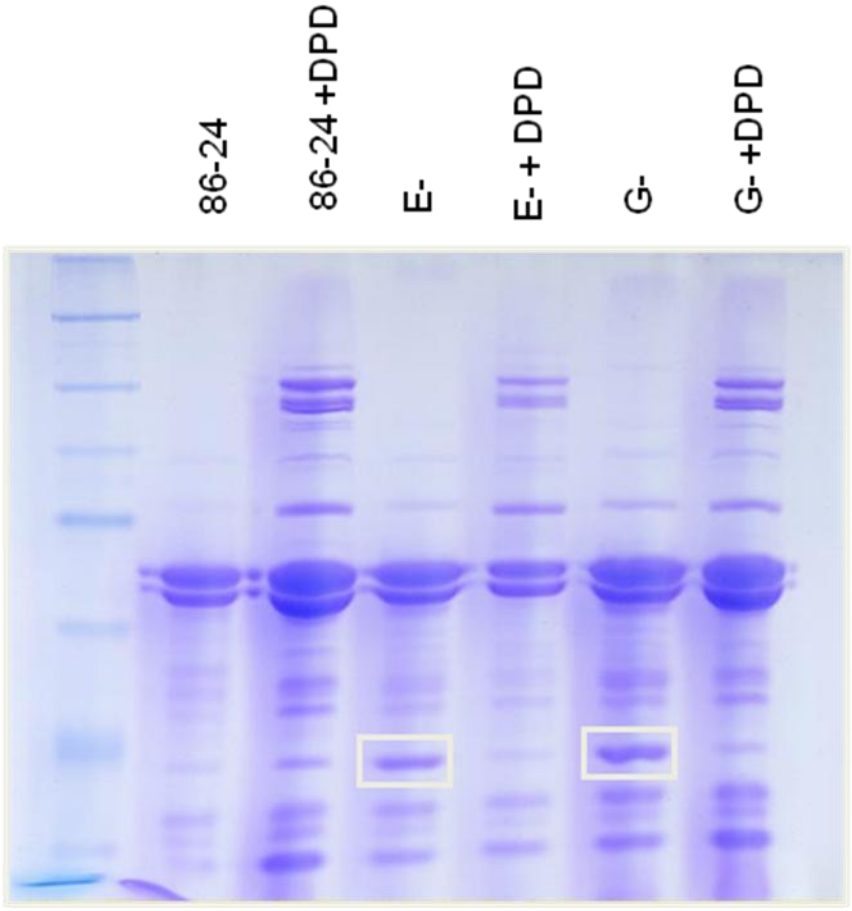


Figure 6.1 – Outer membrane preparations. Commassie stain of outer membrane preparations of WT EHEC, the *qseE* mutant, and the *qseG* mutant and all three with the addition of 350 μM dipyridyl. The boxed protein, OmpW, is overexpressed both in the *qseE* and *qseG* mutants. Figure courtesy of Alfredo Torres.

The second possibility involves QseG as a signaling protein. The existence of an outer membrane protein that recognizes or transports environmental signals into the periplasmic space would facilitate sensing by

sensor kinases. This would answer how sensor kinases located in the inner membrane can sense external signals that cannot diffuse into the cell. Initial studies investigating this possibility, however, have been inconclusive. The *qseG* mutant has no defect in swimming ability or production of flagella indicating that if QseG does act as a signaling protein in EHEC, its effects are not global. In addition, a preliminary experiment to discover whether QseG bound epinephrine using an epinephrine ELISA kit did not show a difference in epinephrine levels between the WT and the mutant strains. These experiments are preliminary, however, and this issue should be investigated in further detail.

In conclusion, through the use of both biochemical and genetic analysis, we have begun an in depth analysis of the QseEFG three-component system. We have identified this system as a new type of two-component system composed of a sensor kinase, response regulator and in addition, a membrane protein. We have shown that these proteins are involved in EHEC's ability to form AE lesions on cells and have identified a target for the regulation of QseF. In addition, we have implicated these proteins in cell metabolism and stress response. Finally, we have also identified several signals that activate QseE and have begun to show how these proteins work together as a system. This study has laid the groundwork for an in depth analysis of the mechanism for QseEFG's role in EHEC pathogenesis and metabolism.

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