THE FUNCTIONAL CHARACTERIZATION OF THE QUORUM SENSING *E. COLI* REGULATORS B AND C IN EHEC

APPROVED BY SUPERVISORY COMMITTEE

Vanessa Sperandio, Ph.D. _____

Melanie Cobb, Ph.D. _____

Eric Hansen, Ph.D. _____

Kevin McIver, Ph.D. _____

DEDICATION

To my husband Daniel Clarke.
For sharing your life with me and challenging me to be the person that you know I can become.

To my parents Frederick and Beth Cunningham, my brother Brian Cunningham,
my grandparents Cecelia Cunningham and Marjorie and Robert Gelczer,
and my second family William, Christine, Kathryn, and Douglas Clarke.
For your unwavering encouragement, support, and love.

Also in memory of my grandfather, Frederick Cunningham, Sr.

THE FUNCTIONAL CHARACTERIZATION OF THE QUORUM SENSING *E. COLI* REGULATORS B AND C IN EHEC

by

MARCIE B. CLARKE

DISSERTATION

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

August, 2005

THE FUNCTIONAL CHARACTERIZATION OF THE QUORUM SENSING *E. COLI* REGULATORS B AND C IN EHEC

Publication No. _____

Marcie B. Clarke, Ph.D.

The University of Texas Southwestern Medical Center at Dallas, 2005

Supervising Professor: Vanessa Sperandio, Ph.D.

Enterohemorrhagic *E. coli* (EHEC) is the causative agent of hemorrhagic colitis. During an infection, EHEC can sense and respond to environmental cues, including the cell density of the intestinal normal flora (through the floral-derived AI-3 signal) and the epinephrine/norepinephrine produced naturally by the host. This cell-to-cell signaling may aid in colonization and disease by allowing EHEC to up-regulate its flagella and motility genes to swim closer to the intestinal epithelium. Previously, Sperandio *et al.* (2002) have shown that the quorum sensing *E. coli* regulators B and C (QseB&C), a two-component system in EHEC, are responsible for the regulation of the master regulator of flagella and motility genes, *flhDC*, in response to cell-to-cell signaling [1].

Here, we show that QseC, the membrane-bound sensor kinase, can autophosphorylate itself in response to AI-3 or epinephrine/norepinephrine and then transfer this phosphate to the response regulator, QseB. The autophosphorylation of QseC is not affected by the addition of autoinducer-2 or intestinal hormones, including

gastrin, galanin, and secretin. Additionally, autophosphorylation can be antagonized upon the addition of phentolamine, an α-adrenergic receptor antagonist. Given that enterocytes harbor α-adrenergic receptors, it would be consistent for a microbial adrenergic sensor (QseC) to mostly resemble (in an orthologous and not a homologous fashion) an α- and not a β-adrenergic receptor. Taken together, these results suggest that QseC may be a microbial adrenergic receptor conserved amongst different bacterial and fungal species.

After QseC has autophosphorylated and transferred its phosphate to QseB, QseB acts as a transcription factor to activate the expression of *flhDC*, the master regulator of flagella and motility genes. Nested deletion analyses of the *flhDC* promoter suggest that QseB may bind to three promoter regions, to either repress or activate transcription. Further transcriptional studies suggest that phosphorylated QseB autoregulates its own transcription in a similar manner. These analyses have identified a QseB consensus binding sequence, which was utilized in an *in silico* search to identify novel potential targets of QseB. Through the use of both biochemistry and genetics, a comprehensive model of the QseB&C signaling cascade was generated.

Table of Contents

PRIOR PUBLICATIONS

Clarke, M.B. and Sperandio, V. (2005) Cell-to-cell signaling amongst microbial flora, host and pathogens: there is a whole lot of talking going on. *American Journal of Physiology - Gastrointestinal and Liver Physiology.* **288**(6):1105-1109.

Clarke, M.B. and Sperandio, V. (2005) Regulation of Flagella and Motility by Quorum Sensing *E. coli* Regulators B and C (QseBC) and FliA (σ^{28}). *Molecular Microbiology.* **In Press.**

Clarke, M.B. and Sperandio, V. (2005) Transcriptional Autoregulation by Quorum Sensing *E. coli* Regulators B and C (QseBC) in EHEC. *Molecular Microbiology.* **In Press.**

Clarke, M.B. and Sperandio, V. (2005) *Chapter 7: LuxS-dependent regulation of Escherichia coli virulence* in Quorum Sensing and Bacterial Cell-to-Cell Communication. Cambridge University Press: ed. D.R. Demuth and R. Lamont. **In Press**.

Sperandio, V., Zhu, C., Rios, H., Davis, K., Clarke, M.B., and Boedeker, E.C. (2005) Quorum Sensing *qseC* and *qseA* Rabbit Enteropathogenic *E. coli* (REPEC) Mutants have Altered Virulence in Rabbits. Manuscript in Preparation for *Infection and Immunity.*

Clarke, M.B. and Sperandio, V. (2005) QseC, A Bacterial Adrenergic Receptor. Manuscript in Preparation.

List of Figures

List of Tables and Appendices

LIST OF ABBREVIATIONS

86-24	Enterohemorrhagic *E. coli* wild-type strain 86-24
AE	Attaching and effacing
AHL	Acyl-homoserine lactone
AI	Autoinducer
Ala	Alanine
Asp	Aspartate
bp	Base pair
CDC	Centers for Disease Control and Prevention
CFU	Colony forming unit
CNS	Central nervous system
DAEC	Diffusely adherent *E. coli*
DEPC	Diethyl pyrocarbonate
DHCP	4,5-dihydroxy-2-cyclopentan-1
DMEM	Dulbecos modified Eagle's medium
DMHF	4-hydroxy-2,5-dimethyl-furanone
DNA	Deoxyribonucleic acid
dNTP	dideoxy-nucleotide triphosphate
DPD	4,5-dihydrody-2,3-pentanedione
DTT	Dithiothreitol
EAEC	Enteroaggregative *E. coli*
E. coli	*Escherichia coli*

EDTA	Ethylenediamine-tetraacetic acid
EHEC	Enterohemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EMSA	Electrophoretic mobility shift assay (gel shift)
ENS	Enteric nervous system
EPEC	Enteropathogenic *E. coli*
Epi	Epinephrine
Epi/NE	Epinephrine / norepinephrine
ETEC	Enterotoxigenic *E. coli*
GI	Gastrointestinal Tract
Glu	Glutamate
HF	Homofuraneol
His	Histidine
HK	Histidine kinase
HUS	Hemolytic Uremic Syndrome
IL	Interleukin
IPTG	β-D-thiogalactopyranoside
kDa	kiloDalton
LB	Luria-Bertani broth
LEE	Locus of Enterocyte Effacement
Ler	Lee-encoded regulator
LPS	Lipopolysaccharide
MHF	4-hydroxy-5-methyl-furanone

NE	Norepinephrine
O.D.	Optical density
PAGE	Polyacrylamide gel electrophoresis
PAI	Pathogenicity Island
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PE	Phentolamine
PNK	Polynucleotide kinase
PO	Propranolol
QS	Quorum Sensing
Qse	Quorum Sensing *E. coli* Regulator
QseB	Quorum Sensing *E. coli* Regulator B (the response regulator)
QseBC	Quorum Sensing *E. coli* Regulators B and C (the two-component system)
QseC	Quorum Sensing *E. coli* Regulator C (the sensor kinase)
REPEC	Rabbit enteropathogenic *E. coli*
RNA	Ribonucleic acid
RR	Response regulator
R-THMF	(2R,2S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran
RT-PCR	Reverse transcriptase PCR
SAM	S-adenosyl-methionine
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel
Stx	Shiga toxin

Tir	Translocated intimin receptor
TSS	Transcriptional start site
TTSS	Type III secretion system
UPEC	Uropathogenic *E. coli*
X-gal	5-bromo-4-chloro-3-indolyl β-galactopyranoside

CHAPTER ONE

LITERATURE REVIEW

TAXONOMY

The genus *Escherichia* is named after Theodor Escherich, who first isolated and characterized it in 1885. *E. coli* belongs to the Enterobacteriaceae Family, whose name is derived from the Greek worked enterikos, which pertains to the intestine. *Escherichia coli* is a gram negative bacillus, which occurs singly or in pairs. *E. coli* is the major facultative anaerobe inhabitant of the large intestine of all animals, including humans, and is one of the most common causes of everyday bacterial infections. Enterohemorrhagic *Escherichia coli,* or EHEC, are an uncommon variety of *E. coli* that produces severe damage to the intestinal epithelium (hemorrhagic colitis). As described later, EHEC was first recognized in a 1983 report by Riley *et al* [2]. The "O" in Enterohemorrhagic *E. coli* O157:H7 refers to the specific component of LPS chains, while the "H" refers to the flagellar antigen.

HISTORICAL PERSPECTIVE OF ENTEROHEMORRHAGIC *E. COLI*

Escherichia coli (*E. coli*) are the most abundant nonpathogenic facultative anaerobes found in the human intestinal microbial flora. This commensal organism resides in the mucus layer of the mammalian colon and typically colonizes the gastrointestinal tract of humans within a few hours of birth. However, there are several clones of *E. coli* that have acquired virulence traits that allow them to cause a broad spectrum of disease, even in healthy human hosts. These virulence traits are usually encoded within mobile genetic elements, such as plasmids and pathogenicity islands, which have evolved to be stable within these clones. There are six well-described categories among the intestinal pathogens: enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), and diffusely adherent *E. coli* (DAEC) [3]. This dissertation research focuses on EHEC, an emerging pathogen of worldwide public health importance.

Enterohemorrhagic *E. coli* (EHEC) O157:H7 is a motile, gram-negative bacillus that is responsible for major outbreaks of bloody diarrhea and hemolytic uremic syndrome (HUS) throughout the world. Each year, EHEC causes an estimated 73,000 illnesses, 2,000 hospitalizations, and 69 deaths in the United States alone. One of the major contributing factors to EHEC outbreaks is its very low infectious dose, estimated to be as few as 50 colony forming units (cfu). Treatment and intervention strategies for EHEC infections are still controversial, with conventional antibiotics usually having little clinical effect and possibly even being harmful (by increasing the chances of patients

developing hemolytic uremic syndrome [HUS]) [4, 5]. HUS is characterized by hemolytic anemia, thrombocytopenia, and renal injury and contributes to the morbidity and mortality observed in the young and elderly [6].

The recognition of EHEC as an important pathogen is a fairly recent occurrence, with the first report of a major outbreak being published by Riley *et al.* in 1983 [2]. The authors reported that at least 47 people came down with an illness characterized by bloody diarrhea and little or no fever after eating undercooked hamburger meat at a chain of fast-food restaurants. Stool cultures from these patients revealed a previously rare isolate of *E. coli*, O157:H7. Almost simultaneously, Karmali *et al.* [7] published a report that associated cases of hemolytic uremic syndrome (HUS) with the presence of cytotoxin-producing *E. coli* in stool samples. Taken together, these two scientific observations were the first recognition of an emerging class of an enteric pathogen that was responsible for causing gastrointestinal and renal disease.

EPIDEMIOLOGY

Escherichia coli O157:H7 annually causes approximately 73,000 illnesses, leading to approximately 2,100 hospitalizations and 61 deaths in the United States alone [8]. Although recognized as a pathogen in 1983, it was not until a large multi-state outbreak linked to undercooked hamburger patties from a fast-food restaurant chain occurred in 1993 that EHEC became widely recognized as a threatening emerging pathogen [9]. In 1994, the Centers for Disease Control and Prevention (CDC)

determined EHEC a nationally notifiable infection, and by 2000, reporting was mandatory in the continental United States [9]. Figure 1.1 represents the typical yearly incidence of reported EHEC infections in the United States (Figure courtesy of CDC, 1997), with most cases occurring during the summer months from May through November. Of the reported outbreaks between 1982 and 2002, Minnesota reported the most outbreaks (43), followed by Washington, New York, California, and Oregon [8].

Out of all cases reported to the CDC, transmission routes for most EHEC outbreaks (52%) have been food-borne [8]. The first EHEC outbreak, reported in 1982, and the outbreak from a fast-food restaurant chain that garnered national attention in 1993, were both linked to undercooked ground beef, which remains the most common food-borne vehicle [8]. However, produce-associated outbreaks (lettuce, apple juice, salad, coleslaw, melons, sprouts and grapes) have also been a prominent food vehicle, accounting for 34% of food-borne illnesses [8]. Recently, EHEC infection has been receiving media attention due to several outbreaks resulting from animal contact at petting zoos, farms, or county fairs. First reported in 1996, outbreaks due to animal contact are one of the newest routes of EHEC transmission. As recently as April of 2005, twenty-six persons at a central Florida state fair petting zoo were infected in an outbreak of EHEC. The remaining outbreaks have been unknown (21%), person-to-person transmission (14%), water (9%), and laboratory related (0.3%) [8].

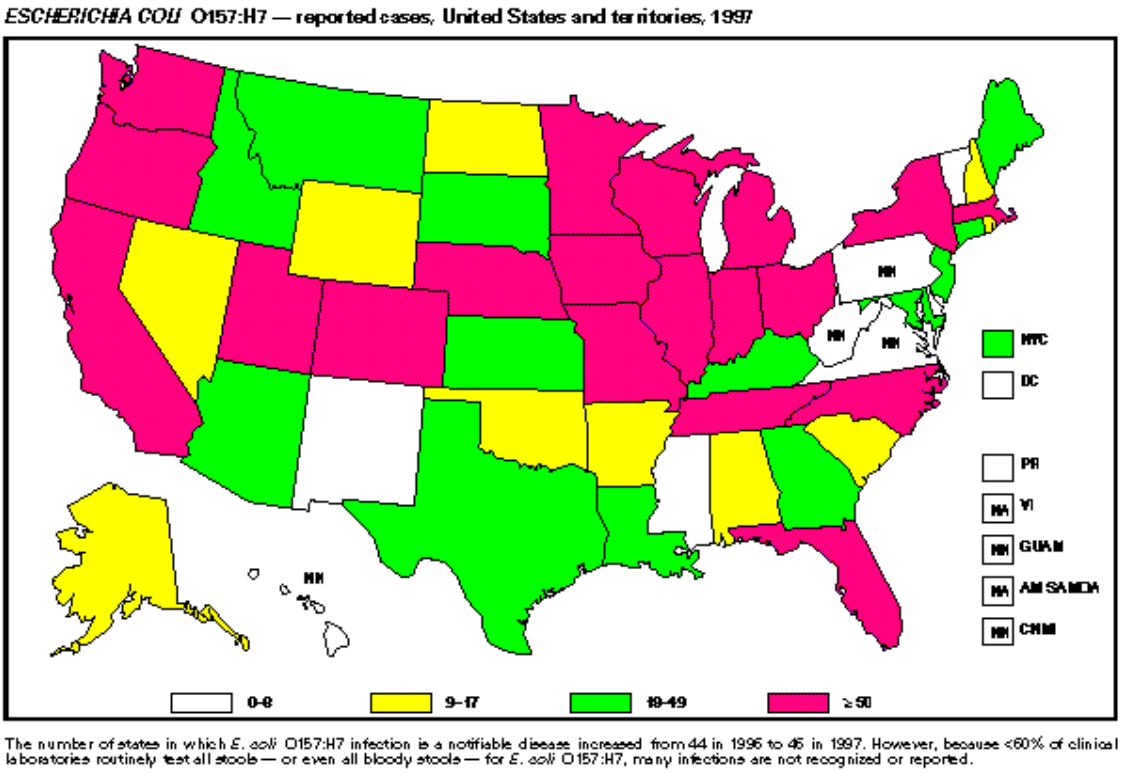


Figure 1.1 – Epidemiology of EHEC infections in the United States (1997). Figure courtesy of Centers for Disease Control & Prevention.

Among the reported cases of EHEC infection, a total of 354 cases (4% of all reported infections) of hemolytic uremic syndrome have also been reported [9]. Between 1982 and 2002, forty reported deaths occurred due to EHEC infection, with approximately 63% of deaths occurring in persons with HUS. Notably, the reported age ranges at death were all 1-4 years and 61-91 years, indicating EHEC infection may play a large role in the morbidity and mortality of the very young and the elderly. The fatality rate did not appear to vary by transmission route [9].

Investigations into the 1993 EHEC outbreak resulting from undercooked hamburgers have led to major improvements in regulation of the fast-food industry [10]. Additionally, trace-back investigations in recent years have led to the identification of

contaminated lots of beef, leading to large recalls and the prevention of many human infections [11]. Outbreaks associated with animal contact, however, represent a new transmission route for EHEC infections in the United States. Recent strategies have been published to help reduce transmission from animals, including the addition of adequate hand washing facilities [12]. Taken together, these changes have led to a small reduction in EHEC outbreak size since 1982 [8]; however, EHEC remains an important emerging pathogen in the United States.

CLINICAL PRESENTATION, DIAGNOSIS, AND TREATMENT

Symptoms of enterohemorrhagic *E. coli* infection begin between two and twelve days after exposure to the pathogen, with a mean incubation period of three days [2]. Normally, EHEC infections cause one or two days of watery diarrhea followed by several days of bloody diarrhea [13]. Figure 1.2 shows the typical progression of EHEC infection. Approximately ten percent of patients develop hemolytic uremic syndrome, which is characterized by hemolytic anemia, thrombocytopenia, and renal injury [8].

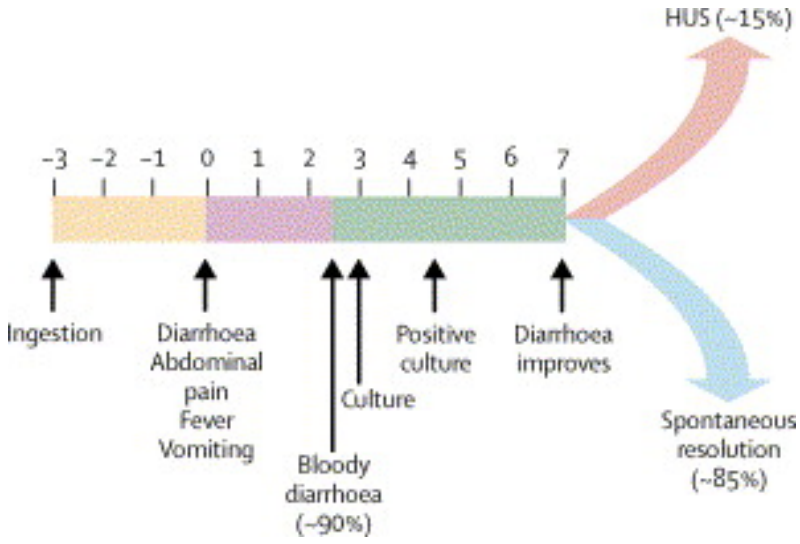


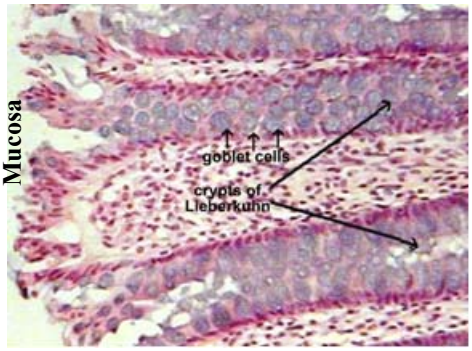
Figure 1.2 – Progression of EHEC infection in days [13].

Diagnosis of EHEC infection usually occurs during the phase of bloody diarrhea. The patient typically presents with severe abdominal pain, bloody stool, and infrequent vomiting. Treatment has several purposes: (i) to decrease severity and duration of symptoms, (ii) to prevent complications such as HUS, and (iii) to prevent further transmission [14]. The current focus of treatment is to alleviate specific diarrheal symptoms through the use of antidiarrheal agents and fluid and electrolyte replacement. Antibiotic treatment is currently fairly controversial, as several studies have presented conflicting results about their efficacy. A study from Washington State [15] reported that treatment of EHEC infection with antibiotics had no benefit and did not prevent the development of HUS. In a Canadian study [16], however, antimicrobial therapy was implicated in the high rate of complications, including the development of HUS. Therefore, the use of antibiotics to treat EHEC infection is currently discouraged.

HISTOPATHOLOGY

The overall intestinal histopathology due to EHEC infection includes hemorrhage and edema in the lamina propria [17]. Many patients show focal necrosis and the infiltration of neutrophils and inflammatory cells. In severe cases, intestinal damage is severe enough to result in severe gastrointestinal complications, including colonic perforation and rectal prolapse [17]. Figure 1.3A shows a cross section of the mucosa of the large intestine, which contains goblet cells responsible for mucus secretion.

Classically, the histopathology of EHEC infection has been referred to as the attaching and effacing (AE) lesion. These lesions are characterized by effacement of the intestinal epithelial microvilli and intimate adherence between each bacterium and the epithelial cell membrane. Beneath the bacterium is the accumulation of cellular actin, which forms a pedestal-like structure (Figure 1.3B), and the accumulation of other cytoskeletal components [18, 19]. The cellular response to the AE lesions formed during EHEC infection may lead to an increase in the intracellular level of calcium, which leads to the secretion of chloride, resulting in diarrhea [20].

A. 

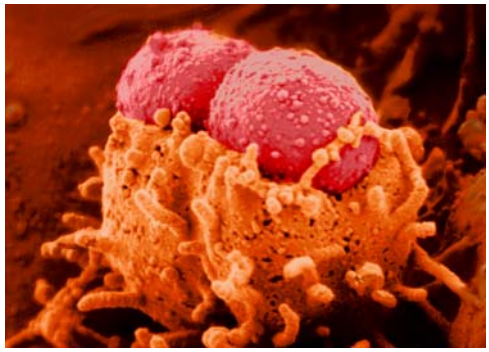
B. 

Figure 1.3 – Intestinal Structure and AE lesions. A.) Cross section of the large intestine mucosa. B.) Two individual EHEC bacteria sit in their pedestal-like structure on the intestinal epithelium.

IMMUNE INVOLVEMENT

Very little is known about the immune response during EHEC infection. To date, most immunological studies have been performed using EPEC, as there is no good animal model for EHEC pathogenesis. It is known in EPEC, however, that bacterial attachment to epithelial cells causes the recruitment of PMNs to the epithelial monolayer, and that this migration could be blocked by the addition of neutralizing antibodies to interleukin-8 (IL-8) [21]. Increased levels of IL-8 have also been found in an EHEC rabbit model [22]. This model was also used to show a large infiltration of PMNs during infection, which could be inhibited by an antibody to CD18, a leukocyte adhesion molecule [22].

A recent study has utilized human patient sera in order to determine the role of the adaptive immune response during EHEC infection [23]. Data from this study indicate that there is a strong antibody response to the translocated intimin receptor (Tir), suggesting that this is a potential candidate for vaccine development against EHEC infection.

VACCINOLOGY

One approach to the control of EHEC disease in humans has been the vaccination of cattle, the main reservoir of infection. This would allow an attack on the non-human reservoir of infection (contaminated beef) and avoid patient compliance issues. Several studies have focused on the vaccination of cattle with intimin, a protein involved in the

attachment of EHEC to host epithelial cells [24]. There are several cons to this approach, however, including cost and the sheer size of the bovine population. Therefore, several methods of active human vaccination in at-risk populations are currently being considered. These methods include the production of conjugate vaccines against Stx, which have proved highly successful in the prevention of disease caused by *Haemophilus influenzae* type B [25], toxin-based vaccines, and live-vector vaccines [14]. In general, however, the prevention of EHEC infection may depend on strategies to improve food safety through sanitation of food and water supplies.

GENETIC CONTENT

The EHEC O157:H7 Sakai strain genome [26] and the EDL933 genome [27] were recently sequenced in 2000 and 2001, respectively. EHEC has an average genome size of 5.5 Mbp containing 5361 predicted open reading frames. Compared to nonpathogenic *E. coli* K-12 strains, EHEC has also lost 0.53Mbp of DNA. However, EHEC has gained 1.34 Mbp of DNA, which is not present in nonpathogenic *E. coli* K-12 strains. This additional sequence encodes several factors that contribute to the virulence of EHEC, including potential adhesins, an iron uptake system, a type III secretion system, and Shiga toxin. EHEC has also been shown to be immune to λ phages due to the high concentration of phage naturally present in its genome.

ANIMAL MODELS

The discovery of novel virulence factors in EHEC has been hampered by the lack of a relevant animal model. Streptomycin-treated mice can be colonized by EHEC, although it does not cause attaching and effacing lesions and its toxicity is solely due to Shiga toxin (Stx), as these results could be reproduced using an *E. coli* K-12 strain carrying cloned *stx* genes [28, 29]. Similarly, streptomycin-treated ferrets were also evaluated as a possible animal model for EHEC infection [30]. These animals developed hematuria and/or histological damage of glomeruli or thombocytopenia, but there was no evidence of colitis or AE lesion formation in the intestinal epithelium. Therefore, the ferret may serve as a model for renal disease secondary to intestinal infection with EHEC. EHEC is able to cause the formation of AE lesions in large animals, such as suckling neonatal piglets [31] and neonatal calves [32]. However, these large animals are expensive and difficult to manage for large-scale screening of potential virulence genes. Recently, an infant rabbit model of EHEC infection has been utilized to study genetically defined mutants in EHEC *in vivo* [33].

As an alternative to EHEC animal models, some have utilized natural animal disease models, such as rabbits infected with rabbit enteropathogenic *E. coli* (REPEC) [34-37], which causes AE lesions in the rabbit intestine and possesses the same virulence factors as human EHEC and EPEC strains [38-40].

VIRULENCE FACTORS OF EHEC

It is thought that EHEC uses its flagella to swim through the large intestine during infection, as a *fliA* mutant in EHEC, which is deficient in flagellin production, was shown to have attenuated virulence in cattle [41]. Once EHEC reaches the intestinal epithelium, it causes the development of attaching and effacing (AE) lesions (Figure 1.4). The AE lesion is characterized by the destruction of the microvilli and the rearrangement of the cytoskeleton to form a pedestal-like structure, which cups each bacterium individually [18, 19].

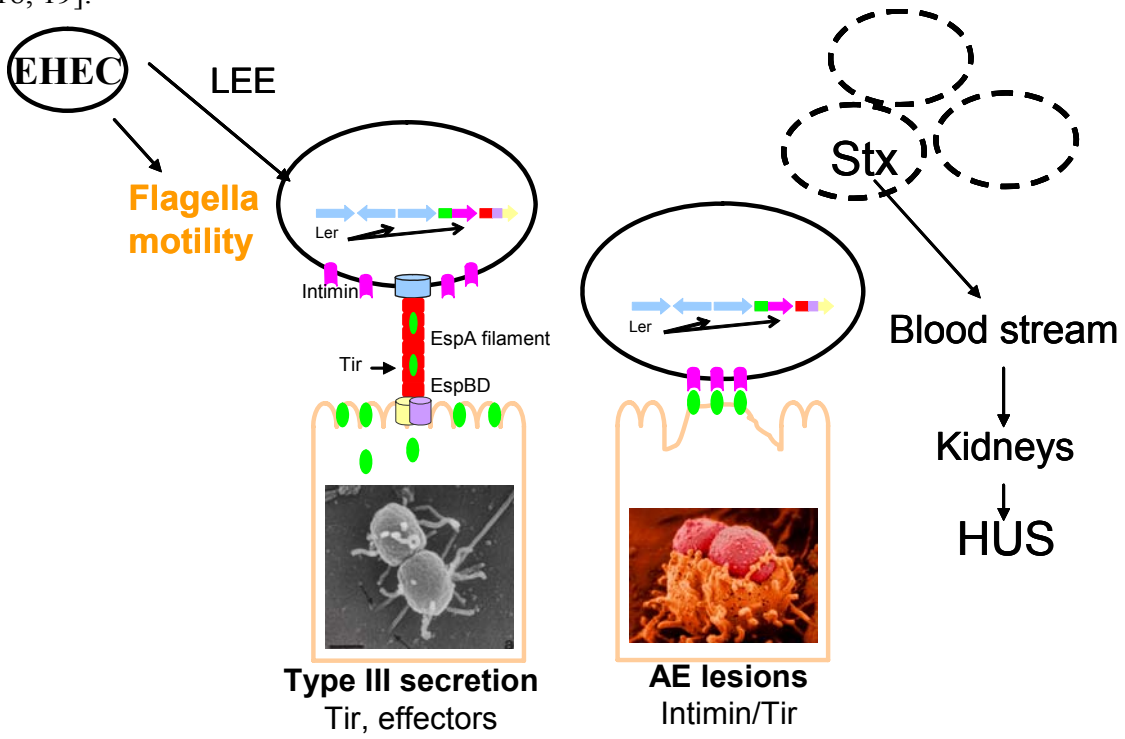


Figure 1.4 – Model of EHEC Pathogenesis. EHEC may activate its flagella regulon in order to swim proficiently through the intestinal mucus layer and get into close contact with the enteric epithelia. EHEC then activates the LEE-encoded type III secretion system, leading to the formation of the attaching and effacing lesions responsible for the beginning of diarrheal disease. During disease, some EHEC cells may become stressed and activate the Stx phage, undergo lysis, and release Shiga toxin. Stx travels to the kidneys, where it causes the development of hemolytic uremic syndrome.

The Locus of Enterocyte Effacement (LEE)

The genes involved in the formation of the AE lesion are encoded within a 35-kb chromosomal pathogenicity island named the Locus of Enterocyte Effacement (LEE) [42]. Much of what is known about the LEE region has been studied in EPEC and inferred to be true for EHEC. The EHEC LEE region contains 41 genes, the majority of which are organized into five major operons: LEE1, LEE2, LEE3, *tir* (LEE5) and LEE4 [43-45], which encode a type III secretion system (TTSS) [46], an adhesin (intimin) [47], and this adhesin's receptor, the translocated intimin receptor (Tir) [48], which is translocated into the epithelial cell through the bacterial TTSS [43, 44] (Figure 1.5). The first gene in the LEE1 operon, the LEE-encoded regulator (Ler) directly activates the transcription of the LEE genes [44, 49-52].

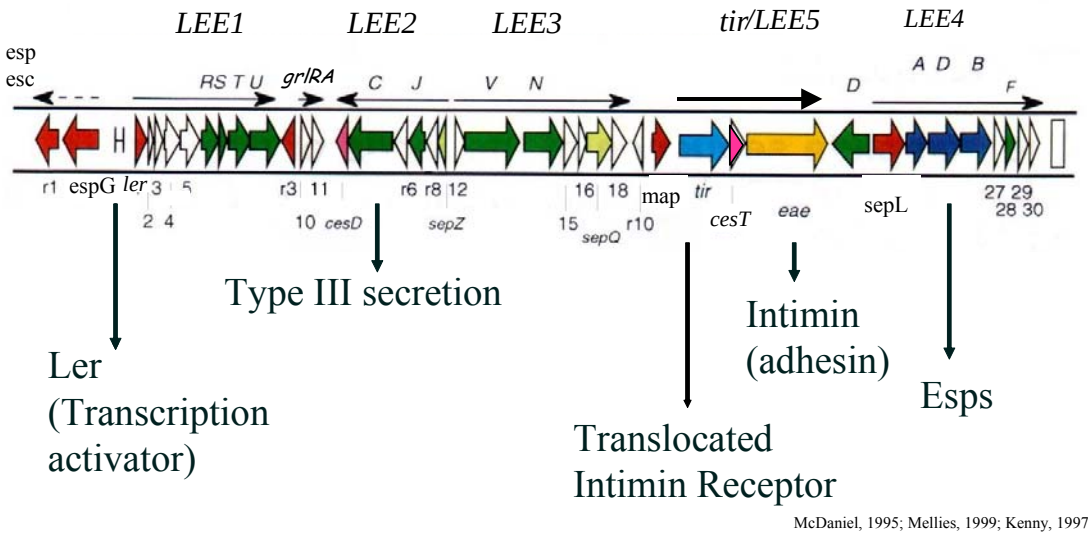


Figure 1.5 – The Locus of Enterocyte Effacement. The LEE is a pathogenicity island found in EHEC, which encodes factors responsible for type III secretion and pedestal formation. *LEE1* encodes for *ler*, the LEE-encoded regulator. *LEE1, LEE2*, and *LEE3* encode for factors involved in type III secretion. *LEE4* encodes for EspA, EspB, and EspD. The *LEE5/tir* operon encodes for intimin and Tir.

The EHEC type III secretion system (TTSS) is a basal apparatus that spans the inner and outer bacterial membranes with a short projecting "needle." Several proteins, including EscD, EscR, EscU, EscV, EscS and EscT span the inner membrane and associate with a cytoplasmic ATPase, EscN, which is required for secretion of proteins [53]. EscC is predicted to form the main protein ring in the outer membrane to which the EscF "needle" is connected [54]. EscF comprises the syringe connected to the filament of the translocon. The translocon consists of EspA, which creates a sheath around the EscF needle. EspB and EspD are located at the distal end of the TTSS and form 3-5 nm pores in the host cell membrane [55], through which translocated proteins are secreted.

The *eae* gene (*E. coli* attaching and effacing) encodes for intimin, an outer membrane protein that acts as an intestinal adherence factor [47]. Mutants of the *eae* gene are defective in intimate adherence to intestinal epithelial cells, which prevents the concentration of polymerized actin necessary for the development of AE lesions. The translocated intimin receptor (Tir), which is also encoded in the LEE, is translocated from the bacterium through the TTSS into the host cell to serve as a receptor for intimin [56-58]. In the host cell membrane, Tir adopts a hairpin loop conformation and serves as a receptor for the bacterial surface adhesin, intimin [59]. Binding of intimin to Tir promotes the clustering of N- and C-terminal cytoplasmic regions and leads to the initiation of localized actin assembly beneath the plasma membrane [60]. Unlike EPEC, the EHEC Tir is not phosphorylated in the host cell [58, 59], and does not recruit the mammalian adaptor protein, Nck to the sites of adherence [61]. The EHEC Tir does recruit N-WASP [62], which it requires for efficient pedestal formation, possibly through

an interaction with $EspF_U$, another bacterial protein that is translocated into the host cell [63].

Chaperones play an important role in directing secreted proteins from the EHEC bacterial cell, as many secreted proteins lack classical N-terminal secretion signals [46]. CesD, a protein associated with the inner membrane, acts as a chaperone for EspD [64]. Additionally, CesT is the chaperone that is responsible for the translocation of Tir [65].

The TTSS encoded by the LEE region also translocates LEE-encoded and non-LEE encoded effectors. The mitochondrial associated protein, *map*, affects the integrity of the host mitochondrial membrane [66], is encoded directly upstream of *tir,* and may also be chaperoned by CesT [67]. Another effector, EspF, is responsible for the disruption of intestinal barrier function and induces cell death by unknown methods [68, 69]. The secreted EspG is responsible for the disruption of microtubule formation, and plays a role in virulence in the REPEC mouse model [70], while EspH, which is encoded in *LEE3*, is responsible for the modulation of host cell cytoskeleton through the inhibition of cell cycle signals [71]. Although encoded outside the LEE pathogenicity island, several effector proteins have recently been shown to be secreted through the EHEC TTSS. These include Cif, which induces host cell cycle arrest and reorganization of host actin cytoskeleton [72], and NleA, which has been shown to localize to the Golgi and play a key role in virulence in an animal model [73].

The regulation of the LEE pathogenicity island is complex. Recently, Iyoba *et al.* observed that EHEC encodes specific genes, named *pchA, pchB,* and *pchC*, (PerC homologs) that positively activate the expression of the LEE genes [74]. *LEE1* encodes for *ler*, the LEE-encoded regulator, which was shown to be required for the expression of

other operons within the LEE [44]. Another factor important in the regulation of the LEE pathogenicity island is the integration-host factor, or IHF. IHF has been shown to be required for the expression of the entire LEE through the direct binding and activation of *ler* [75]. Additionally, EtrA and EivF are two negative regulators of the LEE region, possibly through *ler,* which are encoded within a second type III secretion system (ETT2) [76]. The histone-like nucleoid-structuring protein, H-NS, is responsible for the repression of *LEE2, LEE3,* and *LEE5* transcription in the absence of Ler [49, 77]. RpoS, a stationary phase sigma factor, activates the transcription of the *LEE3* operon and the *LEE5* operon [78]. Finally, Hha has been reported to repress the transcription of the *LEE4* operon [79].

Two previously uncharacterized genes in the LEE region, *orf10* and *orf11* were recently renamed GrlR, global regulator of LEE repressor, and GrlA, global regulator of LEE activator [80]. This study suggests that GrlA is responsible for the transcriptional activation of *ler*, while GrlR represses *ler*. Additionally, it is known that Ler activates the transcription of *grlRA* [50], and that GrlRA activates the expression of *LEE2* and *LEE4*, independently of Ler [81].

Shiga toxin

EHEC also produces a powerful Shiga toxin (Stx) that is responsible for the major symptoms of hemorrhagic colitis and HUS. The Stx family contains two subgroups, Stx1 and Stx2. Stx1 shows little sequence variation between strains [82], whereas antigenic divergence has been observed among the Stx2s, including Stx2, Stx2c, Stx2d, and Stx2e

[82-84]. Stx2 has been found to be more associated with severe human disease than Stx1 [85], with Stx2 and Stx2c most frequently found in patients with HUS [86].

Both of the genes encoding Stx1 and Stx2 are located within the late genes of a λ-like bacteriophage and are transcribed when the phage enters its lytic cycle [87]. Once the phage replicates, Shiga toxin is produced, and the phage lyse the bacteria thereby releasing the toxin into the host. The bacteriophage may receive signals to enter its lytic cycle during an SOS response triggered by disturbances in the bacterial membrane, DNA replication, or protein synthesis [4, 5]. These triggers are all common targets of conventional antibiotics and may contribute to the ineffectiveness of antibiotics during an EHEC infection. It is thought that the Stx gains access to the circulation, and is transported to the kidneys. Shiga toxins consist of a 1A:5B noncovalently associated subunit structure [88]. The B subunit of Stx is known to form a pentamer that binds to the eukaryotic glycolipid receptor, globotriaosylceramide, in the kidneys [89-91]. The A subunit is then nicked with trypsin and reduced, resulting in a polypeptide that causes depurination of a residue in the 28S rRNA of 60S ribosomes [92]. This leads to the inhibition of protein synthesis, injury of renal glomerular endothelial cells, and the initiation of a pathophysiological cascade that leads to HUS.

Additional virulence factors

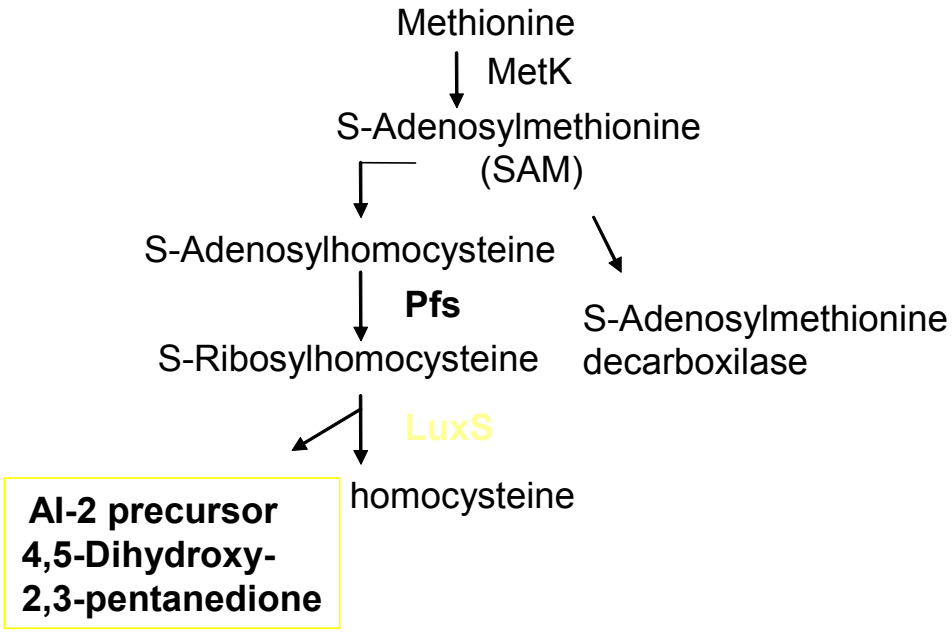
EHEC carries a large 90 kb plasmid, designated pO157 [93], which encodes about 35 proteins and may be involved in the production of an outer membrane protein [94, 95]. It has been shown to contain a hemolysin [94, 96], a type II secretion system [97], and a

serine protease, EspP [98]. The presence of pO157 has been associated with increased adherence to intestinal epithelial cells, although the exact mechanism is unknown [93].

CELL-TO-CELL SIGNALING IN BACTERIA

The cell-to-cell communication system referred to as quorum sensing (QS) is based on the principle that bacteria secrete hormone-like compounds referred to as autoinducers (AI). Upon reaching a threshold concentration, the autoinducer may interact with bacterial transcription factors to regulate gene expression. This phenomenon was first observed in *Vibrio fischeri* and *Vibrio harveyi* to control bioluminescence [99, 100]. The luciferase operon in *Vibrio fischeri* is regulated by LuxI, which is responsible for the production of the acyl-homoserine-lactone (AHL) autoinducer, and LuxR, which is activated by this autoinducer to increase transcription of the luciferase operon [101, 102]. Homologues of this LuxR-LuxI system have been identified in other bacteria, which have been shown to regulate the transcription of antibiotics in *Erwinia,* motility in *Yersinia pseudotuberculosis,* and pathogenesis and biofilm formation in *Pseudomonas aeruginosa* [103-105]. *E. coli* and *Salmonella* have a LuxR homolog, SdiA, [106], but no LuxI homolog, and do not produce AHLs [107, 108]. Recently, Michael *et al.* reported that the role of SdiA in quorum sensing was to sense AHLs produced by other bacterial species, and not an autoinducer produced by *E. coli* itself [108].

The most prevalent quorum sensing system is the *luxS* system, which is present in many bacteria, including *Vibrio harveyi, Salmonella typhimurium,* and *Escherichia coli* O157:H7 [78, 109, 110]. The *luxS* system was originally described as being involved in bioluminescence in *Vibrio harveyi* [111]. LuxS is an enzyme that is involved in the metabolism of S-adenosyl-methionine (SAM); it converts ribose-homocysteine into homocysteine and 4,5-dihydroxy-2,3-pentanedione (DPD) (Figure 1.6). DPD is a highly unstable compound that reacts with water and cyclizes into several furanones, one of which is thought to be the precursor of autoinducer-2 (AI-2) [112].



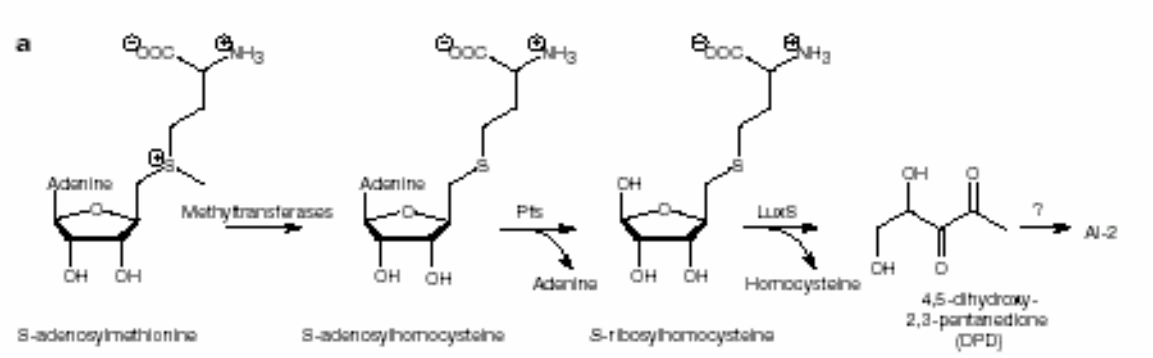


Figure 1.6 – The synthetic pathway of AI-2. The metabolic pathway leads to the production of DPD, the key product of the enzyme LuxS. DPD is the precursor for AI-2. Figure adapted from [113].

Originally, a literature search revealed that the final structure of the AI-2 compound had not been reported, presumably due to its instability. However, several groups had been able to characterize compounds similar to (or derived from) DPD [112],

including 4-hydroxy-2,5-dimethyl-furanone (DMHF), 4-hydroxy-5-methyl-furanone (MHF), homofuraneol (HF), and 4,5-dihydroxy-2-cyclopentan-1 (DHCP). Furthermore, DHCP has been reported to have no AI-2 activity in a *Vibrio harveyi* luminescence assay, and MHF, DMHF, and HF have AI-2 activity at extremely high concentrations [112]. Recently, the final structure of AI-2 from *Vibrio harveyi* has been shown to be a furanosyl-borate diester (Figure 1.7) [114]. This structure was ultimately solved by X-ray crystallography of the autoinducer complexed with its receptor, LuxP [114]. Additionally, the final structure of AI-2 from *Salmonella typhimurium* has been co-crystallized with its receptor, LsrB [113]. LsrB appears to bind a chemically distinct form of the AI-2 signal, (2R,4S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (R-THMF), which lacks boron but is also derived from DPD (Figure 1.7) [113]. These data suggest that different species recognize different forms of the autoinducer signal AI-2, which are both derived from DPD.

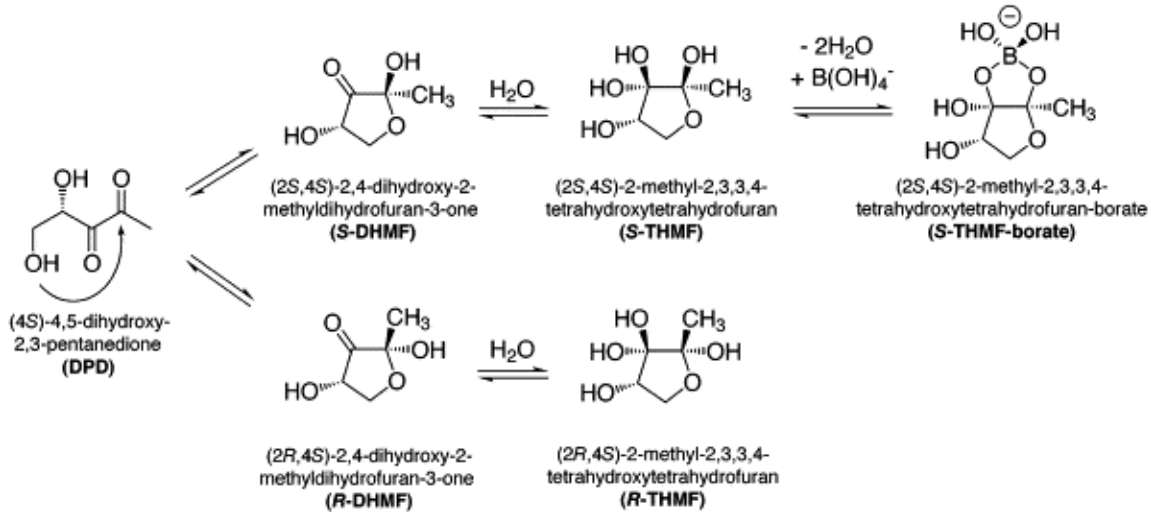


Figure 1.7 – Proposed formation of AI-2 signaling molecules by *V. harveyi* (upper branch) and *S. typhimurium* (lower branch). S-THMF-borate binds to the *V. harveyi* receptor LuxP, while R-THMF binds to the *S. typhimurium* receptor LsrB. Figure adapted from [113].

Initially, diverse roles in signaling were attributed to AI-2 in many bacteria by comparing *luxS* isogenic mutants with wild-type strains. Included in these roles are type III secretion in *V. harveyi* [115], expression of VirB in *Shigella flexneri* [116], and flagella expression and the LEE-encoded type III secretion system in EHEC [78, 117], among others. By far, the most thorough studies on the role of the LuxS system in virulence have been performed in EHEC [1, 78, 117-119]. These studies, however, have demonstrated that the signaling molecule activating type III secretion and flagella in EHEC is not the AI-2 autoinducer, but another autoinducer that is dependent on the presence of the *luxS* gene for its synthesis, AI-3 [119]. This signaling compound, which activates the virulence genes of EHEC, binds to C-18 columns and can only be eluted with methanol. Additionally, electrospray mass spectrometry analysis of AI-3 shows a major peak with a mass of 213.1 Daltons. All of these parameters are different from that of AI-2 [114], suggesting that AI-3 is a unique compound whose presence is dependent on LuxS.

Both AI-3 and AI-2 activities have been observed in spent supernatants from pathogenic organisms such as *E. coli* O26:H11 and O111ac:H9, *Shigella* sp., and *Salmonella* sp. and commensal bacteria, including *E. coli*, *Klebsiella pneumoniae* and *Enterobacter clocae* (Sircili and Sperandio, unpublished results). Additionally, AI-2 and AI-3 activity has been observed in spent supernatants from normal flora cultured from human stools in intestinal simulators [119]. The presence of these autoinducers in both pathogenic and commensal organisms may suggest that interspecies signaling plays a role not only for signaling of intestinal commensal flora, but also in disease caused by pathogenic organisms.

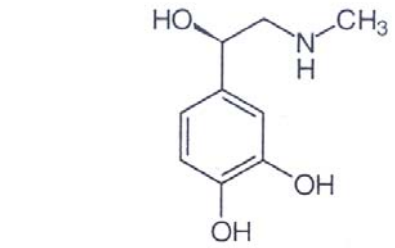
BACTERIA-HOST SIGNALING

Initially, Sperandio *et al*. [78] utilized an isogenic *luxS* mutant in EHEC to show that the transcription of all the LEE operons is activated by autoinducers from EHEC supernatants. *In vitro* analysis in this EHEC *luxS* mutant revealed that type III secretion was dramatically diminished as compared to wild-type and complemented strains [119], and it was expected that this mutant would be unable to form AE lesions on cultured epithelial cells. This type III secretion defect could be restored upon genetic complementation of *luxS* or by the addition of exogenous AI-3. However, studies using the *luxS* mutant incubated with cultured epithelial cells showed that it was able to form attaching and effacing (AE) lesions indistinguishable from those formed by the wild-type EHEC strain [119]. This led to the investigation of whether there was an additional level of cell-to-cell signaling occurring between the bacterial and eukaryotic cells that could activate the expression of EHEC virulence genes. Interestingly, Sperandio *et al.* (2003) found that the incubation of the *luxS* mutant with cell culture medium that had been incubated with HeLa cells for 24 hours and then size-fractioned for smaller than 1kDa also restored the *luxS* mutant phenotype. These experimental results led to the observation that there must be a eukaryotic signaling compound(s) that can complement the defect of the *luxS* quorum sensing mutation.

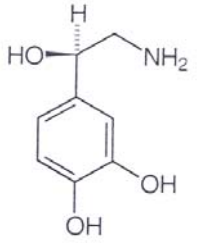
Eukaryotic cell-to-cell signaling occurs through hormones. The three major groups of endocrine hormones include polypeptide hormones, steroid hormones, and hormones derived from the amino acid tyrosine, which include the catecholamines norepinephrine and epinephrine [120]. Interestingly, the acyl-homoserine-lactones and

AI-2, which are gram-negative bacterial autoinducers, are also derived from amino acid metabolism [121]. The catecholamine norepinephrine has previously been demonstrated to induce bacterial growth [122] and to be taken up into bacteria [123]. Ultimately, Sperandio *et al.* (2003) were able to narrow down the eukaryotic signal to the catecholamines norepinephrine and epinephrine, which were found to be present in FBS in epithelial growth cell media. Additionally, purified epinephrine and norepinephrine were used to show that the *luxS* mutant was directly responding to these signals [119].

It has been shown that the hormones epinephrine and norepinephrine (Figure 1.8) are present in considerable amounts in the human intestine [124] and aide in the induction of chloride and potassium secretion and modulate intestinal smooth muscle contraction [125]. Norepinephrine is synthesized by the adrenergic neurons that are present in the enteric nervous system (ENS) [126], while epinephrine is synthesized in the central nervous system (CNS) and adrenal medulla and acts systemically after being released into the bloodstream [127]. To date, nine human adrenergic receptors have been identified and partitioned into three subclasses: α1, α2, and β. Although the crystal structures for α- and β-adrenergic receptors are still unknown, Freddolino *et al.* [128] recently predicted the structure of the human β2 adrenergic receptor based on modeling of the rhodopsin receptor, suggesting that the ligand binding sites for epinephrine and norepinephrine are broadly similar. This evidence suggests that epinephrine and norepinephrine may be recognized by the same receptors in the human gastrointestinal tract.

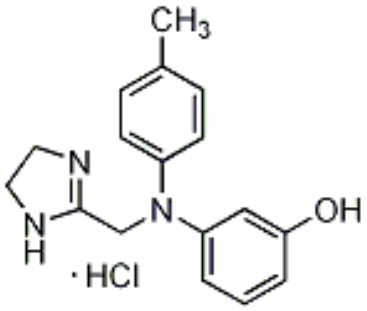


epinephrine

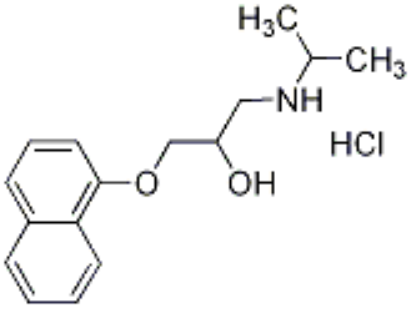


norepinephrine

noraepinephrine



Phentolamine
α-adrenergic antagonist



Propranolol
β-adrenergic antagonist

Figure 1.8 – Chemical Structures of Epinephrine and Norepinephrine, Phentolamine, and Propranolol.

Horger *et al.* (1998) showed that the neuronally-mediated response to these catecholamines in the colon can be suppressed by the β-adrenergic receptor antagonist propranolol (PO) and the α-adrenergic receptor antagonist phentolamine (PE) (Figure 1.8). Sperandio *et al.* (2003) showed that these antagonists were able to block the formation of AE lesions on the *luxS* mutant and wild-type EHEC on cultured epithelial cells, further suggesting that epinephrine and norepinephrine are the specific eukaryotic cross-signaling compounds that were activating the expression of EHEC virulence genes [119]. Moreover, it was shown that epinephrine and norepinephrine can substitute for AI-3 to activate the transcription of the LEE genes, type III secretion, and regulation of the flagella regulon. Taken together, these results suggest that AI-3 and epinephrine/norepinephrine cross-talk and that these compounds may use the same signaling pathway. In this manner, EHEC could respond to both a bacterial quorum sensing signaling system and a eukaryotic signaling system in order to modulate the transcription of virulence genes at different stages of infection at different sites in the gastrointestinal tract.

There are several other examples of prokaryotic-eukaryotic communication in which bacterial signals can modulate the expression of eukaryotic genes [129]. 3-oxo-C12-HSL, an AI of *P. aeruginosa,* has been shown to have immunomodulatory activity by downregulation of tumor necrosis factor alpha and interleukin-12 production [130], and by upregulation of cytokine gamma interferon [131]. The cytolysin of *E. faecalis*, which has been shown to modulate expression of the enterococcal *cyl* operon, also has toxic effects on erythrocytes, retinal tissues, intestinal epithelial cells, neutrophils, and macrophages [132]. On the other hand, eukaryotic factors have also been shown to affect

the transcription of prokaryotic genes. Host cell membranes selectively bind the large subunit of the cytolysin of *E. faecalis*, thereby allowing the small subunit to increase cytolysin expression through a quorum sensing mechanism [132]. Additionally, human airway epithelial cells produce an unidentified substance, which inactivates the 2-oxo-C12-HSL signaling molecule produced by *P. aeruginosa* [133]. Taken together, these examples of prokaryotic-eukaryotic communication provide a foundation for our observation of cross-communication between the bacteria and host.

CELL-TO-CELL SIGNALING IN EHEC

Bacterial cell-to-cell signaling cascades, referred to as quorum sensing (QS), have been studied extensively in organisms such as *Pseudomonas aeruginosa* and *Vibrio harveyi* [104]. The QS regulatory cascade in EHEC has just begun to be described. Sperandio *et al.* [117] utilized an *E. coli* gene array to compare wild-type EHEC to an isogenic *luxS* mutant in EHEC. EHEC has 1.3Mbp of DNA that is absent in *E. coli* K-12, while K-12 has 0.53Mbp of DNA that is absent in EHEC [27]. Approximately 10% of the common genome between EHEC and *E. coli* K-12 was differentially expressed between wild-type and the *luxS* mutant [117]. This high percentage of differentially regulated genes is not surprising when one considers the pleiotropic effect of the *luxS* mutation. As stated earlier, LuxS is a metabolic enzyme that is involved in the conversion of ribosyl-homocysteine into homocysteine and 4,5-dihydroxy-2,3-pentanedione, the precursor of AI-2 [112], and is also somehow involved in the

production of AI-3 [119]. Given that a *luxS* mutant will be required to use salvage pathways in order to compensate for the loss of a metabolic enzyme, amino acid synthetic and catabolic pathways will be changed within the bacterial cell, leading to a large amount of differential gene expression.

These studies led to the identification of several putative genes that were regulated in response to quorum sensing through the AI-3/epinephrine/norepinephrine cell-to-cell signaling system (Figure 1.9). Quorum sensing *E. coli* regulator A (QseA) was identified as a transcriptional regulator from the LysR family [118] that autorepresses its own transcription (Figure 1.9). QseA is transcriptionally activated through quorum sensing and directly activates the transcription of the LEE-encoded regulator (Ler) [118] (F. Sharp and V. Sperandio, unpublished data). Ler is the activator for several genes within the LEE pathogenicity island [44]. Accordingly, a mutation in *qseA* of EHEC shows a reduction in type III secretion, but shows no defect in flagellation or motility.

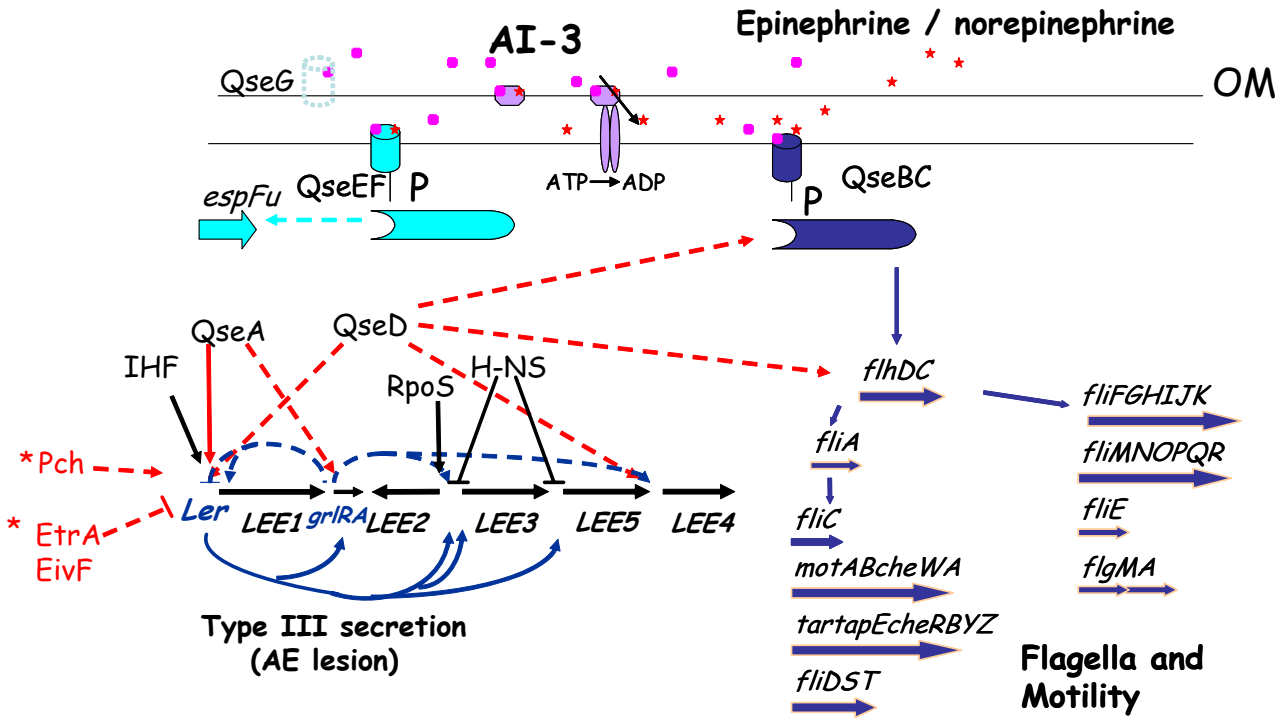


Figure 1.9 – Signaling Model in EHEC. Both AI-3 and Epi/NE seem to be recognized by the same receptor, which is probably in the outer membrane of the bacteria. These signals might be imported into the periplasm where they interact with two major sensor kinases, QseC and QseF, which autophosphorylate. These sensor kinases then transfer the phosphate to their cognate response regulator, which goes on to activate virulence gene transcription. QseB&C may activate the transcription of the flagella and motility, while QseE&F activate the transcription of the LEE region (AE lesions). Dashed arrows indicate that no biochemistry has been performed.

Additionally, a two-component system named quorum sensing *E. coli* regulators B and C (QseB&C) were also identified in the DNA array [117]. Of the 19 putative regulators identified in this study, *qseBC* was shown to be activated 17-fold by quorum sensing (QS), and appear to be organized in an operon. Initial sequence analysis indicated that *qseBC* may belong to the family of two-component systems, with QseC being the predicted sensor kinase, and QseB the putative response regulator. QseB&C are responsible for the transcriptional activation of the flagella regulon in response to quorum sensing [1].

Quorum sensing *E. coli* regulators D, E, and F (QseD, QseE, QseF) were also identified in the DNA array. QseD is another regulator of the LysR family, which may activate the flagella regulon and repress transcription of the LEE genes (F. Sharp and V. Sperandio, unpublished results). QseE and QseF encode a second two-component system that is involved in the regulation of $EspF_U$ (N. Reading and V. Sperandio, unpublished results).

QUORUM SENSING *E. COLI* REGULATORS B AND C (QseB&C)

The quorum sensing *E. coli* regulators B and C (QseB&C) are a two-component system, with QseC being the predicted sensor kinase and QseB the putative response regulator. QseB&C have homologs in UPEC, EPEC, EHEC, *E. coli* K-12, *Shigella flexneri, Salmonella typhimurium, Salmonella typhi, Yersinia pestis, Yersinia enterocolitica, Pasteurella multocida, Haemophilus influenzae, Coxiella burnetti,* and

Francisella tularensis (*qseC* only). QseB has three amino acid changes between EHEC and *E. coli* K-12, while QseC has eight amino acid changes. Additionally, QseB shares a high level of homology with *Salmonella typhimurium* PmrA (46% identity and 62% similarity over 222 amino acids), and QseC shares homology with *Salmonella typhimurium* PmrB (28% identity and 45% similarity over 269 amino acids). PmrA&B are involved in gene regulation in response to extracytoplasmic ferric iron [134] and genes that confer resistance to antimicrobial peptides such as polymyxin [135, 136].

Traditional two-component systems consist of the sensor protein that acts as a histidine kinase to transfer a phosphoryl group upon sensing of an environmental signal to an aspartate residue of its cognate response regulator, which goes on to act as a transcription factor (Figure 1.10). Both QseB and QseC contain conserved domains characteristic of a two-component system. QseC, the putative sensor kinase, has two conserved transmembrane domains and a conserved histidine kinase domain, indicating that its membrane location may allow autophosphorylation upon the recognition of its specific environmental cue. QseC also contains an ATPase domain, which may allow it to exhibit phosphatase activity toward QseB. Additionally, QseC has a conserved EAL domain, commonly found in signaling proteins, which consists of several acidic residues that could be important for metal binding and may make up an active site for a phosphodiesterase of cyclic diguanylate (c-di-GMP), a cyclic nucleotide [137, 138]. The EAL domain of the VieA response regulator in *V. cholerae* has recently been implicated in the formation of biofilms by controlling c-di-GMP concentration in *V. cholerae* [138]. QseB contains typical response regulator and DNA binding domains, which may allow it to receive a phosphate from QseC and go on to regulate gene transcription.

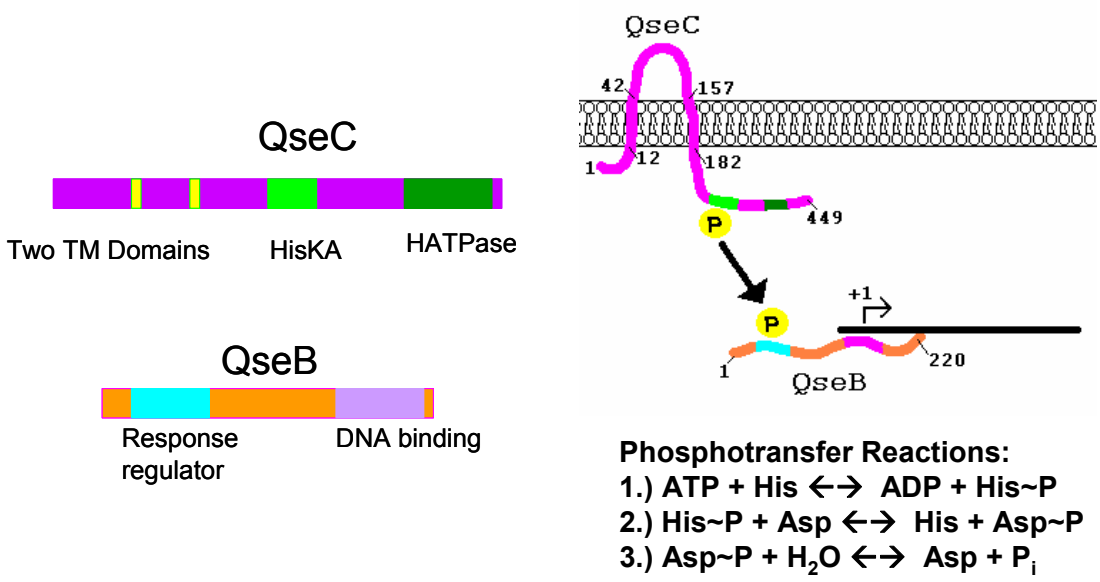


Figure 1.10 – The QseB&C two-component system. QseC is the membrane-bound sensor kinase that binds to environmental signals and autophosphorylates on a conserved histidine residue. QseC then transfers its phosphate to QseB, its cognate response regulator. Once phosphorylated, QseB may go on to regulate transcription of virulence genes.

QSEC, A BACTERIAL ADRENERGIC RECEPTOR

Currently, little is known about the signaling interactions between pathogenic bacteria and the host. It has been hypothesized that bacteria must sense and recognize that they are within the host in order to activate genes essential for colonization. EHEC 0157:H7, a pathogenic bacterium capable of colonizing the human intestine and causing the development of hemorrhagic colitis and hemolytic uremic syndrome [3] can be used to test these host/bacterium signaling interactions. Sperandio *et al.* previously used this model to show that EHEC can activate its virulence genes in response to the autoinducer

AI-3, produced by the bacteria itself, and human hormones produced naturally by the host, epinephrine/norepinephrine [119]. These results suggest that there may be potential cross-communication between the AI-3 bacterial system and the epinephrine/norepinephrine host signaling system.

The transcription of *flhDC* is activated by both epinephrine and AI-3 in the *luxS* mutant [119]. However, motility and *flhDC* transcription in a *qseC* sensor kinase mutant are unable to respond to the presence of either AI-3 or epinephrine (Figure 1.11), indicating that QseC may possibly be sensing the presence of these cross-signaling compounds [119]. Since an isogenic mutant in *qseC*, the sensor kinase, is unable to recognize both bacterial AI-3 and host epinephrine, we hypothesize that QseC may be acting as a bacterial adrenergic receptor that interacts directly with these compounds [119]. This hypothesis is the focus of Chapter 4.

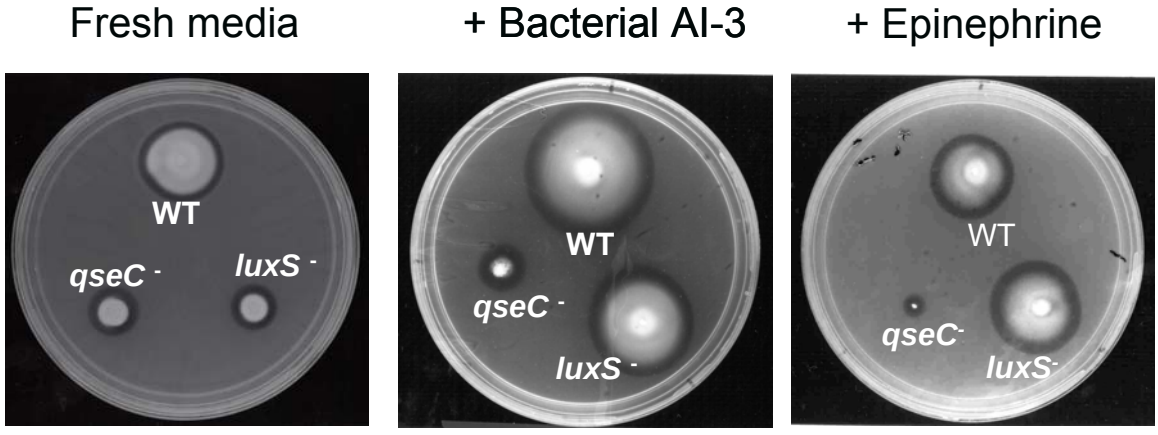


Figure 1.11 – Motility plates of wild-type, *qseC* mutant, and *luxS* mutant [119].

THE FLAGELLAR REGULON

Sperandio *et al.* recently reported that QseBC are involved in activating the transcription of the flagellar regulon in *E. coli* [1]. A *qseC* mutant in EHEC showed reduced flagellin production and motility, and reduced transcription of flagellar genes [1]. The flagella and motility genes comprise a large and complex regulon, with more than 50 genes organized into at least 17 operons [139]. Within this regulon, the operons are classified into three hierarchical transcriptional classes, class 1, class 2 and class 3 (Figure 1.12) [140]. The class 1 genes are the master regulator of flagella and motility, *flhDC*. In *Salmonella enterica*, six transcriptional start sites have been mapped for the *flhDC* promoter [141], indicating the complexity of its regulation. FlhDC are responsible for the activation of the class 2 genes [140, 142], through binding to a conserved 56bp consensus region [143]. The class 2 genes encode the proteins responsible for the formation of the flagellar motor intermediate structure, termed the hook-basal body [144]; the alternative sigma factor, FliA (σ^{28}) [145]; and the anti-sigma factor, FlgM [146]. During the formation of the hook-basal body, the anti-sigma factor FlgM binds to FliA (σ^{28}) to inhibit its interaction with RNA polymerase [147]. However, upon completion of the hook-basal body structure, FlgM is exported through this apparatus, and FliA (σ^{28}) is able to interact with RNA polymerase. FliA (σ^{28}) is necessary to recognize the promoters and to initiate transcription of the class 3 genes, which include *fliC* (the external filament, flagellin) [148]and the *mot* operon [149].

Most of what is known about the regulation of flagella has been performed in *Salmonella* and inferred to be true in *E. coli.* It has been shown that the synthesis and

expression of the flagellar and motility genes are regulated by several cues besides quorum sensing, including temperature [150], osmolarity [151], cAMP-CRP [152, 153], cell cycle control [154], RcsCDB [155], H-NS [156], and IHF [157]. Sperandio *et al.* have recently reported that the expression of flagella and motility in EHEC and *E. coli* K12 is regulated by quorum sensing through QseB&C [1]. Transcriptional fusions of flagella class 1 (*flhDC*), class 2 (*fliA*), and class 3 (*fliC* and *motA*) genes were reduced in an isogenic *qseC* mutant as compared to wild-type. These data may suggest that QseB&C act to regulate flagellar expression through the master regulator, *flhDC*. The molecular mechanisms by which QseBC regulate *flhDC* transcription are the subject of the studies in Chapter 5.

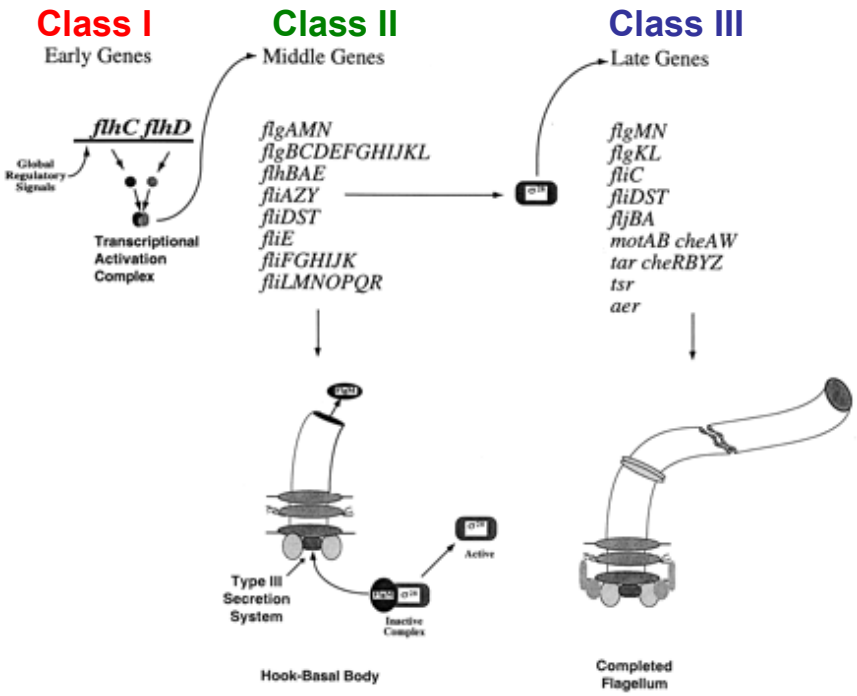


Figure 1.12 – The flagellar regulon of *E. coli* K-12. The Class I genes (red), *flhDC*, are the master regulator of flagella and motility. FlhDC activate the transcription of the Class II genes (green), which encodes proteins that make up the hook-basal body, a sigma factor, and an anti-sigma factor. Once the hook-basal body is completely formed, the anti-sigma factor is exported and the sigma factor is free to activate the transcription of the Class III genes (blue). The Class III genes encode proteins that form the external flagellar filament and the motility proteins. Figure adapted from [149].

QseB&C TRANSCRIPTIONAL AUTOREGULATION

The genomic organization of the QseB&C two-component system gives us some clues as to its regulation. The translational stop site of *qseB* overlaps with the translational start codon of *qseC* (Figure 1.13), suggesting that the *qseBC* genes may be transcribed in an operon. Studies that investigate the possibility that *qseBC* are transcribed in an operon are described in Chapter 6. Additionally, it is well known that many two-component systems act to positively regulate their own transcription [158], a theory that was investigated in Chapter 6.

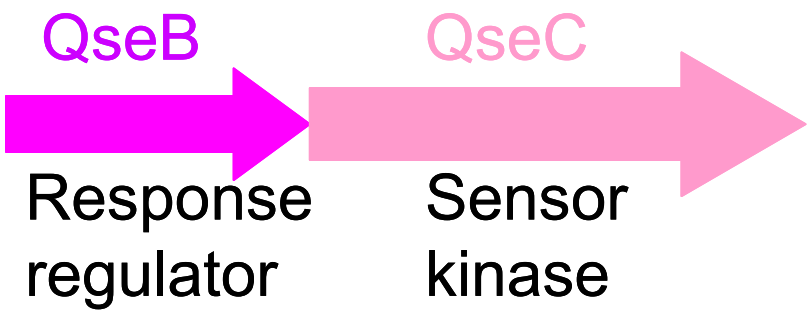


Figure 1.13 – Genetic Organization of *qseBC*. The translational stop site of *qseB* overlaps with the translational start codon of *qseC*, suggesting that *qseBC* may be transcribed in an operon.

CHAPTER TWO

OVERALL OBJECTIVE AND SYNOPSIS

EHEC is a human pathogen that colonizes the large intestine, causing the development of hemorrhagic colitis. During infection, EHEC can sense and respond to environmental cues, such as the cell density of the normal flora in the intestine and the epinephrine/norepinephrine produced by the host. This recognition, termed quorum sensing or cell-to-cell signaling, allows EHEC to activate production of its flagella in order to swim closer to the intestinal epithelium, aiding in colonization and disease. Sperandio *et al.* have previously shown that the quorum sensing *E. coli* regulators B and C (QseB&C), a two-component system in EHEC, are responsible for the regulation of *flhDC,* the master regulator of flagellar and motility genes, in response to quorum sensing.

In order to better understand the signaling role of the QseB&C two-component system in EHEC, gene regulation studies with QseC, the membrane-bound sensor kinase, and QseB, the response regulator, were undertaken. The data generated in these studies indicate that QseC autophosphorylates itself on a conserved histidine residue in response to epinephrine, a host signaling compound, or AI-3, a compound produced by the normal flora. QseC is then able to transfer its phosphate to a conserved aspartate residue of the

response regulator, QseB. This phosphorylation is the first indication that a bacterial quorum sensing component may be directly responding to a host factor during infection.

Following phosphorylation, QseB acts as a transcription factor to directly regulate the expression of *flhDC*, the master regulator of flagella and motility genes. Comprehensive deletion analyses and electrophoretic mobility shift assays using the *flhDC* promoter region suggest that QseB binds the promoter at both high- and low-affinity sites. Binding at both *flhDC* promoter sites may expose the FliA (σ^{28}) promoter, which was mapped to be dependent on QseB&C. FliA (σ^{28}) may subsequently be able to interact with RNA polymerase in order to initiate *flhDC* transcription through the FliA (σ^{28}) promoter. Additionally, our data suggest that an unknown repressor is acting on the central region of the *flhDC* promoter. DNaseI footprint analysis using the *flhDC* promoter regions was also performed. These data allowed the proposal of a consensus sequence to which phosphorylated QseB binds in order to regulate gene transcription.

As it is known that many two-component systems act to autoregulate their own transcription, additional transcriptional analyses were performed, indicating that QseB also autoregulates its own transcription. Using the *qseBC* promoter and QseB, electrophoretic mobility shift assays and DNaseI footprint analyses were again executed. The results of these analyses, combined with the data from the *flhDC* promoter, allowed the proposal of a potential consensus sequence to which QseB binds in order to regulate transcription. Using this sequence in an *in silico* search allowed us to identify potential novel targets of QseB regulation.

Through the use of both genetic and biochemical methodologies, a comprehensive functional analysis of the mechanism by which the QseB&C two-component system regulates the transcription of both itself and the flagellar master regulator, *flhDC*, in EHEC was performed. An in-depth understanding of how this two-component system regulates gene expression may allow us to gain insight into the regulation of virulence in enterohemorrhagic *E. coli.*

CHAPTER THREE

MATERIALS AND METHODS

Bacterial strains, plasmids, and media

All bacterial strains and plasmids utilized in this study are listed in Table 3.1. *E. coli* strains were grown aerobically in either LB (EMD Science) or tryptone medium (EMD Science) at 37^{o}C. Antibiotics were added, as necessary, at the following concentrations: 100 μg ml $^{-1}$ ampicillin, 30 μg ml^{-1} chloramphenicol, 50 μg ml $^{-1}$ kanamycin, and 25 μg ml^{-1} tetracycline. The enterohemorrhagic *E. coli* O157:H7 wild-type strain, 86-24, is streptomycin-resistant and is referred to as "wild-type" in all studies. *E. coli* DH5α (New England Biolabs) was used as a host for all plasmid constructions and protein purifications.

Table 3.1 - Bacterial strains and plasmids used in this study.

Strain/Plasmid	Genotype/Description	Reference
Strain		
86-24	Wild-type EHEC strain (serotype O157:H7)	[17]
DH5α	*supE44 lacU169 (80 lacZ M15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Stratagene
MC4100	*araD139 (argF-lac)U169 rpsL150 relA1 fblB3501 deoC1 ptsF25 rbsR*	[159]
MC1000	*araD139 D(araABC-leu)7679 galU galK D(lac)X74 rpsL thi*	[160]
VS138	86-24 *qseC* mutant	[1]
VS179	VS138 with plasmid pVS178	[1]
VS184	MC1000 *qseC* mutant	[1]
VS185	VS184 with plasmid pVS178	[1]
MC475	86-24 *fliA* mutant	[161]
MC548	MC475 with plasmid pMC546	[161]
MC265	Single-copy *flhDC::lacZ* (+50bp to –100bp) in MC1000	[161]
MC289	Single-copy *flhDC::lacZ* (+50bp to –100bp) in VS184	[161]
MC309	Single-copy *flhDC::lacZ* (+50bp to –100bp) in VS185	[161]
MC262	Single-copy *flhDC::lacZ* (+50bp to –200bp) in MC1000	[161]
MC228	Single-copy *flhDC::lacZ* (+50bp to –200bp) in VS184	[161]
MC303	Single-copy *flhDC::lacZ* (+50bp to –200bp) in VS185	[161]
MC328	Single-copy *flhDC::lacZ* (+50bp to –300bp) in MC1000	[161]
MC291	Single-copy *flhDC::lacZ* (+50bp to –300bp) in VS184	[161]

Strain/Plasmid	Genotype/Description	Reference
Strain		
MC311	Single-copy *flhDC::lacZ* (+50bp to –300bp) in VS185	[161]
MC331	Single-copy *flhDC::lacZ* (+50bp to –450bp) in MC1000	[161]
MC292	Single-copy *flhDC::lacZ* (+50bp to –450bp) in VS184	[161]
MC313	Single-copy *flhDC::lacZ* (+50bp to –450bp) in VS185	[161]
MC322	Single-copy *flhDC::lacZ* (+50bp to –550bp) in MC1000	[161]
MC347	Single-copy *flhDC::lacZ* (+50bp to –550bp) in VS184	[161]
MC222	Single-copy *flhDC::lacZ* (+50bp to –650bp) in MC1000	[161]
MC299	Single-copy *flhDC::lacZ* (+50bp to –650bp) in VS184	[161]
MC315	Single-copy *flhDC::lacZ* (+50bp to –650bp) in VS185	[161]
MC217	Single-copy *flhDC::lacZ* (+50bp to –800bp) in MC1000	[161]
MC238	Single-copy *flhDC::lacZ* (+50bp to –800bp) in VS184	[161]
MC305	Single-copy *flhDC::lacZ* (+50bp to –800bp) in VS185	[161]
MC471A	Single-copy *flhDC::lacZ* (+50bp to –900bp) in MC1000	[161]
MC437	Single-copy *flhDC::lacZ* (+50bp to –900bp) in VS184	[161]
MC463	Single-copy *flhDC::lacZ* (+50bp to –900bp) in VS185	[161]
MC468	Single-copy *qseBC::lacZ* (-10bp to +130bp) in MC1000	[162]
MC469	Single-copy *qseBC::lacZ* (-10bp to +130bp) in VS184	[162]
MC497	Single-copy *qseBC::lacZ* (-10bp to +130bp) in VS185	[162]
MC389	Single-copy *qseBC::lacZ* (-120bp to +130bp) in MC1000	[162]
MC438	Single-copy *qseBC::lacZ* (-120bp to +130bp) in VS184	[162]
MC465	Single-copy *qseBC::lacZ* (-120bp to +130bp) in VS185	[162]

Strain/Plasmid	Genotype/Description	Reference
Strain		
MC466	Single-copy *qseBC::lacZ* (-240bp to +130bp) in MC1000	[162]
MC398	Single-copy *qseBC::lacZ* (-240bp to +130bp) in VS184	[162]
MC464	Single-copy *qseBC::lacZ* (-240bp to +130bp) in VS185	[162]
MC385	Single-copy *qseBC::lacZ* (-360bp to +130bp) in MC1000	[162]
MC298	Single-copy *qseBC::lacZ* (-360bp to +130bp) in VS184	[162]
MC476	Single-copy *qseBC::lacZ* (-360bp to +130bp) in VS185	[162]
MC468	Single-copy *qseBC::lacZ* (-500bp to +130bp) in MC1000	[162]
MC469	Single-copy *qseBC::lacZ* (-500bp to +130bp) in VS184	[162]
MC498	Single-copy *qseBC::lacZ* (-500bp to +130bp) in VS185	[162]
pMC29	*flhDC::lacZ* in pRS551, base pairs (+50 to –200)	[162]
pMC30	*flhDC::lacZ* in pRS551, base pairs (+50 to –300)	[162]
pMC31	*flhDC::lacZ* in pRS551, base pairs (+50 to –450)	[162]
pMC32	*flhDC::lacZ* in pRS551, base pairs (+50 to –550)	[162]
pMC85	*flhDC::lacZ* in pRS551, base pairs (+50 to –650)	[162]
pMC33	*flhDC::lacZ* in pRS551, base pairs (+50 to –800)	[162]
Plasmid		
pRS551	*lacZ* reporter gene fusion vector	[163]
TOPO	Cloning vector with topoisomerase	Invitrogen
pBADMycHis a	C-terminal Myc-His tag vector	Invitrogen
pACYC184	Cloning vector	NEB

Strain/Plasmid	Genotype/Description	Reference
Plasmid		
pBAD33	Cloning vector	[164]
pBR322	Cloning vector	[165]
pKM201	λRed helper plasmid	[166]
pKD3	λRed template plasmid	[166]
pCP20	λRed resolvase plasmid	[166]
pVS154	EHEC *qseB* in pBADMycHis a	[161]
pVS159	*qseBC::lacZ* in pRS551, base pairs (-500 to +130)	[167]
pVS174	EHEC *flhDC* promoter in Topo	[161]
pVS175	EHEC *fliC* promoter in pRS551	[1]
pVS178	EHEC *qseBC* in pBADMycHis a	[1]
pVSAP	*bla::lacZ* in pRS551	[78]
pMC28	*flhDC::lacZ* in pRS551, base pairs (+50 to –100)	[161]
pMC29	*flhDC::lacZ* in pRS551, base pairs (+50 to –200)	[161]
pMC30	*flhDC::lacZ* in pRS551, base pairs (+50 to –300)	[161]
pMC31	*flhDC::lacZ* in pRS551, base pairs (+50 to –450)	[161]
pMC32	*flhDC::lacZ* in pRS551, base pairs (+50 to –550)	[161]
pMC85	*flhDC::lacZ* in pRS551, base pairs (+50 to –650)	[161]
pMC33	*flhDC::lacZ* in pRS551, base pairs (+50 to –800)	[161]
pVS182	*flhDC::lacZ* in pRS551, base pairs (+50 to –900)	[1]
pMC540	*flhDC::lacZ* in pRS551, base pairs (-36 to –900)	[161]
pMC546	EHEC *fliA* in pACYC184	[161]

Strain/Plasmid	Genotype/Description	Reference
Plasmid		
pMC278	*qseBC::lacZ* in pRS551, base pairs (-360 to +130)	[162]
pMC52	*qseBC::lacZ* in pRS551, base pairs (-240 to +130)	[162]
pMC53	*qseBC::lacZ* in pRS551, base pairs (-120 to +130)	[162]
pMC54	*qseBC::lacZ* in pRS551, base pairs (-10 to +130)	[162]
Phage		
λRS45	Specialized transducing phage for constructing *lacZ* operon fusions	[163]

Recombinant DNA techniques

Standard methods were used to perform plasmid purification (Sigma Miniprep Kit) and *E. coli* K12 transformation [165]. EHEC was transformed using electroporation. Briefly, EHEC strains were grown to an $O.D._{600}$ of 0.8, centrifuged down, and the cells were resuspended in cold water. Aliquots of electrocompetent cells were electroporated using the Gene Pulser apparatus set at 200 Ω for 5 seconds. PCR reactions were performed using either *Taq* or *Pfx*-proofreading enzymes according to standard procedures [165]. DNA fragments were purified from agarose gels using the QIAquick gel extraction kit (Qiagen). All oligonucleotide primers are listed in Table 3.2. Ligation and restriction digests were carried out using enzymes purchased from Invitrogen according to manufacturer's instructions.

Plasmid pVS154 was constructed by amplifying the *qseB* gene from the K12 strain MG1655 using *Pfx* DNA polymerase (Gibco BRL) with primers K2164 and K2166 and cloning the resulting PCR product into the *EcoRI-KpnI* cloning site of vector pBADMycHisA (Invitrogen). The QseB protein from the K-12 strain has only three conserved amino acid changes from the EHEC QseB. Plasmid pVS155 was constructed by amplifying the *qseC* gene from the K12 strain MG1655 using *Pfx* DNA polymerase (Gibco BRL) with primers K2163 and K2165 and cloning the resulting PCR product into the *EcoRI-KpnI* cloning site of vector pBADMycHisA (Invitrogen). Plasmid pVS174 was constructed by amplifying the *flhD* regulatory region from EHEC using primers FlhD EH-R and FlhD EH-F.

Table 3.2 - Oligonucleotides used in this study.

Primer Name	Sequence
FlhD EH-R	5’ –CGCGGATCCTCAGCAACTCGGAGGTATGC- 3’
FlhD EH-F	5' -CCGGAATTCCAAGCATCGGCGCAGCTAAT- 3'
K2164	5' -CGGGGTACCCCTGATAGAAGATGACATGCTG- 3'
K2166	5' -CCGGAATTCCCTTTCTCACCTAATGTGTAACC- 3'
FlhDF-A	5' -CCGGAATTCACCAAAAAGTGGCTCTGCT- 3'
FlhDF-B	5' -CCGGAATTCAAAATCGCAGCCCCCCTCCG- 3'
FlhDF-C	5' -CGGGAATTCTTTGCTTGCTAGCGTAGCGA- 3'
FlhDF-D	5' -CCGGAATTCGTTGTGCGGTAAGTGTCTGT- 3'
FlhDF-E	5' -CCGGAATTCATGTACTGATTCCCCGCATT- 3'
FlhDF-F	5' -CCGGAATTCTGGAGAAACGACGCAATCCC- 3'
FlhDF-G	5' -CCGGAATTGGCTTTTGCCAGCAGTTGCTG- 3'
FliA-F	5’ –GTCGACCTGTAAATGTGAACTCCGCG- 3’
FliA-R	5’ –TCTAGACTGTTTCAGTGTAGAGCCA- 3’
FliAλRed-F long	5’ –TCATTTCACCCACTAATCGTCCGATTAAAAACCCTGC AGAAACGGATAATCATGCCGATAACTCATATAACGCAG GGCTGGTGTAGGCTGGAGCTGCTTCG- 3’
FliAλRed-R long	5’ –GATAGCGGCTTAATGGCGGTCTTTTCAGGTGCTGCAC CATCATTAAGAACTCCTGGTAGTCAAAGTTAAAGTGCGG CCATATGAATATCCTCCTTA- 3’
K2181	5' -CCGGAATTCTCCAGTGTCAGGTTTGTCAT- 3'
K2182	5' -CGCGGATCCCCGTCGCCAATCAGCATGTC- 3'
QseBF-A	5’ –CCGGAATTCCCAGTCTTTATCGACTTCACCC -3’
QseBF-B	5’ -CCGGAATTCGAGATCGTCAGAGATGCGTT -3’
QseBF-C	5’ -CCGGAATTCCTGACTTTGCGTTGCCGATG -3’
QseBF-D	5’ -CCGGAATTCATGTCTGTTTCCGAGCATTT -3’
K2164	5' -CGGGGTACCCCTGATAGAAGATGACATGCTG- 3'
K2166	5' -CCGGAATTCCCTTTCTCACCTAATGTGTAACC- 3'
ApF	5’ -GGAATTCGAAAGGGCCTCGTGATACGC -3’
ApR	5’ –CGGGATCCGGTGAGCAAAAACAGGAAGG -3’
K2026	5’ -CCGGAATTCAGCCTGACGCGCAGACTAAG -3’
K2164	5’ -CGGGGTACCCCTGATAGAAGATGACATGCTG -3’
360R	5’ – GTGAAGTCGATAAAGACTGG -3’
QseBλRed-F long	5’ –GTCCTTAACAACTTCTTAAGGGAAAAAAATAAAATT TAGTGCTGTACAGAGCGCGTTACAACACGGTTTACTGGCA GCGTGTAGGCTGGAGCTGCTTCG - 3’
QseBλRed-R long	5’ –AAAAGATTAGCGTCAGCCTGACGCGCAGACTAAGAC GTTGGGTAAATTTCATTTCTCACCTAATGTGTAACCAATA CCATGCACCATATGAATATCCTCCTTA - 3’

RNA purification

RNA purification was performed according to manufacturer's instructions using the TRIzol reagent (Invitrogen). RNA was isolated from strains 86-24, VS138, and VS179 grown in LB aerobically at 37^{o}C to an O.D.$_{600}$ of 0.8. Briefly, these cells were centrifuged and resuspended in TRIzol solution. After incubation at 65^{o}C for 10 minutes, 800 µl of chloroform was added, and the solution was centrifuged. The aqueous colorless phase was extracted, and the RNA was washed and precipitated using isopropanol. Samples were resuspended in water and stored at -80^{o}C until used.

Primer extension analysis and sequencing ladders

Primer extension analysis was performed as described previously [44]. Briefly, for determination of the *flhDC* transcriptional start site responsive to QseB&C, primer FlhD EH-R, located 10bp downstream of the ATG (Table 3.2) was end-labeled using γ^{32}P dATP. A total of 35 ug 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 FlhD EH-R and the plasmid pVS174.

In order to determine the transcriptional start site for *qseBC* responsive to QseBC, primer K2182, located 40bp upstream of the ATG (Table 3.2), was utilized. The corresponding sequencing ladder was generated using primer K2182 and the plasmid template pVS159. Mapping of the *qseBC* constitutive promoter was performed in the

same manner, using primer K2182 (Table 2) and RNA purified from strain VS138 containing plasmid pVS154 in multi-copy.

Reverse-transcriptase polymerase chain reaction (RT-PCR)

Eight micrograms of total RNA was used for cDNA synthesis using the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's instructions. The resulting cDNA was utilized for PCR with gene-specific primers targeting *qseB* (K2026) and *qseC* (K2164) (Table 3.2). In addition, a positive control with genomic EHEC DNA (strain 86-24), and a negative control without the addition of reverse-transcriptase were used.

Construction of operon fusions with *lacZ*

Construction of transcriptional operon fusions with a promoterless *lacZ* were created by PCR amplifying the regulatory regions using *Pfx* polymerase (Invitrogen) and cloning the resulting fragments into the *Eco* RI / *Bam* HI (Invitrogen) restriction sites of plasmid pRS551 [163].

For the *flhDC* promoter, pMC28 was constructed by amplifying the regulatory region upstream of *flhDC* (+50bp to –100bp) using the primers FlhD EH-R and FlhDF-A. Plasmid pMC29 was constructed by amplifying the regulatory region upstream of *flhDC* (+50bp to –200bp) using the primers FlhD EH-R and FlhDF-B. Plasmid pMC30 was constructed by amplifying the regulatory region upstream of *flhDC* (+50bp to –300bp) using the primers FlhD EH-R and FlhDF-C. Plasmid pMC31 was constructed by

amplifying the regulatory region upstream of *flhDC* (+50bp to –450bp) using the primers FlhD EH-R and FlhDF-D. Plasmid pMC32 was constructed by amplifying the regulatory region upstream of *flhDC* (+50bp to –550bp) using the primers FlhD EH-R and FlhDF-E. Plasmid pMC85 was constructed by amplifying the regulatory region upstream of *flhDC* (+50bp to –650bp) using the primers FlhD EH-R and FlhDF-F. Plasmid pMC33 was constructed by amplifying the regulatory region upstream of *flhDC* (+50bp to –800bp) using the primers FlhD EH-R and FlhDF-G. These plasmids were introduced into wild-type (86-24), *qseC*⁻ (VS138), and complemented (VS179) EHEC strains.

For the *qseBC* promoter, plasmid pMC278 was constructed by amplifying the regulatory region upstream of *qseBC* (-360bp to +130bp) using primers QseBF-A and K1282. Plasmid pMC52 was constructed by amplifying the regulatory region upstream of *qseBC* (-240bp to +130bp) using primers QseBF-B and K2182. Plasmid pMC53 was constructed by amplifying the regulatory region of *qseBC* (-120bp to +130bp) using primers QseBF-C and K2182. Plasmid pMC54 was constructed by amplifying the regulatory region of *qseBC* (-10bp to +130bp) using primers QseBF-D and K2182. All primer sequences are listed in Table 3.2.

To generate single-copy chromosomal *lacZ* fusions, the constructs in pRS551 were transferred into the chromosome of MC1000 (*recA*+) using the specialized transducing phage λRS45 [163]. Overnight cultures of these donor strains were resuspended in 0.5 volumes 0.01 M $MgSO_4$ and combined with 1 x 10^7 pfu. Phage particles were allowed to absorb for 15 minutes at 27°C, placed in 2.5 mL top agar, and overlaid on LB plates that were incubated overnight at 37°C. Phage lysates were then harvested. In order to transfer fusions to single-copy into the chromosome, these phage

lysates were combined with an equal volume of the recipient strains, MC1000 and VS184 in 0.01 M $MgSO_4$, and absorbed for 15 minutes at room temperature. At this point, 2 mL LB supplemented with 2 mg/ml maltose was added, incubated for two hours at 37^{o}C, and plated on LB agar containing kanamycin and X-gal. All resulting transductants were streak purified, screened for loss of ampicillin resistance, and assayed for β-galactosidase activity. The resulting transductants are listed in Table 3.1.

β-galactosidase assays

Bacteria containing *lacZ* fusions were grown overnight at either 37^{o}C in LB or 30^{o}C in tryptone media containing the appropriate selective antibiotic. Cultures were then diluted 1:100 and grown in LB supplemented with 0.2% arabinose to an $O.D._{600}$ of 0.8. These cultures were then diluted 1:10 in Z buffer (60 mM $Na_2HPO_4 \bullet 7H_20$, 40 mM $NaH_2PO_4 \bullet H_20$, 10 mM KCl, 1 mM $MgSO_4 \bullet 7H_20$, 50 mM β-mercaptoethanol) and assayed for β-galactosidase activity using o-nitrophenyl-β-D-galactopyranoside (ONPG) as a substrate as described previously [168].

Purification of QseB-His and QseC-His under native conditions

In order to purify the His-tagged QseB protein, the *E. coli* strain containing pVS154 was grown at 37^{o}C in LB to an $O.D._{600}$ of 0.7, at which point arabinose was added to a final volume of 0.2% and allowed to induce for three hours. QseC-His was purified in the same fashion from a strain containing pVS155. Protein purification was then performed using nickel columns according to manufacturer's instructions (Qiagen). Briefly, bacterial cells were pelleted and run through an Emulsiflex high-pressure

homogenizer. 5 mL of NiNTA slurry (Qiagen) was then added to the cell lysate mixture for one hour at 4^{o}C. The lysates/NiNTA mixture was then run through a nickel column (Qiagen), washed with lysis buffer (50 mM NaH_2PO_4 pH 8.0, 300 mM NaCl, 20 mM imidazole), and eluted with elution buffer (50 mM NaH_2PO_4 pH 8.0, 300 mM NaCl, 250 mM imidazole). Protein purity was verified by running on an SDS-PAGE gel. Both QseB-His and QseC-His are termed "QseB" or "QseC" throughout this dissertation.

Electrophoretic mobility shift assays (EMSAs)

In order to study the binding of QseB to the *flhDC* promoter, EMSAs were performed using purified QseB-His and PCR-amplified DNA probes. Probes were end-labeled with [γ-^{32}P]-ATP (NEB) using T4 polynucleotide kinase using standard procedures [165], and gel-purified using the Qiagen PCR purification kit. EMSAs were performed by adding increasing amounts of purified QseB-His protein (0 to 10 μg) to end-labeled probe (10 ng) in binding buffer (500 μg ml^{-1} BSA (NEB), 50 ng poly-dIdC, 60 mM HEPES pH 7.5, 5 mM EDTA, 3 mM DTT, 300 mM KCl, 25 mM $MgCl_2$) with or without 0.1 M acetyl phosphate for 20 minutes at 4^{o}C. Immediately before loading, a 5% ficol solution was added to the mixtures. The reactions were electrophoresed for approximately 8 hours at 180 V on a 6% polyacrylamide gel, dried, and exposed to KODAK X-OMAT film. Double EMSAs were performed in the same manner, using identical concentrations of each end-labeled probe (5 ng). Competition reactions were performed by adding increasing amounts of unlabeled probe to EMSA reactions.

In order to study the direct binding of QseB to its own promoter, electrophoretic mobility shift assays were performed using the purified QseB-His and PCR amplified

DNA probes. *Pfx* (Invitrogen) was used to amplify the *qseBC* DNA probe from pMC53 (-120bp to +130bp of *qseBC*) using primers QseB-B and K2182. The *bla* region, used as a negative control, was amplified from pBR322 using primers ApR and ApF. DNA probes were then end-labeled with [γ-^{32}P]-ATP (NEB) using T4 polynucleotide kinase using standard procedures [165]. End-labeled fragments were run on a 6% polyacrylamide gel, excised, and purified using the Qiagen PCR purification kit.

DNaseI footprinting

DNaseI footprints were performed as described previously [52]. In order to footprint the *flhDC* promoter, PCR primers flhDC-C and flhDFR-F were end-labeled with [γ-^{32}P]-ATP (NEB) using T4 polynucleotide kinase using standard procedures [165] and utilized in a PCR reaction with unlabeled primers flhD EH-R and flhDF-F, respectively, to create single-end labeled PCR probes. The binding reactions were performed as described for EMSAs with purified, phosphorylated QseB-His (0 to 5μg) for 20 minutes at 4^{o}C. A 1:500 dilution of DNaseI (Invitrogen), 5 mM $MgCl_2$ and 1 mM $CaCl_2$ were added and incubated at room temperature for 2 minutes. The reaction was stopped by adding 100 μl of stop solution (200 mM NaCl, 2 mM EDTA, and 1% SDS). The protein was then subject to phenol-chloroform extraction, and the DNA was precipitated using 7.5 M NH_4OAc, pH 7.5, 100% EtOH, and 1 μ1 glycogen. The DNaseI reactions were run on a 6% polyacrylamide gel next to a sequencing reaction (Sequenase kit, USB) from pVS182 using primers flhD EH-R and flhDF-F.

In order to footprint the *qseBC* promoter, primers K2181 and K2182 were end labeled by standard procedures [165] using [γ-^{32}P]-ATP (NEB). The resulting labeled

primers were utilized in a PCR reaction with unlabeled primers 360R and QseBF-C (Table 3.2), respectively. The resulting single-end labeled PCR products were used in binding reactions as described for EMSAs with purified, phosphorylated QseB-His (0 to 5 μg) for 20 minutes at 4^{o}C and as described above. Once again, the DNaseI reactions were run on a 6% polyacrylamide gel next to a sequencing reaction (Sequenase kit, USB) from pVS159 using primers K2181 and K2182.

fliA isogenic mutant construction

Construction of an isogenic *fliA* mutant was carried out as previously described [166]. Briefly, 86-24 cells containing pKM201 were prepared for electroporation, as described for DNA methods. A *fliA* PCR product was generated using primers FliAλRed-F long and FliAλRed-R long and pKD3 as a template (Table 2) and gel purified. Electroporation of the PCR product into these cells was performed, cells were incubated at 37^{o}C for 2 hours and the expression of lambda Red Recombinase was induced. These cells were then plated on media containing 30 μg ml^{-1}chloramphenicol overnight at 37^{o}C. Resulting colonies were patched for chloramphenicol resistance, to select for recombination, and ampicillin sensitivity, to select for loss of the plasmid expressing the recombinase. Positive clones were then verified by PCR for the absence of the gene. The chloramphenicol cassette was then resolved from the mutant in order to create a nonpolar, isogenic *fliA* mutant. Plasmid pCP20, encoding a resolvase, was electroporated into the mutant strain in order to eliminate the chloramphenicol cassette. The resulting colonies were patched for chloramphenicol sensitivity, and sequenced in order to verify that the loss of the gene did not result in a frame shift mutation. The

nonpolar *fliA* mutant was then complemented with vector pMC546 that was constructed by amplifying the *fliA* gene from EHEC genomic DNA using primers FliA-F and FliA-R and cloning the resulting fragment into the *Sal I* / *Xba I* sites of vector pACYC184.

qseB isogenic mutant construction

Construction of an isogenic *qseB* mutant in EHEC was carried out as described above for *fliA* [166]. The *qseB* PCR product was generated using primers QseBλRed-R long and QseBλRed-F long (Table 3.2). The resulting colonies were PCR verified and sequenced for the absence of the gene.

Western blotting

Polycolonal anti-flagellin antiserum for western blot experiments was obtained from Dr. James B. Kaper at the University of Maryland. Total protein extracts were prepared from strains 86-24, MC475, and MC548 grown in either LB or tryptone broth to an $O.D._{600}$ of 0.8. The same amount of protein (determined using the Biorad protein assay) was added in each lane of the SDS-PAGE. A cross-reactive band was utilized as a loading control.

Polycolonal anti-Myc antiserum for western blot experiments was obtained from Invitrogen and used at a dilution of 1:5000. Liposome fractions containing reconstituted QseC-His protein were blotted according to standard procedures [165].

Motility assays

Motility assays were performed at 37^{o}C on 0.3% agar plates containing tryptone media (1% tryptone and 0.25% NaCl) or LB. Overnight cultures were stabbed into the motility agar, and motility halos were measured at 4, 8, and 16 hours.

Reconstitution of QseC-His into liposomes

Liposomes were reconstituted as described by Janausch *et al* [169]. Briefly, 50 mg of *E. coli* phospholipids (Avanti Polar Lipids, 20 mg/ml in chloroform) were evaporated and then dissolved into 5 ml potassium phosphate buffer containing 80 mg N-octyl-β-D-glucopyranoside. The solution was dialyzed overnight against potassium phosphate buffer. The resulting liposome suspension was subjected to freeze/thaw in liquid N_2. The liposomes were then destabilized by the addition of 26.1 mg dodecylmaltoside, and 2.5 mg of QseC-His was added, followed by stirring at room temperature for 10 minutes. 261 mg of Biobeads were then added to remove the detergent, and the resulting solution was allowed to incubate at 4^{o}C overnight. The supernatant was then incubated with fresh Biobeads for 1 hour in the morning. The resulting liposomes containing reconstituted QseC-His were frozen in liquid N_2 and stored at -80^{o}C until used.

Phosphorylation of QseC-His in liposomes

20 µl of the liposomes containing QseC-His were adjusted to 10 mM $MgCl_2$ and 1 mM DTT, and various concentrations of agonist or antagonist (see below), frozen and

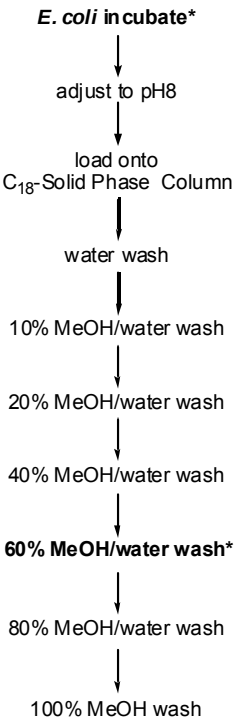
thawed rapidly in liquid N_2, and kept at room temperature for 1 hour. 0.625 µl of [γ^{32}P] dATP (110 TBq/mmol) was added to each reaction. To some reactions, 10 µg of QseB-His was added. At each time point (0, 10, 30, 60, or 120 minutes), 20 µl of SDS loading buffer was added. For all experiments involving QseC alone, a time point of 10 minutes was used. The samples were run on SDS-PAGE according to standard procedures [165] and visualized via phosphorimager. The bands were quantitated using ImageQuant version 5.0 (Amersham) software.

Agonists and antagonists

Various concentrations of agonist or antagonist were (Sigma) added to each of the liposome experiments, resulting in final concentrations as follows: 5 µM or 50 µM epinephrine, 50 µM norepinephrine , 50 µM or 500 µM phentolamine, 50 µM or 500 µM propranolol, 50 µM gastrin, 50 µM galanin, 50 µM secretin, 50 µM clonidine, and 500 µM yohimbine. Synthetic AI-2 (1:10) and purified AI-3 (1:10) were purified as described previously [119]. Briefly, AI-2 was synthesized *in vitro* using His-tagged purified Pfs, LuxS, and S-adenosyl-homocysteine substrate. The AI-2 reaction mixtures contained 1 mM substrate, 10 mM sodium phosphate buffer, and 1 mg/ml of each fusion protein. After incubation, reactions were run through Biomax-5 ultrafree centrifugation filters (Millipore) to remove protein from the reaction products. AI-3 was purified by growing EHEC strain 86-24, which produces AI-3, in DMEM at 37^0C to an OD_{600} of 1.0 (Figure 3.1). The bacteria were pelleted by centrifugation and the supernatant was filtered to ensure that it was free of bacterial cells. With the assistance of Bhavani Sangres and J.R. Falck (Department of Biochemistry, UT Southwestern Medical Center),

we then performed size exclusion through a 3 kDa filter, adjusted the supernatants to pH 3, and passed the supernatant through a C18-sold phase extraction column. The column was then washed with methanol, pH 8 buffered, and sequentially washed with water and increasing portions of methanol. AI-3 was selectively removed in the 60% Methanol/water fraction, evaporated, and purified by RP-HPLC (Figure 3.1). Tritiated norepinephrine was obtained from Amersham Biosciences and used at a final concentration of 5 μM or 10 μM.

Figure 2. C_{18}-Solid Phase Extraction Scheme



**biologically active fraction*

Figure 3.1 – AI-3 purification scheme. *E. coli* are grown and the supernatant is collected. Size exclusion is then performed through a 3kDa filter, and the supernatants are adjusted to pH 3 and passed through a C18-sold phase extraction column. The column is then washed with methanol, pH 8 buffered, and sequentially washed with water and increasing portions of methanol. AI-3 is selectively removed in the 60% Methanol/water fraction, evaporated, and purified by RP-HPLC.

Determination of tritiated ligand binding

In order to determine the concentration of tritiated norepinephrine that was bound to QseC-His in the liposomes, 20 µl of the liposome containing QseC-His were adjusted to 10 mM $MgCl_2$ 1 mM DTT, and either 5 µM tritiated norepinephrine, 10 µM tritiated norepinephrine or 5 µM tritiated norepinephrine plus 50 µM phentolamine. The liposomes were frozen and thawed rapidly in liquid N_2, and kept at room temperature for 1 hour. 0.625 µl of [γ^{32}P] dATP (110 TBq/mmol) was added to each reaction. After 10 minutes, SDS loading dye was added, and the samples were run on SDS-PAGE according to standard procedures [165] and visualized via phosphorimager. The bands containing phosphorylated QseC-His were precisely excised and then counted in a scintillation counter.

CHAPTER FOUR

QseC, A BACTERIAL ADRENERGIC RECEPTOR

INTRODUCTION

It is estimated that the total microbial population within the gastrointestinal (GI) tract (approximately 10^{14} cells) exceeds the total number of mammalian cells (approximately 10^{13}) by an order of magnitude [170]. The density of bacteria can vary greatly along the GI tract, the site of the largest and most complex environment in the mammalian host. It is estimated that the majority of the normal flora, which co-exist intimately with their host, reside in the colon [170]. The bacterial flora is extremely important in human development, as well as in shaping the innate immune system [171]. With the enormous number and diversity of bacteria present in the GI tract, it is not surprising that the members of this community may communicate amongst themselves and with the host to coordinate various processes.

The intestine plays host to a broad range and high density of normal bacterial flora that are beneficial in human development. Detrimental interactions with pathogenic bacteria, however, can lead to the development of disease. Given these polar relationships, it is important to study communication between prokaryotes and their eukaryotic host. It has been shown that the epithelia from the GI tract maintain an inflammatory hyporesponsiveness toward the prokaryotic normal flora [172], and that bacterial autoinducers have immunomodulatory activities, including the production of IL-8 [173], the inhibition of lymphocyte proliferation, TNFα, and IL-12 production [130].

Currently, little is known about the signaling interactions between pathogenic bacteria and the host. It has been hypothesized that bacteria must sense and recognize the host environment in order to activate genes essential for colonization. We tested this idea at a molecular level using EHEC 0157:H7, a pathogenic bacterium capable of colonizing the human intestine and causing the development of hemorrhagic colitis and hemolytic uremic syndrome after ingestion of contaminated food or water [3]. Sperandio *et al.* previously reported that EHEC activates its virulence genes in response to the autoinducer AI-3, produced by the endogenous GI bacterial flora, and human hormones produced naturally by the host, epinephrine/norepinephrine [119]. An isogenic mutant in *qseC*, a sensor kinase in the predicted QseBC two-component system in EHEC, was unable to recognize both bacterial AI-3 and host epinephrine [119], suggesting that QseC may be acting as a bacterial adrenergic receptor that interacts directly with these compounds. This hypothesis, which we test here, suggests that there may be potential cross-communication between the AI-3 bacterial signaling system and the epinephrine/norepinephrine signaling system through QseC.

RESULTS

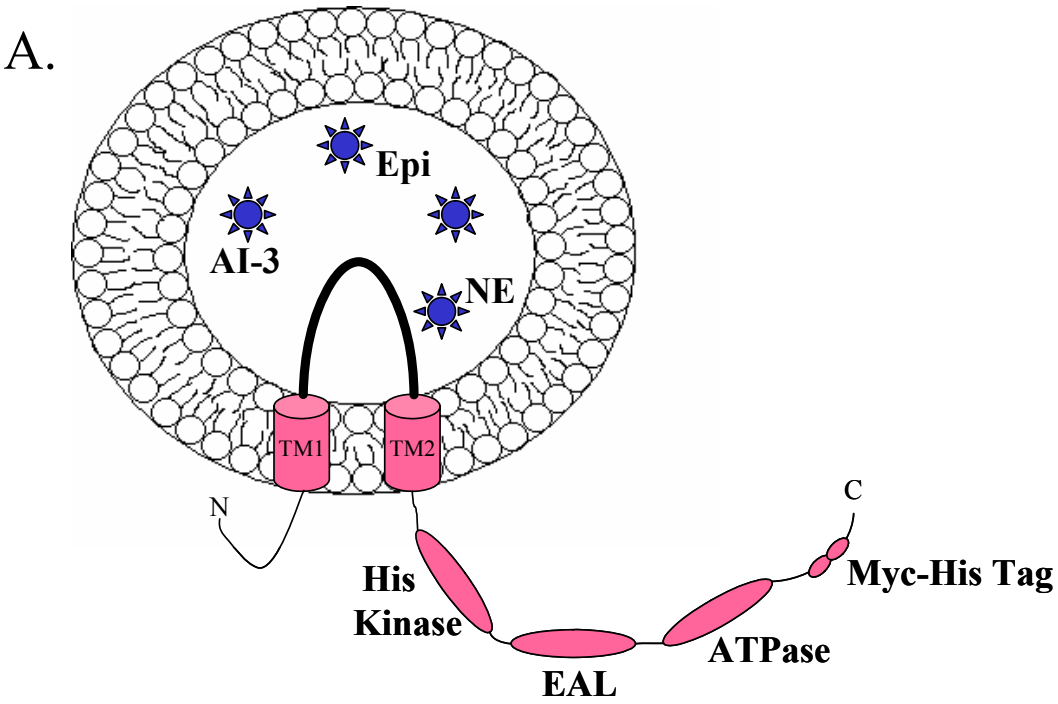
Reconstitution of QseC into liposomes

In order to determine the role of the QseB&C two-component system in signaling, *qseC* from EHEC was cloned into a C-terminal Myc-His vector, overexpressed, and purified under native conditions on a nickel-affinity column. We were not able to observe QseC autophosphorylation in solution under various buffer conditions *in vitro* (data not shown). This is not unusual, as most studies of sensor kinases were gained with soluble domains of the protein obtained by genetic truncation [174-180]. However, Janausch *et al.* were able to study the phosphorylation and signaling of the DcuSR two-component system by solubilizing DcuS and reconstituting it into an artificial membrane system, liposomes [181]. As most sensor kinases are membrane-bound, we also endeavored to reconstitute purified QseC-MycHis protein into "*in vitro*" synthesized liposomes.

Liposomes afford several advantages over other *in vitro* phosphorylation studies, especially considering the fact that the QseC sensor kinase is membrane-bound *in vivo*. Firstly, functionality can be easily assessed due to the fact that QseC-MycHis adopts an inside-out orientation (Figure 4.1A); that is to say that the stimulus binding domain is inside the liposomes while the kinase domain is located outside. Orientation has been established by previous groups [174] and can be concluded from the accessibility of ATP to the kinase site without disruption of the liposomes. Additionally, this system allows us

to “load” the liposome with agonists and antagonists so that we can determine the effect of specific compounds on the auto-phosphorylation of QseC.

To create the liposomes, 50 mg of *E. coli* phospholipids were evaporated and dialyzed. The resulting liposomes were incubated with small amounts of dodecylmaltoside to achieve “onset solubilization” [182]. QseC-MycHis was then mixed into the solution, and the detergent was removed by treatment with BioBeads, resulting in the successful incorporation of QseC-MycHis into liposomes. In order to verify protein incorporation, western blot analysis using anti-Myc antibody was performed (Figure 4.1B). QseC-MycHis, hereafter referred to as QseC, was successfully incorporated into liposomes in all cases.



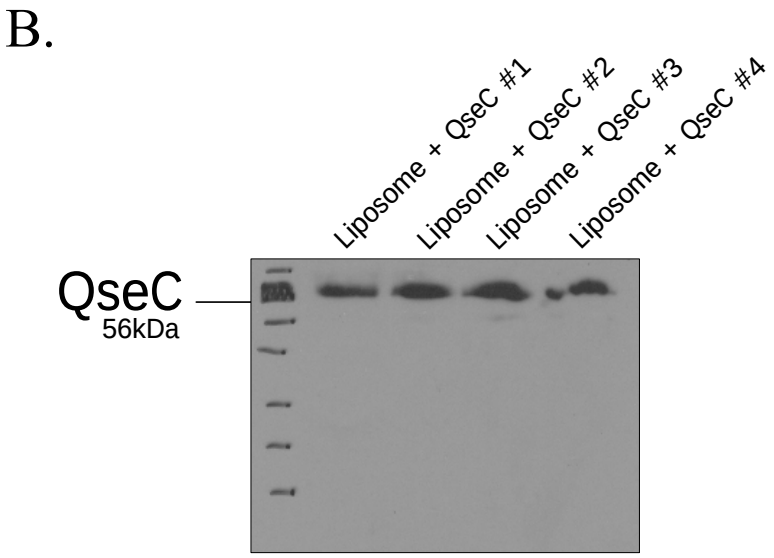


Figure 4.1 – QseC-MycHis Reconstituted into Liposomes. A.) Graphic depiction of the "inside out" orientation of QseC-MycHis in the liposome. B.) Western blot of QseC-MycHis liposomes using an anti-myc antibody.

QseC autophosphorylation in response to agonists and antagonists

Because it has previously been shown that a *qseC* mutant is unable to respond to the addition of epinephrine/norepinephrine, we hypothesized that QseC may be directly sensing these signaling compounds. To test this theory, we reconstituted QseC into the liposome system, with or without loading the liposomes with 5-50 μM epinephrine. Figure 4.2A shows that, in the presence of epinephrine, QseC appears to increase its level of autophosphorylation as compared to liposomes to which no epinephrine was added. This suggests that QseC is a bacterial protein that may be directly responding to the presence of the human hormone, epinephrine. In order to further determine the specificity of the response of QseC to epinephrine/norepinephrine, we tested whether QseC could sense and respond to other intestinal hormones. Figure 4.2B shows that QseC autophosphorylation increases with the addition of both 5 μM and 50 μM epinephrine, although the effect is only statistically significant with 50 μM epinephrine. The addition of 50 μM norepinephrine (NE) also acts to increase the autophosphorylation of QseC (data not shown). The addition of other intestinal hormones, 50 μM gastrin, 50 μM secretin, or 50 μM galanin, had no effect on the amount of QseC autophosphorylation (Figure 4.2B). Each graph represents results from three separate experiments, on which we performed a student's T-test in order to determine whether the results were statistically significant. The level of phosphorylation was not significantly different between any of the intestinal hormones (secretin, gastrin, and galanin) and the control where no signal was added, suggesting that these hormones do not play a role in signaling

through QseC. However, these results show that there is a statistically significant response to epinephrine (Figure 4.2A) and NE (data not shown).

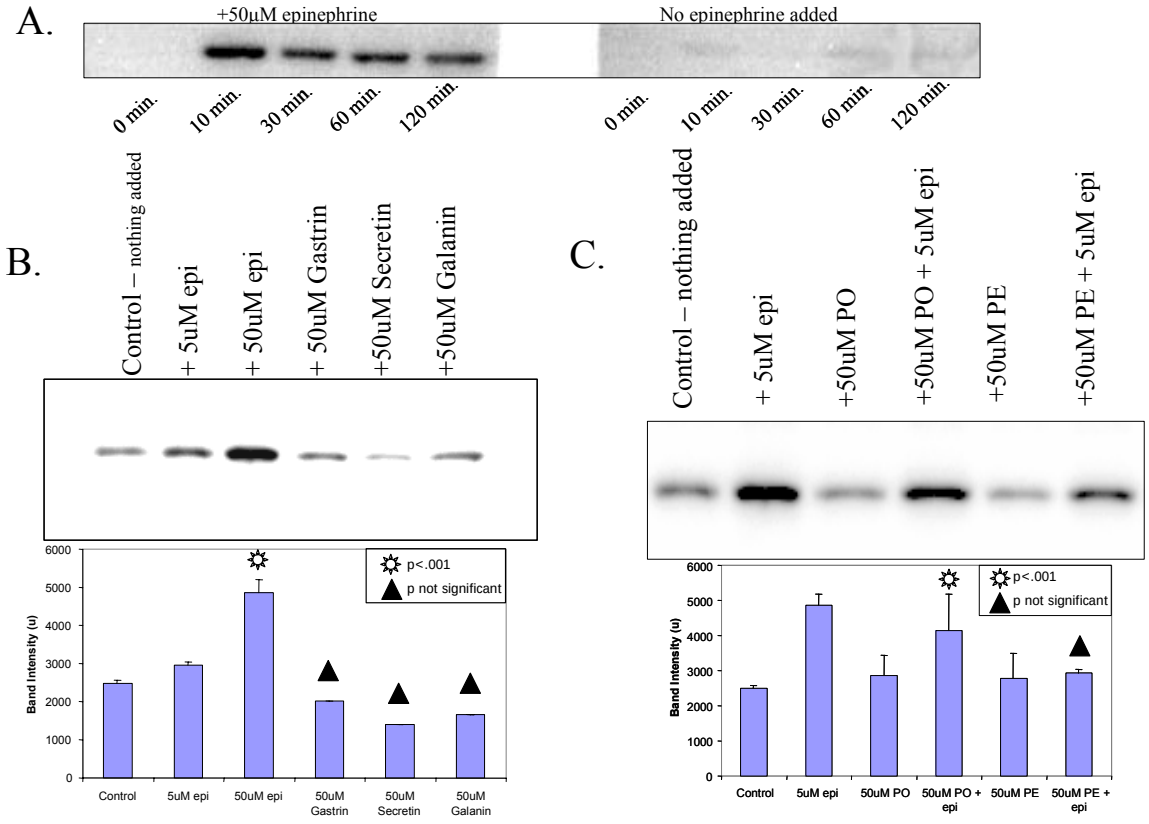


Figure 4.2 – QseC autophosphorylation in response to agonists/antagonists. A.) QseC increases its autophosphorylation in response to epinephrine. B.) QseC does not increase its autophosphorylation in response to other intestinal hormones, including gastrin, galanin, or secretin. C.) The autophosphorylation of QseC is inhibited by the α -adrenergic antagonist, phentolamine (PE) but not the β -adrenergic antagonist, propranolol (PO).

It is known that there is a considerable amount of epinephrine and NE in the human intestinal tract, and that its neuronal response in the colon can be blocked by the β-adrenergic receptor antagonist propranolol (PO) and the α-adrenergic receptor agonist phentolamine (PE). Due to the fact that QseC autophosphorylation appears to be responding to the addition of Epi/NE, we investigated whether we could block this response using PO or PE. Figure 4.2C shows that the addition of PO or PE alone appears to have no effect on QseC autophosphorylation. According to our previous experiments, the addition of 5 μM Epi induced QseC autophosphorylation. To address whether PO and PE could act as antagonists of Epi for QseC autophosphorylation, these experiments were performed in the presence of 5 μM Epi and an excess concentration (50 μM) of PE or PO. Autophosphorylation of QseC is increased by Epi in the presence of PO (Figure 4.2C), suggesting that PO cannot antagonize the recognition of Epi by QseC. However, in the presence of PE, QseC was unable to respond to Epi (Figure 4.2C), suggesting that this α-adrenergic antagonist can block Epi recognition by QseC.

We have previously shown that epinephrine/NE appear to cross-signal with the bacterial autoinducer AI-3 and not AI-2 [119]. In order to test whether QseC could respond to either of these autoinducer compounds, we performed the liposome experiment, adding either a 1:10 dilution of synthesized AI-2 (roughly 100 μM) or a 1:10 dilution of purified AI-3 (roughly 100 nM). Figure 4.3 shows that the addition of synthesized AI-2 appears to have no statistically significant difference in QseC autophosphorylation as compared to our negative control, where no signal was added to the liposome. The addition of a 1:10 dilution of purified AI-3, however, did significantly

increase the amount of QseC autophosphorylation to a level similar to that obtained with epinephrine.

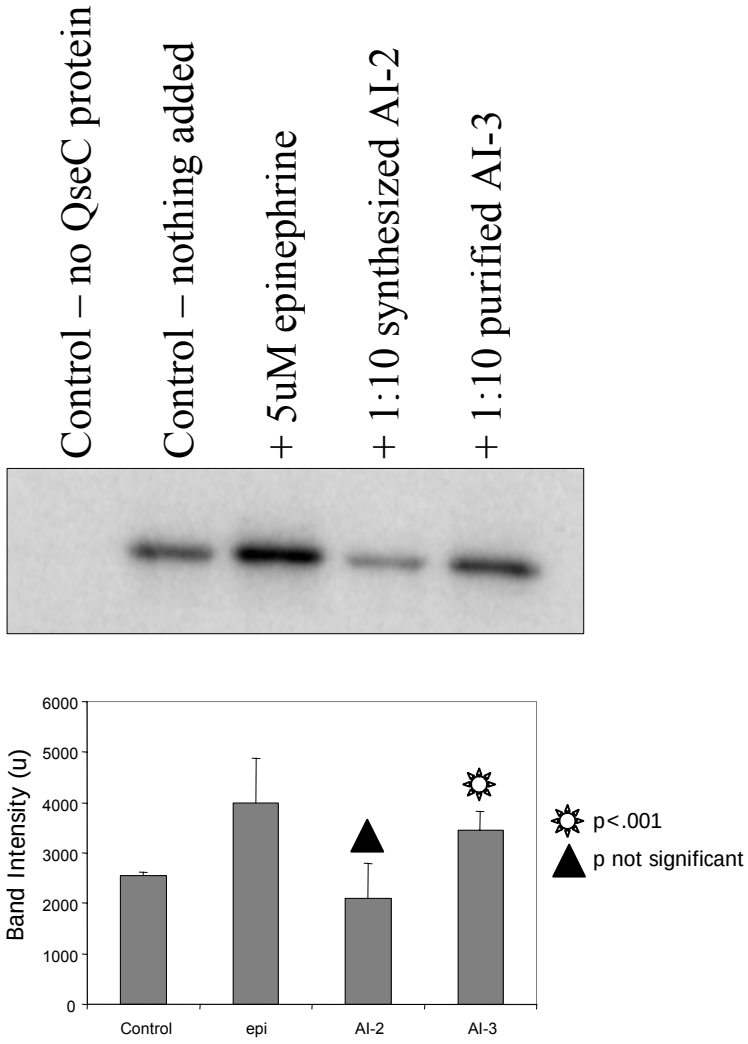


Figure 4.3 – QseC autophosphorylates in response to AI-3 but not AI-2.

The DcuS sensor kinase does not respond to adrenergic agonists

In order to verify the specificity of the response of QseC to AI-3 and epi/NE, we constructed a hybrid protein as a negative control. The hybrid was created by swapping out the periplasmic sensing domain of QseC for the periplasmic sensing domain of EnvZ, a well-studied two-component signaling protein. EnvZ is a membrane-bound sensor histidine kinase in *E. coli,* which has been shown to play a pivotal role in bacterial cell adaptation to changes in low extracellular osmolarity [183]. We were able to successfully create this hybrid protein and observe its complementation of the motility phenotype of a *qseC* mutant in EHEC in response to low osmolarity in tryptone media (Figure 4.4A). Motility of the *qseC* mutant complemented with the hybrid protein, however, was not restored in high osmolarity in LB media (Figure 4.4B).

Although we were able to observe the complementation of the motility phenotype by the hybrid sensing osmolarity in motility plates, we were not able to observe any autophosphorylation of the hybrid protein in the *in vitro* liposome assay (data not shown). It is possible that no phosphorylation was observed due to the fact that the specific signal for EnvZ is unknown. Although the soluble cytoplasmic histidine kinase domain of EnvZ has been extensively studied, little is known about the structure of its periplasmic domain, which has been implicated in the sensing of specific signals that underlies its osmosensing function.

The only sensor kinase in *E. coli* other than QseC that has had its signal identified biochemically is the two-component sensor kinase DcuS. We decided to utilize this protein as a negative control for AI-3/Epi/NE sensing in future studies. DcuS of *E. coli* is

a membrane-bound sensor kinase that has successfully been reconstituted into liposomes [169]. Additionally, Janausch *et al.* have previously shown that DcuS increases its phosphorylation in liposomes in response to the signals fumarate and succinate. Thus, future studies will utilize DcuS as a negative control to observe the effect of AI-3 and epi/NE on its autophosphorylation and signaling.

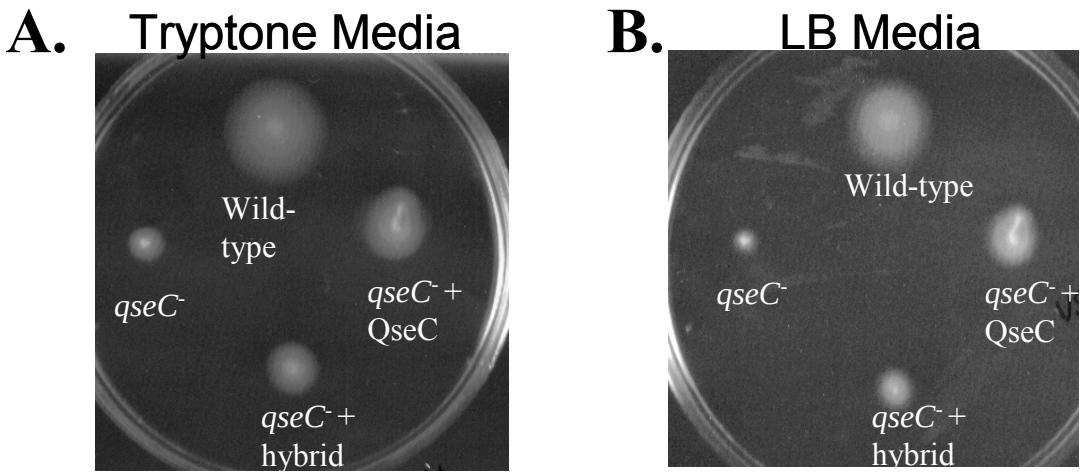


Figure 4.4 - Motility plate of wild-type, *qseC* mutant, and the *qseC* mutant complemented with the QseC/EnvZ hybrid protein. A.) Tryptone media alone (low osmolarity). B.) LB media alone (high osmolarity).

QseC binds to tritiated norepinephrine

Because we observed that the autophosphorylation of QseC increased in response to Epi, NE, and AI-3, we hypothesized that these compounds were directly interacting with the membrane-bound QseC sensor kinase to stimulate signaling. In order to study binding, we again utilized the QseC liposome system. 5 μM and 10 μM tritiated norepinephrine (Amersham) alone or with 50 μM phentolamine were "loaded" into QseC-MycHis liposomes. Next, [γ^{32}P] dATP was added to the mixture for ten minutes.

Figure 4.5A reveals that QseC autophosphorylation increases with the addition of norepinephrine, as expected. The addition of 5 μM norepinephrine plus 50 μM phentolamine appears to show no significant increase in the amount of QseC autophosphorylation as compared to liposomes with no signal added, again suggesting that phentolamine antagonizes NE recognition. In order to determine whether or not the tritiated norepinephrine was binding to QseC, we then proceeded to excise the bands that contained phosphorylated QseC protein. The bands were resuspended in scintillation fluid and counted, which allows the differential counting of ^{3}H-NE (to assess binding) and γ^{32}P-ATP (to assess autophosphorylation). Figure 4.5B graphically shows the results of the study. When 5 μM norepinephrine was added to the QseC liposome, we observed a 2.25-fold increase in the amount of tritium sample, which correlates with an increase in QseC autophosphorylation. When 10 μM norepinephrine was loaded into the liposome, we observed a 4-fold increase in the amount of tritium in the sample but no significant increase in the amount of QseC autophosphorylation. This could be due to the fact that QseC is very sensitive, and even a small amount of signal activates full autophosphorylation for a quick response. Finally, when 5 μM NE and 50 μM PE were both loaded into the liposome, we observe a dramatic, 6-fold decrease in the amount of tritiated NE bound to QseC. These data also correlate with the fact that there is no statistically significant increase in QseC autophosphorylation in the presence of excess PE, again suggesting that PE antagonizes the recognition of NE by QseC. These data suggest that NE is directly interacting with QseC in order to increase autophosphorylation and that phentolamine antagonizes NE recognition by QseC.

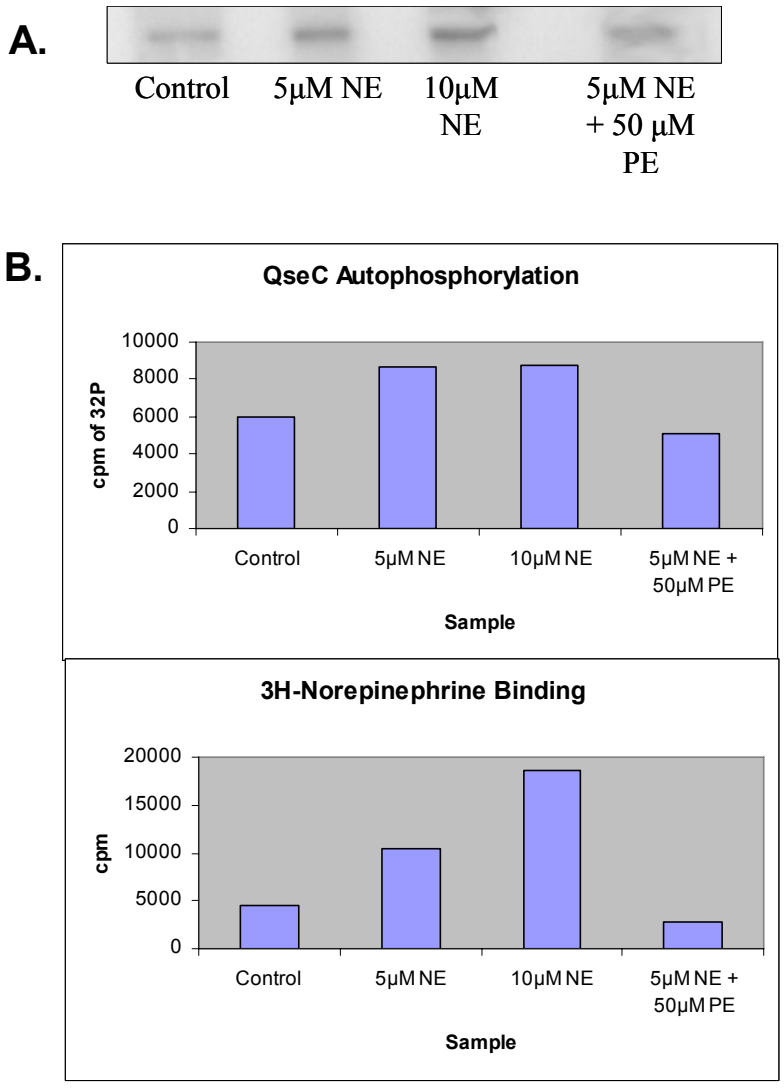


Figure 4.5 – QseC increases autophosphorylation after a direct interaction with tritiated norepinephrine. A.) Autophosphorylation of QseC increases in response to NE and but is inhibited by PE. B.) Graphical representation of the level of QseC autophosphorylation (^{32}P) and tritiated NE binding (^{3}H).

QseC transfers a phosphate to its cognate response regulator, QseB

QseB is predicted to be the cognate response regulator for QseC. In order to determine whether QseB receives a phosphate from the QseC sensor kinase, we again utilized QseC-His liposomes loaded with 50 μM epinephrine. 5 μg of purified QseB-His and 250 μCi of γ^{32}P dATP were also added to the reaction, after the liposomes had been stabilized. Figure 4.6 shows that the QseC sensor kinase begins to autophosphorylate after ten minutes of incubation in the presence of Epi and transfers its phosphate to its cognate response regulator, QseB, following sixty minutes of incubation. After 120 minutes of incubation, it appears that QseC has transferred most of is phosphate to QseB. These data confirm that QseB&C do comprise a two-component system where QseC autophosphorylates itself and transfers its phosphate to its cognate response regulator, QseB.

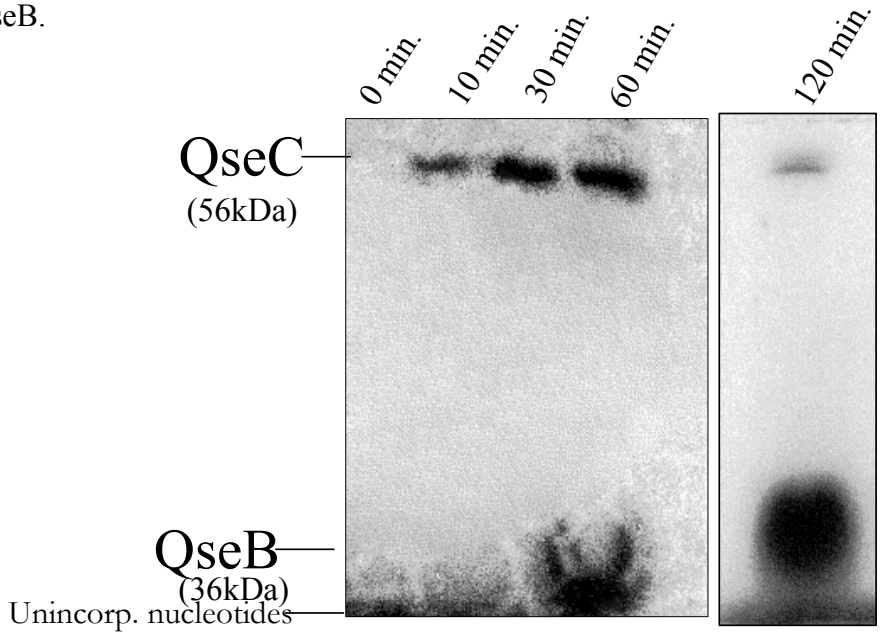


Figure 4.6 – QseC transfers its phosphate to QseB, its cognate response regulator.

DISCUSSION

Given the fact that the human GI tract is colonized by hundreds of different commensal bacteria, it is not surprising that some form of bacteria-host communication should occur. The recent discovery of a new bacterial signaling molecule named AI-3, which cross-signals with the host epi/NE hormones, has led to a breakthrough in the study of cell-to-cell signaling between the host and pathogenic organisms [119]. AI-3 activity has been found to be present in supernatants from several bacteria species, including EHEC, enteropathogenic *E. coli, Shigella* sp., *Salmonella* sp., *E. coli* K-12, *Klebsiella pneumoniae,* and *Enterobacter cloacae* (Sircili and Sperandio, unpublished results). These data may suggest that AI-3 is an important signaling molecule that is present in many species of bacteria. Thus, the possibility arises that EHEC has utilized its AI-3 QS system to exploit host signaling by "hijacking" Epi/NE compounds to activate its virulence genes and colonize the host.

Histidine kinases (HK) are arguably one of the most widely used sensors of all signal-transduction enzymes in nature, being present in the Bacteria, Archaea and Eukarya [184, 185]. Bacteria utilize these HKs in order to coordinate responses to environmental cues. Although there are no known HK present in animals, eukaryotes such as yeasts, fungi, plants, and protozoa also use histidine kinases in order to regulate hormone-dependent developmental processes [185]. Thus, it has been suggested that HK originated in bacteria and were later transferred into eukaryotes and archaea [186]. Consequently, HKs have a complex evolutionary history that has only begun to be understood.

In this study, we have utilized an *in vitro* liposome system that allowed us to reconstitute purified QseC protein into a membranous environment. Additionally, the liposomes can be "loaded" with signaling compounds in order to gauge the response of QseC to specific agonists and antagonists. We were able to successfully observe an increase in QseC auto-phosphorylation (in response to AI-3 and epi/NE) and transfer of phosphate to QseB (Figures 4.1 – 4.6). Furthermore, we demonstrated that QseC auto-phosphorylation could be blocked by the addition of the α-adrenergic antagonist, phentolamine (PE). This suggests that QseC's recognition of signaling compounds may more closely resemble that of an α-adrenergic receptor.

Very few signals or inhibitors for two-component systems are currently known. Roychoudhury [187] was the first to identify an inhibitory compound of the AlgR21 two-component system of *Pseudomonas aeruginosa*. AlgR21 regulates the transcription of alginate, which inhibits access of antimicrobials to the site of infection in cystic fibrosis patients [188]. Of the 25,000 synthetic and natural compounds screened, only 15 inhibitors were identified, many of which contained aromatic rings [187]. Additionally, inhibitors have been identified through large screens, including several inhibitors for the KinA/SpoOF two-component system in *B. subtilis* [189, 190]. While many compounds have been reported as inhibitors of two-component systems, their mechanism of action and actual stimulatory signals are largely unknown [191]. Even less is known about signaling compounds that activate HKs. One of the few systems for which a defined signal has been identified is the DcuS&R two-component system of *E. coli,* which controls the expression of genes of C(4)-dicarboxylate metabolism [192]. Janausch *et al.*

have shown that the autophosphorylation of DcuS, reconstituted into liposomes, is stimulated by the signals fumarate and succinate [181].

During infection, EHEC may utilize the QseB&C two-component signaling system to activate transcription of virulence genes. Our datum suggests that both the AI-3 produced by the normal flora and the Epi/NE produced naturally by the host are sensed by QseC, which then autophosphorylates upon a conserved histidine residue. QseC subsequently transfers its phosphate to QseB, which goes on to regulate both is own transcription and the transcription of the flagellar and motility genes. EHEC may then be able to swim to the intestinal epithelium, activate its type III secretion system, and form attaching and effacing lesions. This knowledge may ultimately lead to the development of therapeutic drugs that may aid in the treatment of EHEC infections. Furthermore, these studies may also yield a greater understanding of the communication between microbes and their host.

CHAPTER FIVE

TRANSCRIPTIONAL REGULATION OF flhDC BY QseB&C AND σ^{28} (FliA) IN EHEC

INTRODUCTION

During infection, EHEC can sense and respond to environmental cues, such as the cell density of the normal flora in the intestine and the epinephrine/norepinephrine produced by the host. This cell-to-cell signaling allows EHEC to activate its flagella in order to swim closer to the intestinal epithelium, aiding in colonization and disease.

Sperandio *et al.* have previously shown that the quorum sensing *E. coli* regulators B and C (QseB&C), a two-component system in EHEC, are responsible for the regulation of the master regulator of flagella and motility genes, *flhDC*, in response to the AI-3/epinephrine/norepinephrine cell-to-cell signaling system [1, 119]. An isogenic *qseC* mutant had reduced flagellin production and motility, and reduced transcription of flagellar genes [1].

In this chapter, we describe the mechanism by which QseB&C regulates the transcription of *flhDC*. We first mapped the transcriptional start site of *flhDC* responsive

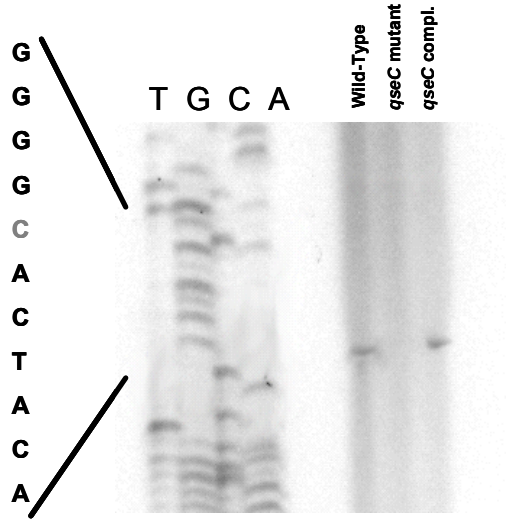
to QseB&C. This transcriptional start site contains a class 2, FliA (σ^{28}) promoter consensus sequence. FliA-dependent *flhDC* transcription was confirmed using a nonpolar *fliA* mutant. Additionally, we determined the minimal regions necessary for QseB&C activation of *flhDC* and QseB binding sites within the *flhDC* promoter and have utilized DNaseI footprinting techniques to define a QseB binding consensus sequence. Taken together, these data allowed us to propose a model of *flhDC* transcriptional regulation via QseB&C and FliA (σ^{28}).

RESULTS

Primer extension analysis of the *flhDC* transcriptional start site

In order to identify the specific *flhDC* promoter through which QseB&C regulate transcription, we performed primer extension analysis. A primer was designed approximately 10 base pairs downstream from the translational start codon of the *flhDC* genes. Primer extension analysis was then performed using cDNA synthesized from RNA that was purified from 86-24 (the wild-type EHEC strain), VS138 (the *qseC* isogenic mutant), and VS179 (complemented strain) grown in LB at 37^{o}C to an O.D.$_{600}$ of 0.8 (Figure 5.1). The transcriptional start site (TSS) of *flhDC* responsive to QseB&C was mapped to 51 base pairs upstream of the *flhDC* ATG translational start codon determined by Soutorina *et al.* 1999. This TSS was present in both wild-type and complemented strains but not in the *qseC*$^{-}$ strain, suggesting that this transcriptional

regulation is lost in the absence of *qseC*. A search for–10 and –35 consensus boxes led to the identification of a conserved FliA (σ^{28})–dependent consensus promoter sequence (Figure 5.1). The *flhDC* promoter consisted of a potential FliA (σ^{28}) –10 box (6/8 bp match to the consensus sequence) and a –35 box (6/8 bp match to the consensus sequence) [193]. The presence of this FliA (σ^{28})–dependent consensus promoter sequence suggested that σ^{28} (FliA) may play a role in activating the transcription of the master regulator of flagella and motility genes, *flhDC*.



***fliA* (σ^{28}) Consensus Sequence:**

-35 -10

TAAAGTTT--C---T-C-TGCCGATAAT
C G CAAC T T C G CA A A C TC
T T CA G A

***flhDC* Promoter Sequence:**

-35 -10 +1

TCTAATGCTAAT**TA**TT**TTTT**AC**C**GGGGCTT**CCCG**G**C**G**AC**ATCA**C**GGGGTGC
GGTGAAACCGCATAAAAATAAAGTTGGTTATTCTGGGTGGGAATA**ATG**

Figure 5.1 - The transcriptional start site of *flhDC* responsive to QseB&C. Primer extension analysis was performed with RNA from wild-type (86-24), *qseC*⁻ (VS138), and complemented (VS179) strains grown at 37°C to and O.D.$_{600}$ of 0.8. The FliA (σ^{28}) consensus sequence is shown, with the most highly conserved nucleotides depicted as larger letters and the less conserved nucleotides depicted as smaller letters [193]. The *flhDC* promoter sequence is also shown, with bold letters indicating nucleotides that match the consensus. The *flhDC* FliA (σ^{28}) –10 region has 6bp of 8bp conserved, and the –35 region has 6bp of 8bp conserved.

FliA-dependent *flhDC* transcription

In order to test the hypothesis that FliA (σ^{28}) plays a role in *flhDC* transcriptional initiation, a nonpolar *fliA* mutant was constructed in EHEC. To confirm that the *fliA* mutant exhibited the same phenotypes as previously constructed *fliA* mutants from *E. coli* K-12 strains [194], a motility assay was performed (Figure 5.2A). As expected, the motility of the *fliA* mutant is drastically reduced as compared to wild-type and complemented strains. Additionally, we constructed a *fliC::lacZ* transcriptional fusion in order to confirm that the transcription of *fliC* (a flagellar class 3 gene) was decreased in the *fliA* mutant. Figure 5.2B shows that the *fliA* mutant has a 3.5-fold reduction in *fliC* transcription, as compared to wild-type and complemented strains. Finally, a western blot using an anti-flagellin antibody was performed (Figure 5.2C), again suggesting that the production of flagellin is drastically reduced in the *fliA* mutant as compared to wild-type and complemented strains. These results again confirm that the nonpolar *fliA* mutant in EHEC shows an identical phenotype to the *fliA* mutant of *E. coli* K-12 strains and that all of these phenotypes can be rescued upon complementation with *fliA in trans.*

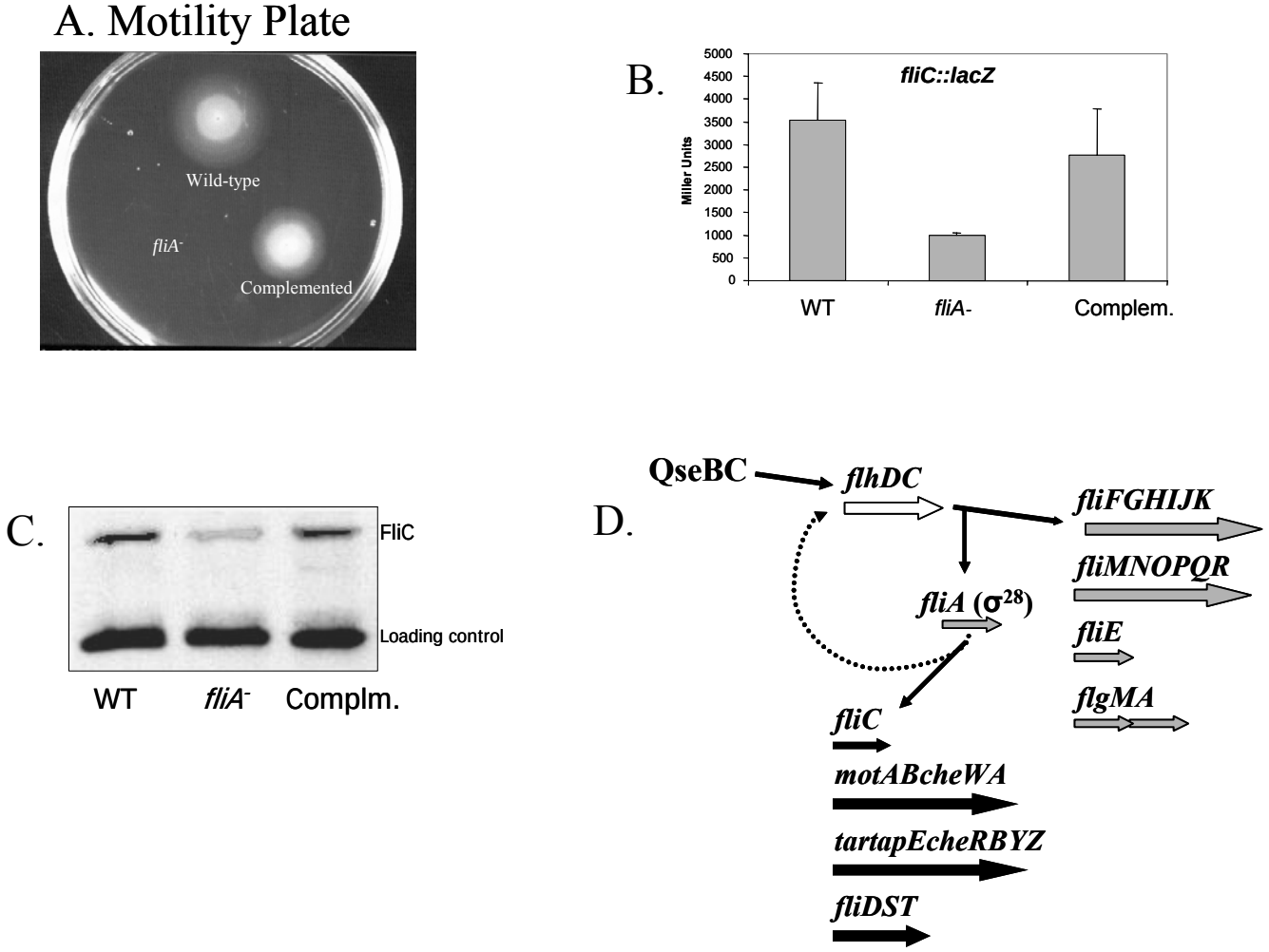


Figure 5.2 - *fliA* mutant phenotypes. A.) Tryptone motility plate with wild-type, *fliA* mutant, and complemented strains. B.) β-galactosidase assays were performed in multi-copy on a *fliC::lacZ* promoter fusion in wild-type (86-24), *fliA*$^-$ (MC475) and complemented (MC548) strains. C.) Western blot of wild-type (86-24), *fliA*$^-$ (MC475) and complemented (MC548) strains using an anti-flagellin antibody. D.) Diagram of the flagella regulon hierarchy. *flhDC*, the class 1 genes, are represented by a white arrow. FlhDC regulate the class 2 genes, represented as gray arrows. FliA (σ^{28}) goes on to regulate the transcription of class 3 genes, represented by black arrows. Our data indicate that FliA (σ^{28}) may act to regulate the transcription of *flhDC*, a class 1 gene (dashed line).

Shin and Park (1995) previously mapped an ambiguous *flhDC* transcriptional start site, while studying *flhDC* regulation by OmpR in *E. coli* K-12, using strains grown in tryptone broth to an $O.D._{560}$ of 0.4. Soutourina, *et al.* also mapped a σ^{70} *flhDC* transcriptional start site in *E. coli* K-12, responsive to H-NS and CRP, under unspecified conditions at an $O.D._{600}$ of 0.4 [195]. We did not detect either of these TSS using EHEC grown in LB at 37°C to an $O.D._{600}$ of 0.8. However, Shin and Park (1995) also reported a minor band of transcriptional initiation 128bp downstream of their major initiation site, which was not further discussed. This minor site of initiation is the FliA (σ^{28}) transcriptional start site that we found to be responsive to QseB&C in EHEC.

To determine the role of the newly identified FliA (σ^{28}) promoter in *flhDC* transcription in EHEC, we constructed several *flhDC::lacZ* transcriptional fusions and introduced these fusions in wild-type, *fliA*$^{-}$, and complemented strains (Figure 5.3A). The large plasmid construct pVS182 (+50bp to –900bp, containing the σ^{28}, σ^{70}, and ambiguous promoters) showed a 35-fold reduction in transcription in the *fliA* mutant compared to wild-type and complemented strains. These data support the observation that the FliA (σ^{28}) promoter may play an important role in *flhDC* transcription in EHEC.

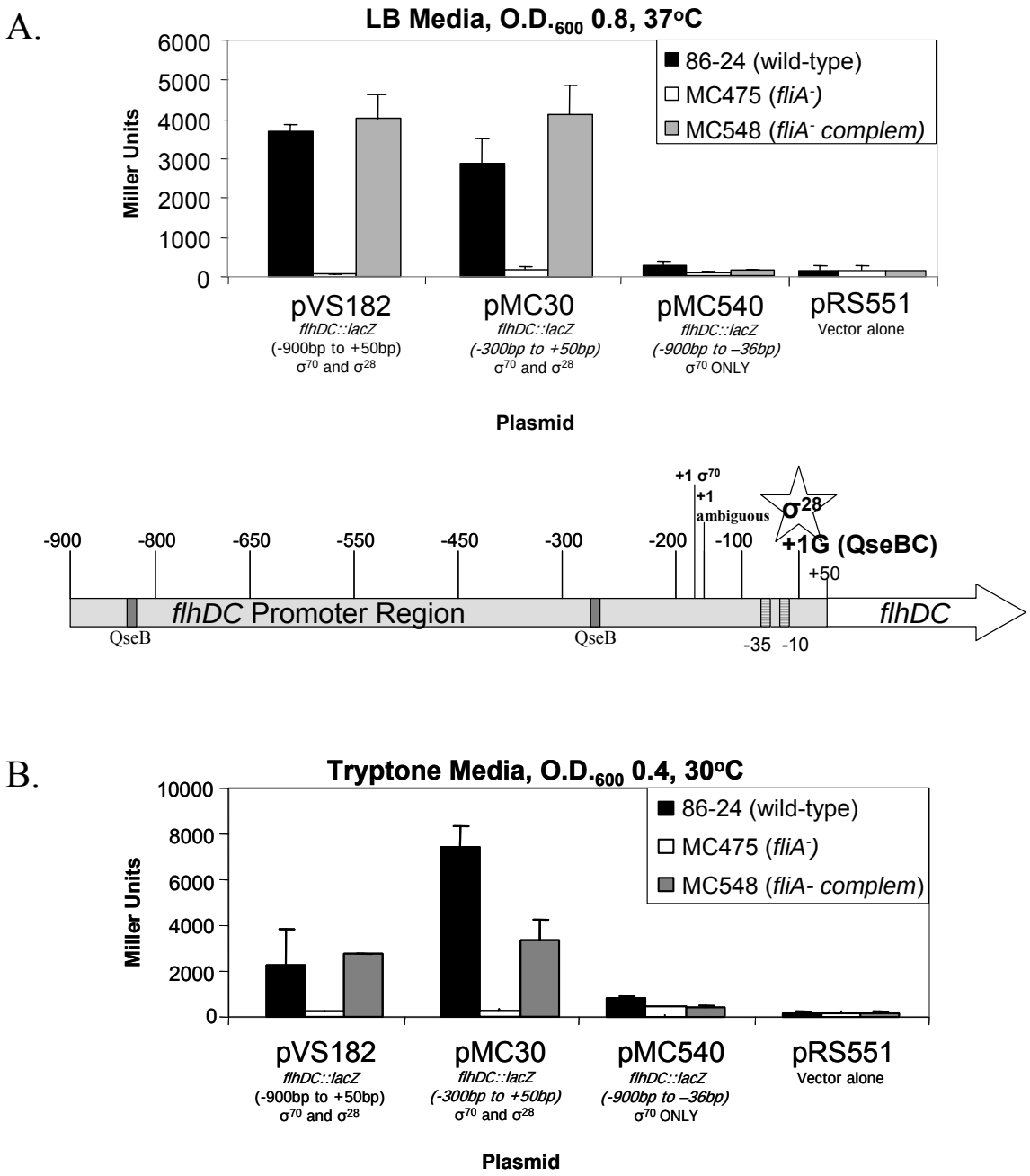


Figure 5.3 - Analysis of *flhDC* transcription in the *fliA* mutant. A.) β-galactosidase assays were performed in multi-copy using *flhDC::lacZ* promoter fusions in wild-type (86-24), *fliA*$^-$ (MC475) and complemented (MC548) strains grown at 37^{o}C to and O.D.$_{600}$ of 0.8. Fusion pMC30 and pVS182 contained both σ^{70} and σ^{28} promoters. pMC540 contains a small truncation of the *flhDC* promoter that eliminates the σ^{28} promoter but leaves the σ^{70} promoters intact. B.) β-galactosidase assays were performed in multi-copy using *flhDC::lacZ* promoter fusions in wild-type (86-24), *fliA*$^-$ (MC475) and complemented (MC548) strains grown at 30^{o}C to and O.D.$_{600}$ of 0.4.

In order to further substantiate this observation, we also demonstrated that a smaller plasmid construct, pMC30 (+50bp to –300bp, containing the σ^{28}, σ^{70}, and ambiguous promoters) also showed a 20-fold reduction in transcription in the *fliA* mutant as compared to wild-type EHEC and complemented strains (Figure 5.3A). Additionally, to specifically determine the role of the σ^{28} promoter in *flhDC* transcription, we constructed plasmid pMC540 (-36bp to –900bp), which contained a small truncation of the *flhDC* promoter, removing the FliA (σ^{28}) promoter and containing only the intact σ^{70} and ambiguous promoters. Using plasmid pMC540, we observed that transcription of *flhDC* through the previously mapped σ^{70} and ambiguous promoters is similar to the background expression levels of the vector-alone negative control (pRS551) in all strains. This result suggests that *flhDC* transcription in EHEC strains grown in LB at 37^{o}C to an O.D.$_{600}$ of 0.8 is primarily initiated through the FliA (σ^{28}) promoter. The σ^{70} and ambiguous promoters may play a more significant role during transcription in *E. coli* K-12 strains grown in tryptone media at 30^{o}C to an O.D.$_{600}$ of 0.4.

In order to determine if growth conditions did play a role in the determination of promoter activity in EHEC, β-galactosidase experiments were performed using the same transcriptional fusions (described above, Figure 5.3A) under the exact conditions that Shin and Park (1995) utilized to map the σ^{70} *flhDC* transcriptional start site in *E. coli* K-12. Our EHEC strains containing the transcriptional fusions were grown in tryptone broth to an O.D.$_{560}$ of 0.4 at 30^{o}C (Figure 5.3B). Under these conditions, the large plasmid construct pVS182 (+50bp to –900bp, containing the σ^{28}, σ^{70}, and ambiguous promoters) again showed a reduction (10-fold) in transcription in the *fliA* mutant compared to wild-type and complemented strains. The smaller plasmid construct,

pMC30 (+50bp to –300bp, containing the σ^{28}, σ^{70}, and ambiguous promoters) also showed a 16-fold reduction in transcription in the *fliA* mutant as compared to wild-type EHEC and complemented strains. *flhDC* transcription from plasmid pMC540 (-36bp to –900bp), which had no FliA (σ^{28}) promoter and contained only the intact σ^{70} and ambiguous promoters was again lower than *flhDC* transcription from plasmids pVS182 and pMC30, which contained the σ^{28} promoter. However, under these growth conditions (tryptone broth to an $O.D._{560}$ of 0.4 at 30^{o}C), pMC540 shows a 4-fold increase in transcription as compared to pRS551 (vector alone) (Figure 5.3B). Although it is difficult to determine whether this effect is FliA-dependent, these data may suggest that the σ^{70} promoter mapped by Soutourina *et al.* [195]) and the ambiguous promoter mapped by Shin and Park [151] may play a role in *flhDC* transcription in EHEC that is grown in tryptone broth to an $O.D._{560}$ of 0.4 at 30^{o}C. However, *flhDC* transcription in EHEC appears to occur mostly through the FliA (σ^{28}) promoter.

Nested deletion analyses of the *flhDC* regulatory region

Sperandio *et al.* have previously shown that a *qseC* mutant has decreased transcription of flagellar class 1, 2, and 3 genes in both EHEC and K-12 *E. coli* strains, and that QseC proteins from either strain can complement this *qseC* mutation, suggesting that QseB&C may function similarly in both strains [1]. In order to map the minimal region of the *flhDC* promoter that is necessary for activation by QseB&C, a series of nested deletions of the EHEC *flhDC* regulatory region were constructed (Figure 5.4A).

These promoter fragments were fused to a promoterless *lacZ* gene in plasmid pRS551 and used for deletion analyses in multi-copy in EHEC strain 86-24 and K-12 strain MC1000, and also for single-copy deletion analysis in K-12 strain MC1000. Unfortunately, a single-copy deletion analysis in EHEC could not be performed due to the fact that EHEC is immune to λ transduction. The multi-copy deletion analyses of the EHEC *flhDC* regulatory region in EHEC and K-12 backgrounds were identical, leading us to assume that the single-copy EHEC *flhDC* deletion analysis in K-12 is representative of the regulation in EHEC, as well.

Figure 5.4A depicts the *flhDC::lacZ* constructs. The multi-copy deletion analysis in the EHEC background is shown in Figure 5.4B. The large *flhDC* promoter construct, pVS182 (+50bp to –900bp) shows a 2.5-fold reduction in transcription in the *qseC* mutant as compared to wild-type and complemented strains. Additionally, two smaller promoter constructs, pMC30 (+50bp to –300bp) and pMC31 (+50bp to –450bp) show a 4.5-fold and 2.5-fold reduction in *flhDC* transcription in the absence of *qseC* as compared to wild-type and complemented strains. All of the other fusions, pMC28 (+50bp to –100bp), pMC29 (+50bp to –200bp), pMC32 (+50bp to –550bp), pMC85 (+50bp to –650bp), and pMC33 (+50bp to –800bp) do not appear to depend on QseB&C for transcriptional activation. Furthermore, although transcription from pMC33 (+50bp to –800bp) did not appear to be dependent on the presence of *qseBC*, β-galactosidase activities were three fold lower than that of the full-length fusion, pVS182 in the wild-type strain (+50bp to –900bp). These data suggest that there may be an unidentified repressor acting in the central *flhDC* promoter region.

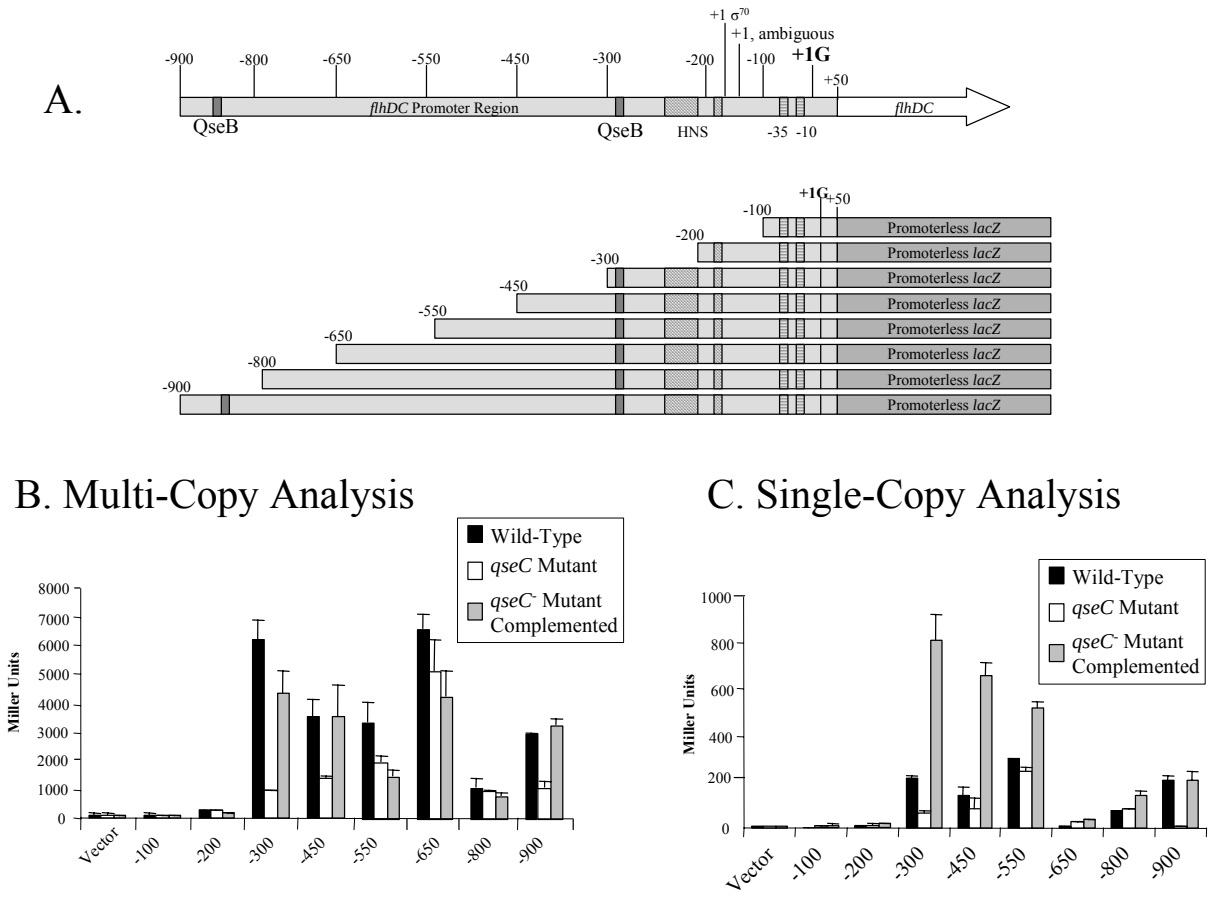


Figure 5.4 - Deletion analysis of *flhDC*. A.) Diagram of *flhDC* promoter fusions in relationship to the FliA (σ^{28}) transcriptional start site and ATG. B.) β-galactosidase assays were performed in multi-copy on a series of *flhDC::lacZ* nested deletion promoter fusions in wild-type (86-24), *qseC*⁻ (VS138) and complemented (VS179) strains. Vector (pRS551) alone was used as a negative control. C.) β-galactosidase assays were performed on single-copy chromosomal *flhDC::lacZ* transcriptional fusions in wild-type (MC1000), *qseC*⁻ (VS184) and complemented (VS185) strains.

To avoid copy number issues, as well as coiling effects, this deletion analysis was also performed in single-copy in the chromosome of K-12 (Figure 5.4C). Given that we observed the identical regulation pattern of the EHEC *flhDC* promoter in the multi-copy analysis in both EHEC and K-12 backgrounds, we assumed that the single-copy analysis is also reflective of transcription in the EHEC background. The large promoter fusion (+50bp to –900bp) and the small promoter fusion (+50bp to –300bp) showed a dependence on the presence of QseB&C for transcriptional activation. The large promoter fusion (+50bp to -900bp) shows a 21-fold decrease, and the small promoter fusion (+50bp to -300bp) shows a three fold decrease in transcription in the absence of *qseC* as compared to wild-type and complemented strains. These results suggest that these two regions may play a role in the transcriptional activation of *flhDC* by QseB&C. Similar to the multi-copy analysis, the other single-copy fusions derived from pMC28 (+50bp to –100bp), pMC29 (+50bp to –200bp), pMC32 (+50bp to –550bp), pMC85 (+50bp to –650bp), and pMC33 (+50bp to –800bp) did not show QseB&C-dependent activation of transcription. The *flhDC* promoter region from +50bp to -800bp (pMC33) appears to be repressed in both single- and multi-copy analyses. However, the fusion derived from pMC85 (+50bp to –650bp) shows low basal levels of transcription in single-copy as compared to multi-copy. This may indicate that this region of the promoter may be under repression that is relieved in multi-copy.

QseB binds to two regions in the *flhDC* promoter

To determine if QseB directly interacts with the *flhDC* promoter region to activate transcription, we purified QseB-His protein under native conditions as described previously [162] to use in electrophoretic mobility shift assays (EMSAs). This purified QseB-His protein should be mostly unphosphorylated, as it was highly over expressed in the strain and its cognate response regulator, QseC, was expressed at normal, wild-type levels. The two QseB&C-dependent *flhDC* promoter regions (-300bp to +50bp and –900bp to –650bp) and a *bla* promoter fragment (negative control) were PCR amplified and end-labeled with γ-^{32}P dATP. EMSAs using these promoter fragments and purified, mostly unphosphorylated QseB did not initially reveal a shift, indicating that unphosphorylated QseB was not binding to these promoter fragments (Figure 5.5A).

It has previously been shown that several response regulators can be phosphorylated by the addition of a small phosphodonor to the reaction [196]. To investigate whether or not the phosphorylation of QseB was necessary for binding to the *flhDC* promoter fragments, we performed EMSAs to which 0.1 M of the phosphodonor acetyl phosphate was added. This phosphorylated QseB was incubated with the two *flhDC* promoter regions that showed QseB&C-dependent activation in the deletion analyses (Figure 5.4B and 5.4D). Figure 5.5B reveals that phosphorylated QseB binds to and shifts the –300bp to +50bp *flhDC* promoter fragment. A negative control (*bla* promoter) was included, and Figure 5.5C shows that phospho-QseB does not bind or shift the *bla* promoter. In order to further verify the specificity of phospho-QseB binding to the –300bp to +50bp *flhDC* promoter, we performed a competition experiment (Figure

5.5D). Upon the addition of unlabeled probe, phospho-QseB binding was competed out by the cold *flhDC* probe (-300bp to +50bp) at a ratio of about 1:2. At a ratio of about 2:1 unlabeled probe to radiolabeled probe, phospho-QseB binding was completely competed out. However, the addition of an unlabeled *bla* probe shows no competition, suggesting that the binding of phospho-QseB to the *flhDC* promoter is specific. Additionally, an EMSA was also performed with the –900bp to –650bp *flhDC* promoter fragment and phospho-QseB (Figure 5.5E). This fragment also binds phospho-QseB, and the shift is again competed out upon the addition of unlabeled *flhDC* (-900bp to –650bp) probe. These results indicate that phosphorylated QseB is specifically binding to the *flhDC* promoter in two regions, the –900bp to –650bp region, and the –300bp to +50bp region.

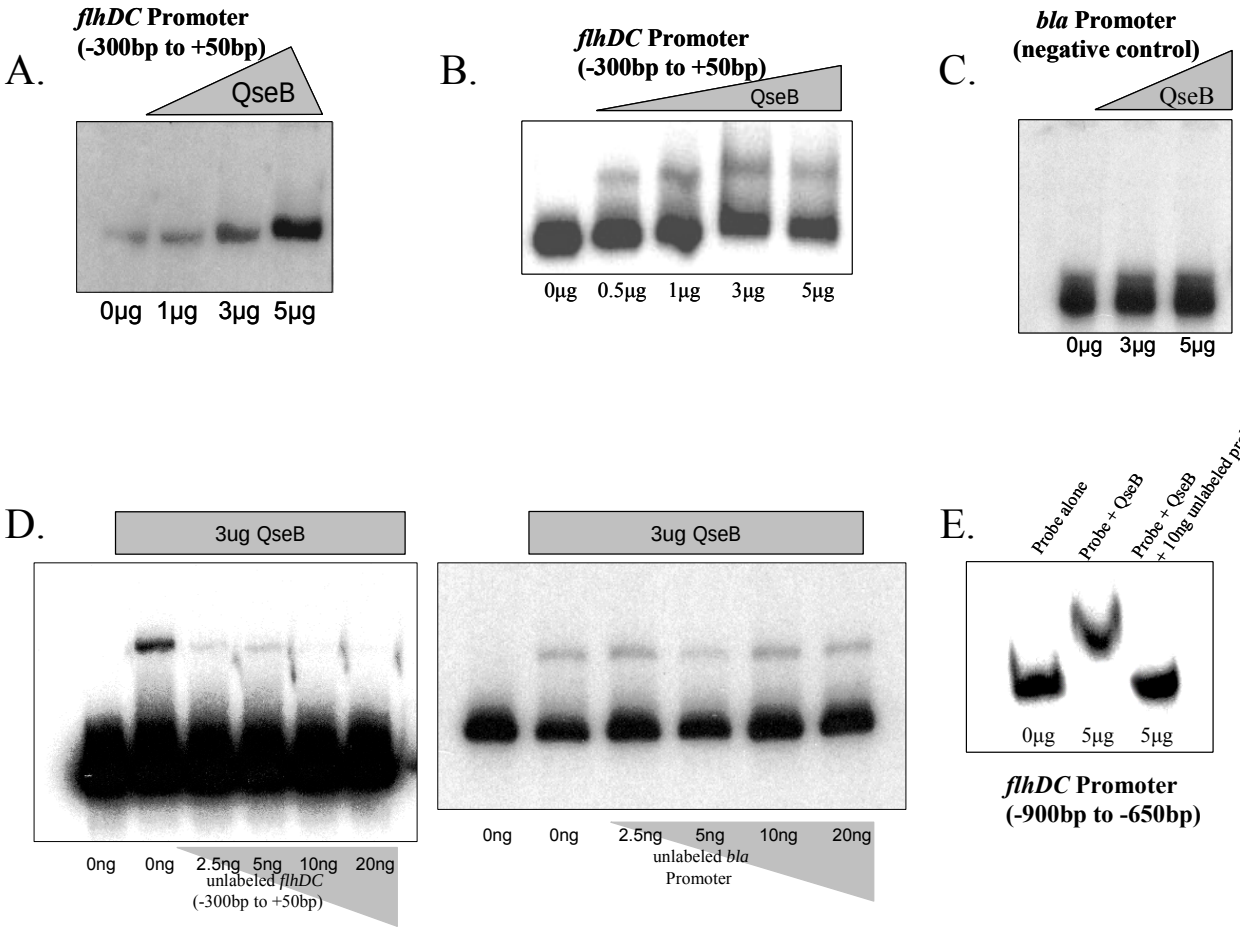


Figure 5.5 - EMSA and competition assays with phosphorylated QseB. A.) An EMSA was performed using purified, mostly unphosphorylated QseB and the *flhDC* (-300bp to +50bp) promoter fragment. B.) An EMSA was performed using phosphorylated QseB and the *flhDC* (-300bp to +50bp) promoter fragment. C.) The bla promoter and phosphorylated QseB were used as a negative control. D.) Competition assays were performed using 3µg of purified, phosphorylated QseB and adding increasing amounts of unlabeled promoter (-300bp to +50bp) probe. E.) An EMSA was performed using purified, phosphorylated QseB and the *flhDC* (-900bp to –650bp) promoter fragment.

Given that phosphorylated QseB appeared to be binding to two separate regions of the *flhDC* promoter, we were interested in whether or not QseB bound cooperatively or in a specific order to the promoter. We performed double EMSAs using identical concentrations (5ng) of the radiolabeled proximal (-300bp to +50bp) and distal (-900bp to –650bp) *flhDC* promoter fragments and increasing concentrations of phosphorylated QseB. The double EMSA results (Figure 5.6) suggest that phosphorylated QseB first binds the smaller, distal promoter fragment (-900bp to –650bp) and secondly to the larger, proximal promoter fragment (-300bp to +50bp). These data suggest that phosphorylated QseB may have a higher-affinity binding site located in the distal *flhDC* promoter (-650bp to –900bp) and a lower-affinity binding site located in the proximal *flhDC* promoter (-300bp to +50bp). Higher concentrations of phosphorylated QseB (8-16μg) appear to saturate both binding sites.

Since binding affinity of phosphorylated QseB to each promoter region may play a role in the regulation of *flhDC*, we designed a competition assay to determine the relative affinity of QseB for the binding sites. Figure 5.6B graphically shows the results of this competition experiment. The end-labeled *flhDC* proximal (-300bp to +50bp) promoter fragment was incubated at identical concentrations (20ng) together with 3μg of purified, phosphorylated QseB protein. Upon the addition of increasing amounts (0ng to 10ng) of unlabeled *flhDC* distal (-650bp to -900bp) probe, QseB binding began to be competed out at a ratio of 1:8 (Figure 5.6B). The unlabeled distal probe completely competes out the labeled proximal probe at a ratio of 1:2, further establishing that phosphorylated QseB has increased binding affinity for the distal region (-650bp to -900bp) of the *flhDC* promoter.

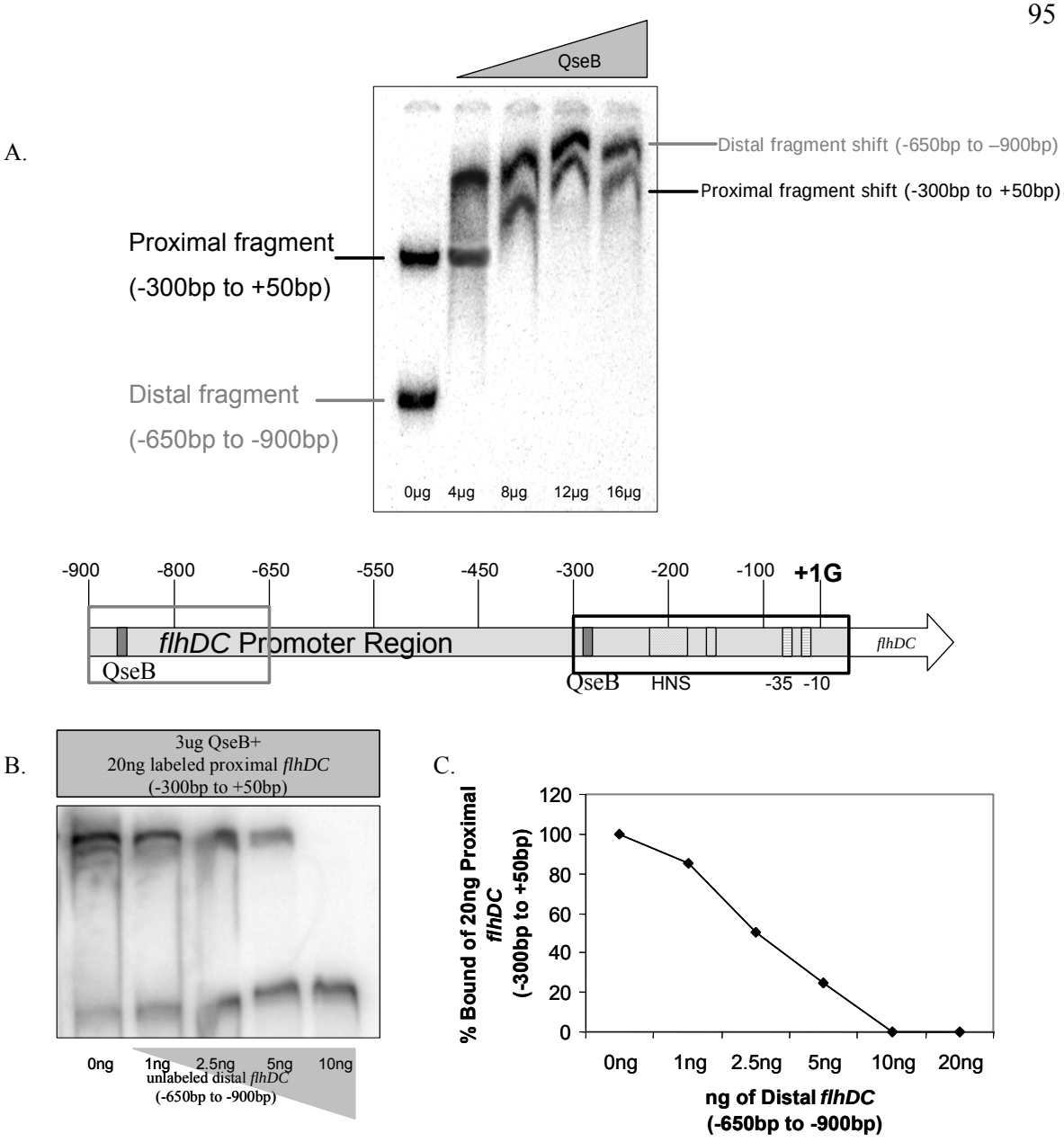


Figure 5.6 - Double EMSA and competition of *flhDC* fragments using phosphorylated QseB. A.) Increasing amounts of QseB (0µg to 16µg) were added to 5ng of the proximal (-300bp to +50bp) and 5ng of the distal (-900bp to –650bp) *flhDC* promoter fragments. B.) The competition assay was performed by adding 3µg of purified, phosphorylated QseB to 20ng of the labeled proximal (-300bp to +50bp) with increasing amounts of unlabeled distal (-900bp to -650bp) probe. C.) The graphical representation of these data is shown here.

DNaseI Footprints

In order to determine the specific binding sites of QseB in the *flhDC* promoter regions, DNaseI footprint experiments were performed. Footprint analysis of the -300bp to +50bp proximal regulatory region of *flhDC* showed that phosphorylated QseB protected a region of about 18 base pairs from -251bp to -233bp (Figure 5.7A). It does not appear that QseB protects any additional sequences in this promoter area. The size of the footprinted region did not appear to change with the amount of phosphorylated QseB (0µg/µl to 5µg/µl) added to the reaction (Figure 5.7A). We did not observe any additional protection of the *flhDC* regulatory region from -300bp to +50bp when QseB was added at higher concentrations (data not shown).

Since we observed that phosphorylated QseB also bound directly to the distal *flhDC* promoter region (-650bp to –900bp), we again performed DNaseI footprint analysis on the distal promoter region. Figure 5.7B shows that phosphorylated QseB, added in increasing concentrations from 0µg/µl to 5µg/µl, protects a region of the distal promoter from -710bp to -723bp. It does not appear that QseB protects any additional sequences in this promoter area. Similar to the proximal promoter fragment, the size of the protected distal *flhDC* promoter region (-650bp to -900bp) did not change with the amount of phosphorylated QseB added to the reaction (Figure 5.7B).

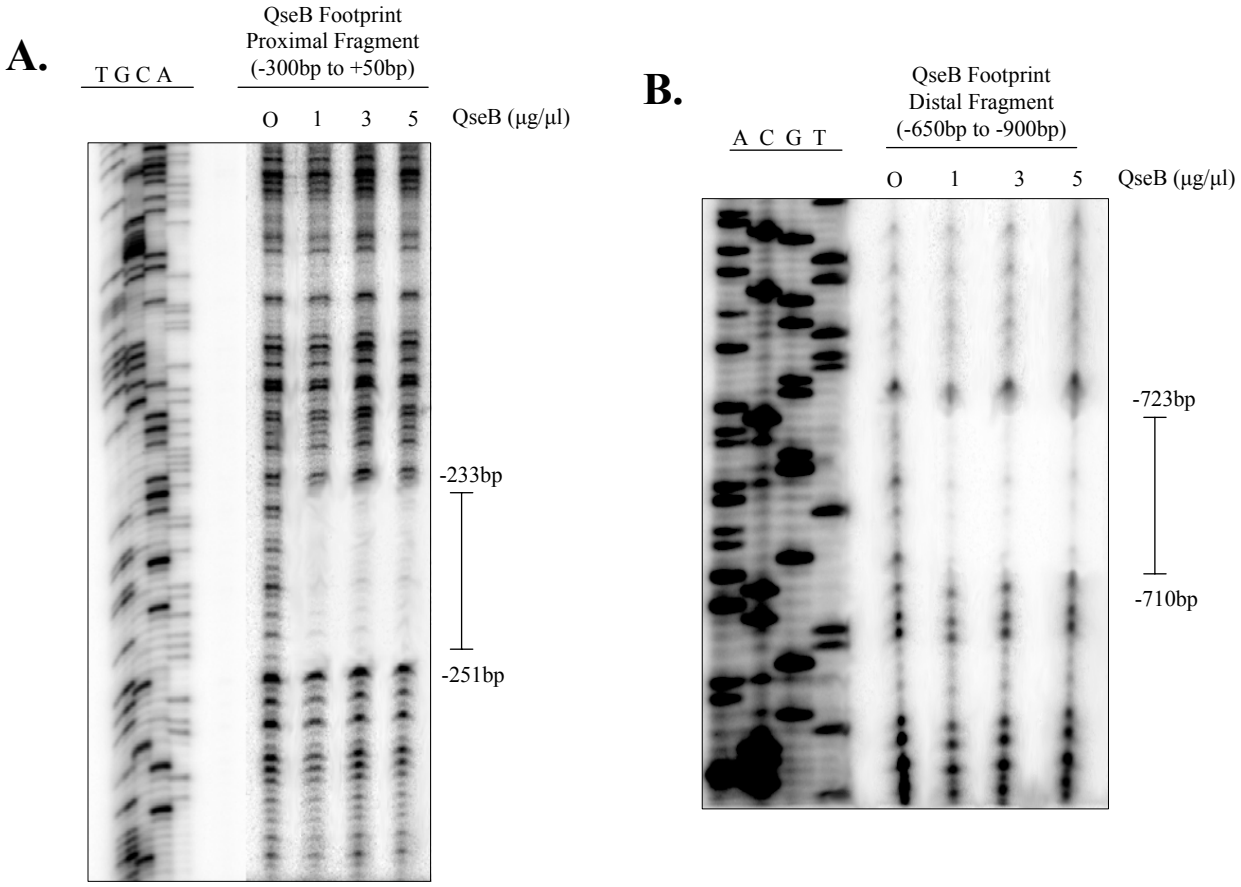


Figure 5.7 - DNaseI footprints of *flhDC*. A.) A DNaseI footprint was performed using the coding strand of the *flhDC* proximal fragment (-300bp to +50bp) and increasing amounts of phosphorylated QseB. B.) A DNaseI footprint was performed using the coding strand of the *flhDC* distal promoter fragment (-900bp to -650bp) and increasing amounts of phosphorylated QseB.

DNaseI footprint analyses have allowed us to determine the specific binding sites of phosphorylated QseB protein to the *flhDC* promoter (Figure 5.7). Alignment of these *flhDC* sequences (Figure 5.8) with sequences bound by QseB in the *qseBC* promoter (Chapter 6 and [162]) yielded a consensus sequence to which phosphorylated QseB binds. This consensus sequence, CAATTACGAATTA, will be useful in the search for additional genes that may be regulated by the QseB&C two-component system in EHEC.

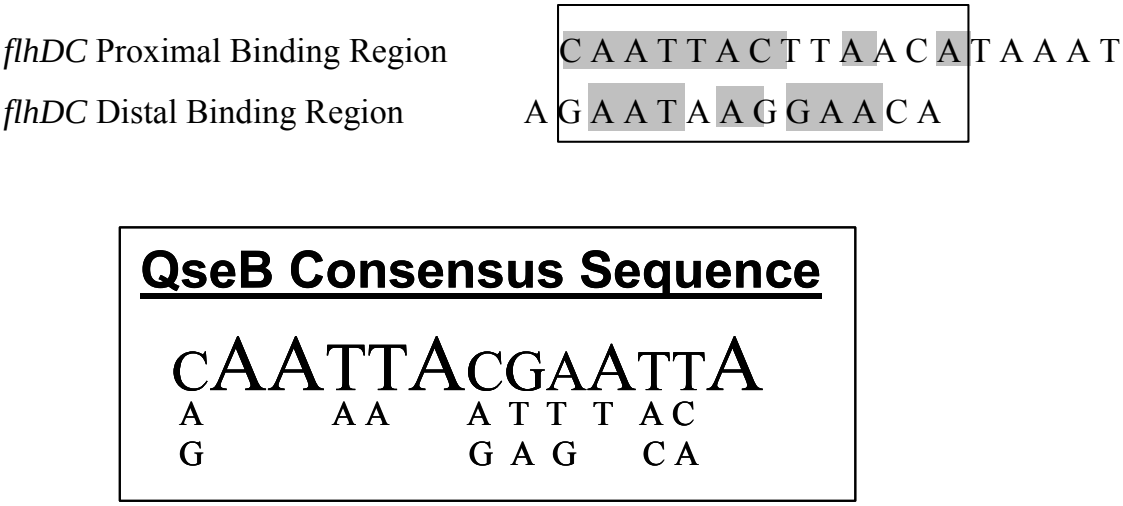


Figure 5.8 - Sequence alignment of QseB binding sites. Larger letters represent the highest level of nucleotide conservation. Smaller letters below represent conserved but less common nucleotides.

DISCUSSION

It has previously been shown that QseB&C act to activate the transcription of *flhDC* [1]. This Chapter endeavored to further understand the mechanism of this regulation. This process has allowed the construction of an overall model of *flhDC* regulation by QseB&C (Figure 5.10). When cell-to-cell signaling is activated, we hypothesize that QseC, a predicted sensor kinase, actively autophosphorylates itself on a conserved histidine residue and transfers its phosphate to a conserved aspartate residue of QseB, its putative response regulator. We cannot rule out the possibility that QseB is phosphorylated by other sensor kinases. However, the chances of any cross-phosphorylation are low, as a study by Yamamoto *et al.* (2005) showed that phosphorylation of non-cognate response regulators by histidine kinases is rare and occurs in only 22 of 692 combinations *in vitro* [197]. Here, we show that phosphorylated QseB is able to bind to both high- and low-affinity sites of the *flhDC* promoter, possibly saturating both sites (Figure 5.8B). Binding at both promoter sites may expose the FliA (σ^{28}) promoter. FliA (σ^{28}) is subsequently able to interact with RNA polymerase in order to initiate *flhDC* transcription through the FliA (σ^{28}) promoter. Additionally, our data suggest that an unknown repressor may be acting on the central region of the *flhDC* promoter. Thus, the activation of flagellar and motility genes may be very sensitive to the phosphorylation state of the QseB&C two-component system.

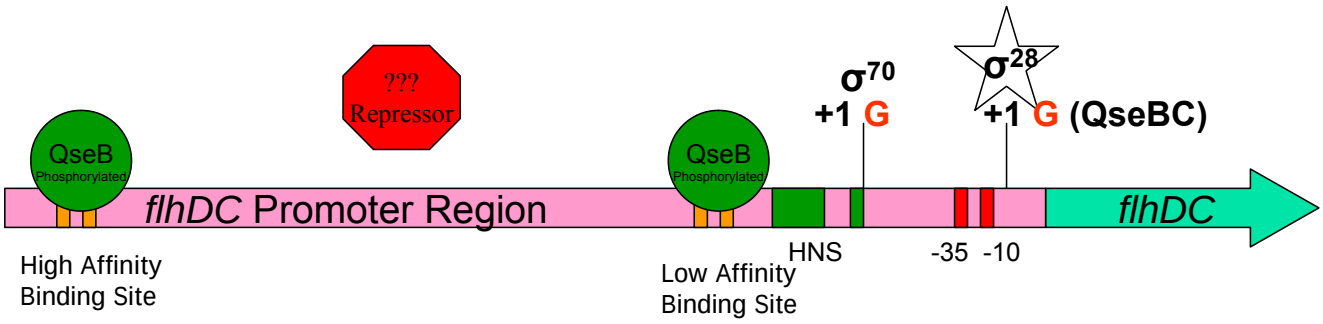


Figure 5.10 – Model of *flhDC* regulation by QseB&C. Phosphorylated QseB binds both the high-and low-affinity binding sites of the *flhDC* promoter. This allows FliA (σ^{28}) to interact with RNA polymerase in order to initiate *flhDC* transcription. An unidentified repressor may be binding to the central region of the promoter.

During this study, we identified a FliA (σ^{28}) consensus sequence at the *flhDC* promoter that responded to QseB&C in EHEC. Kutsukake previously showed that, in *Salmonella typhimurium*, a *flhDC* transcriptional fusion was repressed in the absence of σ^{28} [198], suggesting that σ^{28} may play a role in the activation of *flhDC* transcription in *Salmonella*. These studies indicate that *flhDC* has a FliA (σ^{28}) promoter in EHEC, as well (Figures 5.1-5.3). Thus, a feedback loop in which FliA (σ^{28}), a class 2 protein, activates the transcription of *flhDC* (class 1 genes) may act to sustain and strengthen an environmental response to increase the production of flagella. During infection, EHEC may need to quickly sense and respond to cell-to-cell signaling to express its flagella and motility genes in order to swim closer to the intestinal epithelium [119].

In *Salmonella*, six transcriptional start sites (TSS) have been mapped for *flhDC* [141]. The FliA (σ^{28}) consensus sequence that we showed to be responsive to QseB&C (Figure 3.1) is only the third TSS mapped in *E. coli* to date, to the best of our knowledge. Soutourina *et al.* (1999) mapped a σ^{70} TSS in *E. coli* K-12 that is 145bp upstream of the FliA (σ^{28}) promoter. Shin and Park (1995) mapped an ambiguous TSS in their primer extension experiments, which is 128bp upstream from the FliA (σ^{28}) promoter. They also mapped a TSS coincident with the FliA (σ^{28}) promoter in *E. coli* K-12 [151], indicating that this was a minor TSS, but did not further address it or recognize that there was a FliA (σ^{28}) consensus site located there. Our data indicate that each of these TSS play roles in the regulation of *flhDC* transcription under different growth conditions in EHEC. The presence of multiple TSSs for *flhDC* in *E. coli* concurs with the data suggesting the presence of several TSS in *Salmonella.* Additionally, there may be differences in the regulatory region of *flhDC* between EHEC and *E. coli* K-12 strains. Barker *et al.* (2004) further substantiated this hypothesis when they observed differential expression of *flhDC* due to insertion sequences in this promoter region in *E. coli* K-12 that are not present in EHEC [199]. Strict regulation of *flhDC* transcription by several cellular factors would allow the cell a precise level of control over the energetically expensive process of flagellar expression and synthesis.

Although only a few TSS of *flhDC* have been mapped to date in *E. coli*, it is known that numerous other regulatory factors bind to its promoter. The binding sites of OmpR [151], H-NS and CRP [195], RcsA&B [155] and LrhA [200] to the *flhDC* promoter have been mapped in *E. coli*. In addition, IHF [157] has been shown to play a role in the regulation of *flhDC*. Our single-copy deletion analysis (Figure 5.4C) indicates

that there is some form of repression in the central region of the promoter (-650bp to +50bp and –800bp to +50bp) that is relieved in multi-copy (Figure 5.4B). There are no descriptions of any other regulatory factors binding in this area, given that the previous studies only used smaller (approximately –400bp to +200bp) *flhDC* promoter regions. We could not identify any intrinsically curved AT-rich tracts that may suggest an additional H-NS binding site in this area. This may indicate that there is an additional, yet unidentified, repressor of *flhDC*. Taken together, this again suggests that it may be very important for the bacterial cell to precisely control the regulation of *flhDC*.

These studies demonstrate that QseB is specifically binding to two areas of the *flhDC* promoter (Figure 5.8B), in a similar pattern to the binding of QseB to its own promoter [162]. The identification of multiple binding sites for a response regulator has precedence from studies with another two-component system in *E. coli*, OmpR. OmpR has previously been shown to bind to several different sites (both high- and low-affinity) of the *ompC* promoter in a hierarchical and cooperative style [201]. Figure 5.6 indicates that QseB may have a high-affinity binding site that is located in the more distal promoter region (-650bp to –900bp) and a low-affinity binding site that is located in the proximal promoter region (-300bp to +50bp). Thus, QseB may be acting in a similar manner to OmpR to regulate the transcription of *flhDC*. Additionally, these studies suggest that the distal, high-affinity binding site for QseB is located between –650bp and –900bp on the *flhDC* promoter (Figures 5.4-5.6). This *flhDC* promoter region is also upstream of the divergently transcribed *yecG* gene, suggesting that QseB may also play a role in the regulation of *yecG* expression. However, preliminary studies using a *yecG::lacZ*

transcriptional fusion suggest that there is no QseB&C-dependent regulation of *yecG* (B. Habdas and V. Sperandio, unpublished data).

This chapter describes, for the first time, the mechanism of transcriptional regulation of *flhDC* by QseB&C in EHEC. Additionally, we have identified a FliA (σ^{28}) promoter that may indicate that a feedback loop between the class 2 proteins (FliA) and the class 1 genes (*flhDC)* exists in the flagella regulon in enterohemorrhagic *E. coli*. Determining all of the interactions of the regulation of flagella and motility genes is a complex process that involves many transcriptional factors responding to several environmental cues, amongst which QseB&C seem to respond to cell-to-cell signaling.

CHAPTER SIX

TRANSCRIPTIONAL AUTOREGULATION BY QUORUM SENSING E. COLI REGULATORS B AND C (QseB&C) IN EHEC

INTRODUCTION

A recent report suggests that the production of flagella and motility in EHEC and K12 strains by QS is controlled by quorum-sensing *E. coli* regulators B and C (QseB&C) [167]. Specifically, we suggested that QseB&C may be responsive to the AI-3/epinephrine/norepinephrine cell-to-cell signaling system [202]. QseB&C are a putative two-component system with homology to the PmrA&B two-component system of *Salmonella typhimurium.* QseB is predicted to be the response regulator, while QseC shares domain homology with sensor kinases. Previously, an isogenic mutant in *qseC* showed reduced motility and decreased flagellin production as compared to wild-type and complemented strains. Transcriptional fusions also indicated that the QseB&C two-component system regulated transcription of the flagella regulon through the master regulator, *flhDC* [167].

In this chapter, we report that the QseB&C two-component system that is involved in the regulation of *flhDC,* is also responsible for its own autoregulation. We show that *qseBC* are transcribed in an operon and have identified the minimal region necessary for QseB to activate *qseBC* transcription. We also demonstrate that QseB binds directly to its own promoter. These studies suggest a mechanism by which QseB binds directly to two sites in its own promoter in order to activate its own transcription.

RESULTS

The QseB&C two-component system is transcribed in an operon and autoregulates its own transcription

The predicted response regulator, QseB, shares domain homology with typical response regulators, and also appears to contain a conserved helix-turn-helix DNA-binding domain (Figure 6.1A). QseC, the sensor kinase, has two conserved transmembrane domains and a conserved histidine kinase domain, indicating that its membrane location may allow autophosphorylation upon sensing its specific environmental cue. QseC also contains an ATPase domain, which may allow it to exhibit phosphatase activity toward QseB, and a conserved EAL domain, which has been implicated in the formation of biofilms in *V. cholerae* [138]. (Figure 6.1A). The translational stop codon of *qseB* overlaps with the translational start codon of *qseC*. This

genomic organization of *qseBC* suggests that these genes may be transcribed in an operon (Figure 6.1B).

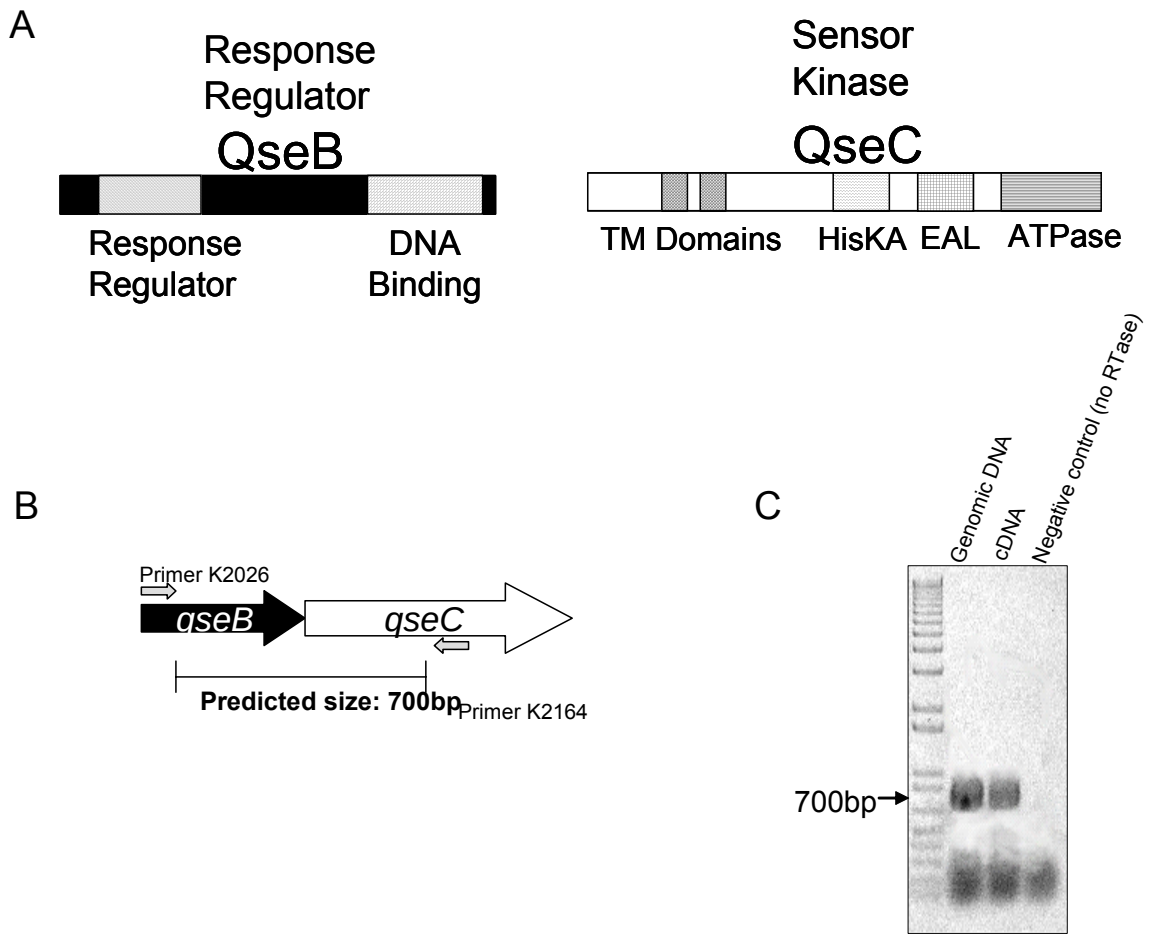


Figure 6.1 - Genomic organization of the QseB&C two-component system. A.) QseC, the predicted sensor kinase, contains two transmembrane (TM) domains, a conserved histidine kinase domain that may autophosphorylate when responding to its signal, an EAL domain that may be involved in biofilm formation, and an ATPase domain. QseB, the putative response regulator, contains both a typical response regulator domain that may receive the phosphate from QseC and a DNA binding domain that may allow QseB to regulate gene expression. B.) Organization of *qseBC* in the *E. coli* chromosome and primers used for PCR amplification. The predicted product, if *qseBC* are in an operon, is 700bp. C.) RT-PCR results using a genomic DNA positive control, cDNA, and a negative control with no RTase.

In order to determine whether *qseBC* were transcribed in an operon, reverse-transcriptase polymerase chain reaction (RT-PCR) was performed using cDNA synthesized from RNA isolated from the wild-type 86-24 strain. In addition, a positive genomic DNA control and a negative no RT-added control were used (Figure 6.1C). RT-PCR indicated that transcriptional read-through occurred across the intergenic region, further suggesting that these two genes are transcriptionally linked (Figure 6.1C). Additionally, an *in silico* analysis (http://www.tigr.org/software/transterm.html) of the EHEC O157:H7 coding sequence did not identify the presence of any Rho-independent transcriptional terminators in the coding sequence of *qseBC*, further indicating that these genes are transcribed in an operon.

Given that several two-component systems autoregulate their own transcription [158], we hypothesized that the QseB&C two-component system may act the same way. In order to investigate this hypothesis, transcriptional fusions were created between the *qseBC* promoter and a promoterless *lacZ* gene, resulting in plasmid pVS159. The resulting plasmid was electroporated into strains 86-24, VS138 (*qseC*$^-$*)*, and VS179 (*qseC*$^-$ complemented) and β-galactosidase activities were measured in order to determine the level of *qseBC* transcription in the absence of *qseC* versus wild-type and complemented strains. As negative controls, we tested a constitutive promoter (the β-lactamase promoter) and the empty vector (pRS551). As shown in Figure 6.2, the *qseC* mutant shows a 6.5-fold reduction in transcription as compared to wild-type and complemented strains, suggesting that a functional QseB&C two-component system is required for full activation of its own promoter. Datum analysis indicates that these results are statistically significant with a p value of <0.001.

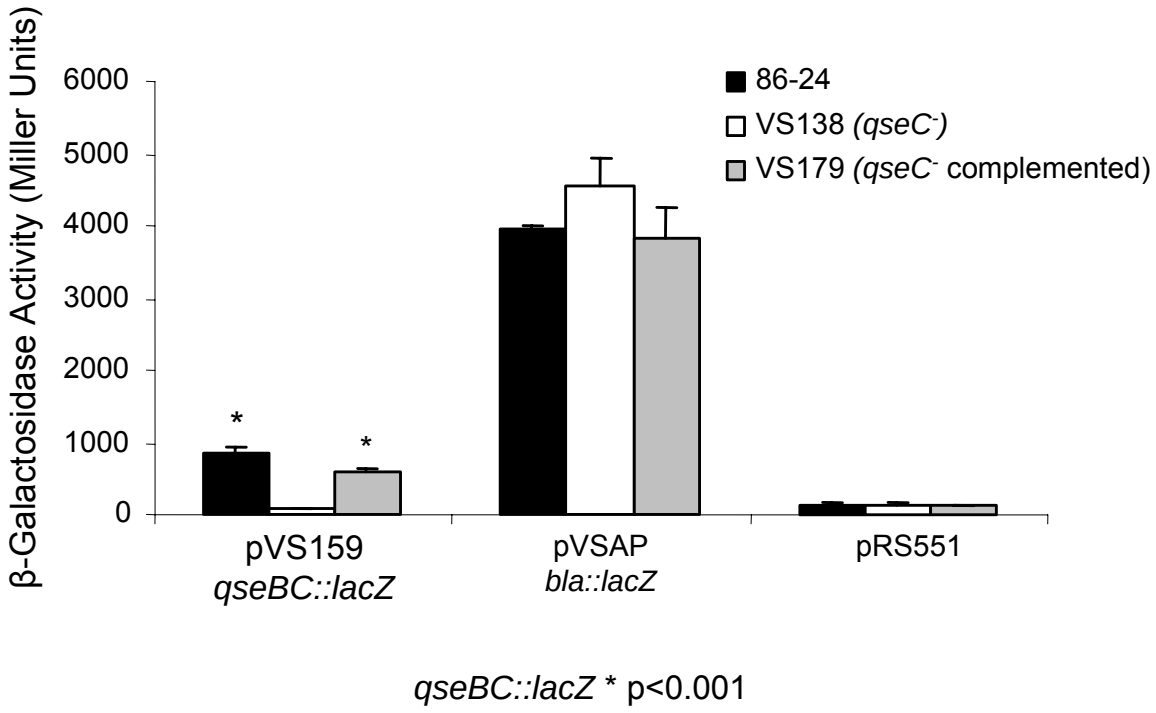
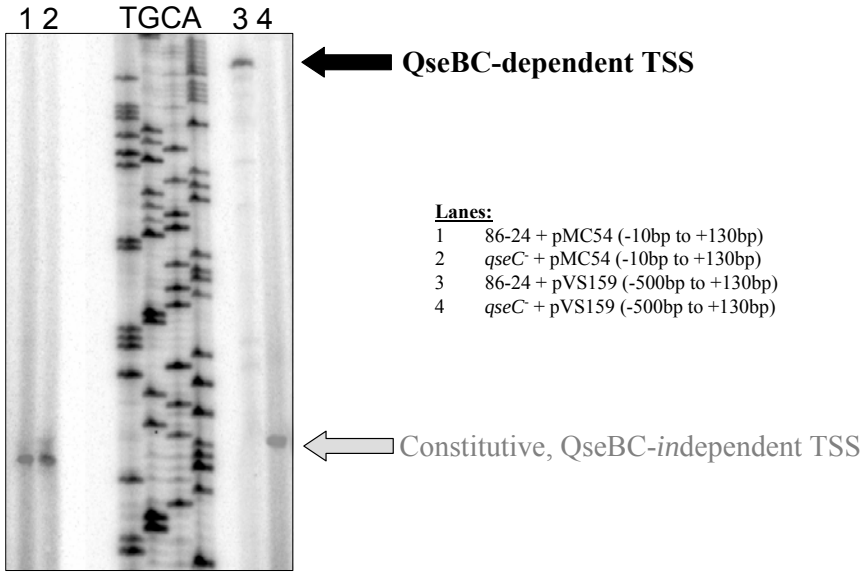


Figure 6.2 - QseB&C autoregulate their own transcription. β-galactosidase assays were performed on a *qseBC::lacZ* fusion (pVS159) in wild-type (86-24), *qseC*$^-$ (VS138) and complemented (VS179) strains. Positive controls were performed using the bla promoter (pVSAP) in all strains, and negative controls were performed with vector (pRS551) alone.

Primer extension analysis of the *qseBC* transcriptional start site

In order to identify the specific promoter through which QseB&C may act to initiate transcription, we performed primer extension analysis. We designed a primer approximately 40 base pairs downstream from the translational start codon of the *qseB* gene, ATG. Primer extension analysis was then performed using RNA isolated from the wild-type strain, 86-24, and the *qseC* mutant strain, VS138, both carrying a plasmid with the complete *qseBC* promoter (pVS159, -500bp to +130bp). These strains were grown in LB at 37^{o}C to an O.D.$_{600}$ of 0.8. As can be observed in Figure 6.3 (Lane 3), a QseB&C-dependent transcriptional start site (TSS) of the *qseBC* operon was mapped to 77 base pairs upstream of the *qseB* translational start site in the wild-type strain. This TSS was not detected in the *qseC*$^{-}$ strain (Figure 6.3, Lane 4), suggesting that autoregulation from this promoter is lost in the absence of *qseC*. Upstream of the QseB&C-dependent TSS we identified a –10 box (3bp of 6bp match the consensus) and –35 box (3bp of 6bp match the consensus), with the σ^{70}–dependent consensus promoter sequence (Figure 6.3). We identified a second TSS (Figure 6.3, Lane 4) that was present in the *qseC* mutant strain but was not present in the wild-type strain (Figure 6.3, Lane 3). It is common for two-component systems to contain a basally active, constitutive promoter in addition to an auto-regulated promoter [158]. Our data may suggest that this second TSS is QseB&C-independent, as it is present only in the *qseC* mutant (Figure 6.3, Lane 4). In the presence of QseC, transcription may occur preferentially from the distal promoter (Figure 6.3, Lane 3).

Figure 3



σ^{70} consensus -35 **TTGACA** -16bp to 18bp- -10 **TATAAT**

qseBC promoter

TTAAC**AAGATA**GTCCTTAACAACTT**CTTAAG**GGAAAAA**A**AATAAAA

TTTAGTGCTGTACAGAGCGCGTACAACACGGTTTACTGGCAGCAA

ATACGGTTATCGCAGGGATGAAAAA**ATG**CGA

Figure 6.3 - The transcriptional start site of *qseBC* responsive to QseB&C. Primer extension analysis was performed using wild-type (86-24) and *qseC*⁻ (VS138) carrying either plasmid pVS159 (-500bp to +130bp) or pMC54 (-10bp to +130bp). The QseB&C-dependent *qseBC* promoter (black arrow and text) sequence, constitutive QseB&C-independent *qseBC* promoter (grey arrow and text) and σ^{70} consensus sequence are shown, indicating the conserved –10 and –35 regions.

Nested deletion analysis of the *qseBC* regulatory region

The *qseBC* regulatory regions between EHEC and K-12 are 99.6% identical [GenBank accession numbers: NC002655 (bp 3976071 to 3976571) and NC000913 (3167344 to 3167844)]. In addition, we have also previously showed that a *qseC* mutant acts to regulate transcription of the flagella regulon in both EHEC and K-12 strains, and that the QseC proteins from either strain can complement the mutation, suggesting that QseB&C may function similarly in both strains [1]. In order to map the minimal region of the *qseBC* promoter that is necessary for activation by QseB&C, a series of deletions of the *qseBC* regulatory region was constructed in EHEC (Figure 6.4A). These deletions were then fused to a promoterless *lacZ* gene and used for both multi-copy deletion analysis in EHEC strain 86-24, K-12 strain MC1000, and λRS45 single-copy deletion analysis in K-12 strain MC1000. The single-copy deletion analysis could not be performed in EHEC due to the fact that EHEC is immune to λ transduction.

The multi-copy deletion analysis was identical in both EHEC and K-12 backgrounds, further indicating that this transcriptional regulation is conserved between these two strains. The EHEC multi-copy deletion analysis is shown in Figure 6.4B. The largest plasmid construct, pVS159 (-500bp to +130p) and a smaller plasmid construct, pMC53 (-120p to +130bp), depicted a dependence upon the presence of QseB&C for transcriptional activation of the *qseBC* operon. In the pVS159 fusion (-500bp to +130bp), transcription of *qseBC* was activated 6.5-fold in the presence of QseB&C as compared to the isogenic mutant strain that lacked *qseC* (Figure 6.4B). Comparatively, the pMC53 fusion (-120bp to +130bp) was only activated 2.5-fold in the presence of

QseB&C. The fusions pMC52 (-240p to +130bp) and pMC54 (-10bp to +130bp) were not dependent on the presence of QseB&C for transcriptional activation and still showed wild-type levels of transcription in all strains. Although the fusion pMC278 (-360bp to +130bp) did not show any QseB&C-dependent activity, β-galactosidase activities were twofold higher than that of the wild-type fusion pVS159 (-500bp to +130bp) in multi-copy.

To avoid coiling and plasmid copy number issues, we also investigated the transcription of *qseBC* using single-copy chromosomal fusions. The same general pattern of regulation was observed in single-copy analysis as in the multi-copy analysis. Figure 6.4C shows that, once again, the largest promoter fusion (-500bp to +130bp) shows a comparable 6.5-fold activation of transcription dependent on the presence of QseB&C. Similarly, the single-copy fusion derived from the pMC53 promoter fragment (-120bp to +130bp), once again showed 2.5-fold QseB&C-dependent transcriptional activation. These results suggest that the regions between –500bp and –360bp and/or –120bp and -10bp may be important for QseB&C transcriptional autoregulation. Additionally, the level of transcription of the largest promoter fragment (-500bp to +130bp) diminishes just 3-fold in single-copy as compared to multi-copy (Figure 6.4B and 6.4C), as compared to other promoter fragments that diminish about ten-fold. These data suggest that QseB&C may auto-activate its transcription more efficiently in single-copy.

As in the multi-copy analysis, the single-copy fusions derived from pMC278 (-360bp to +130bp), pMC52 (-240bp to +130bp), and pMC54 (-10bp to +130bp) showed no QseB&C-dependent activation of transcription. Interestingly, however, the fusions derived from pMC278 (-360bp to +130bp) and pMC52 (-240bp to +130bp) showed very

low levels of transcription in single-copy as compared to multi-copy. In fact, these levels were only two to three-fold the level of vector alone, indicating that these regions may be under transcriptional repression. This repressor may be sensitive to copy number effects, as repression appears to be relieved in multi-copy. Additionally, the promoter fusion derived from pMC54 (-10bp to +130bp) does not contain the complete QseB&C-responsive promoter. However, this construct still exhibits high levels of transcription as compared to the vector negative control, in both single- and multi-copy. These data again support the possibility, mentioned earlier, that there is yet another basally active promoter for *qseBC* that is independent of QseB&C.

In order investigate the existence of an additional QseB&C-independent *qseBC* promoter (Figure 6.3, Lane 4), we performed primer extension analysis using RNA purified from wild-type (86-24) and the isogenic *qseC* mutant (VS138) containing plasmid pMC54 (-10bp to +130bp) in multi-copy. As depicted in Figure 6.3 (Lanes 1 and 2), we again mapped the second, QseB&C-independent TSS to 27bp upstream of the *qseB* translational start site, ATG. Upstream of the transcriptional start site there is a conserved –10 box (4bp of 6bp match the consensus) and –35 box (4bp of 6bp match the consensus), with the σ^{70}–dependent consensus promoter sequence (Figure 6.3). These data further support the hypothesis that there is an additional *qseBC* promoter that is constitutively active and QseB&C-independent.

In order to determine the role of that the constitutive, basally transcribed promoter may play in *qseBC* transcription, we constructed a promoter fusion, pMC576 (-500bp to +5bp), containing the QseB&C-dependent promoter but lacking the QseB&C-independent promoter. Figure 6.4B shows that, in multi-copy, this fusion (-500bp to

+5bp) depends on the presence of QseB&C for transcriptional activation. Transcription of *qseBC* was activated 23-fold in the presence of QseB&C as compared to the isogenic mutant strain that lacked *qseC*. In fact, transcription of this fusion (-500bp to +5bp) was decreased to levels of vector alone in the *qseC* mutant. Thus, the loss of the constitutive, basally-transcribed promoter leads to a decrease in transcription in the *qseC* mutant, but does not appear to affect *qseBC* transcription in a wild-type situation (Figure 6.4B).

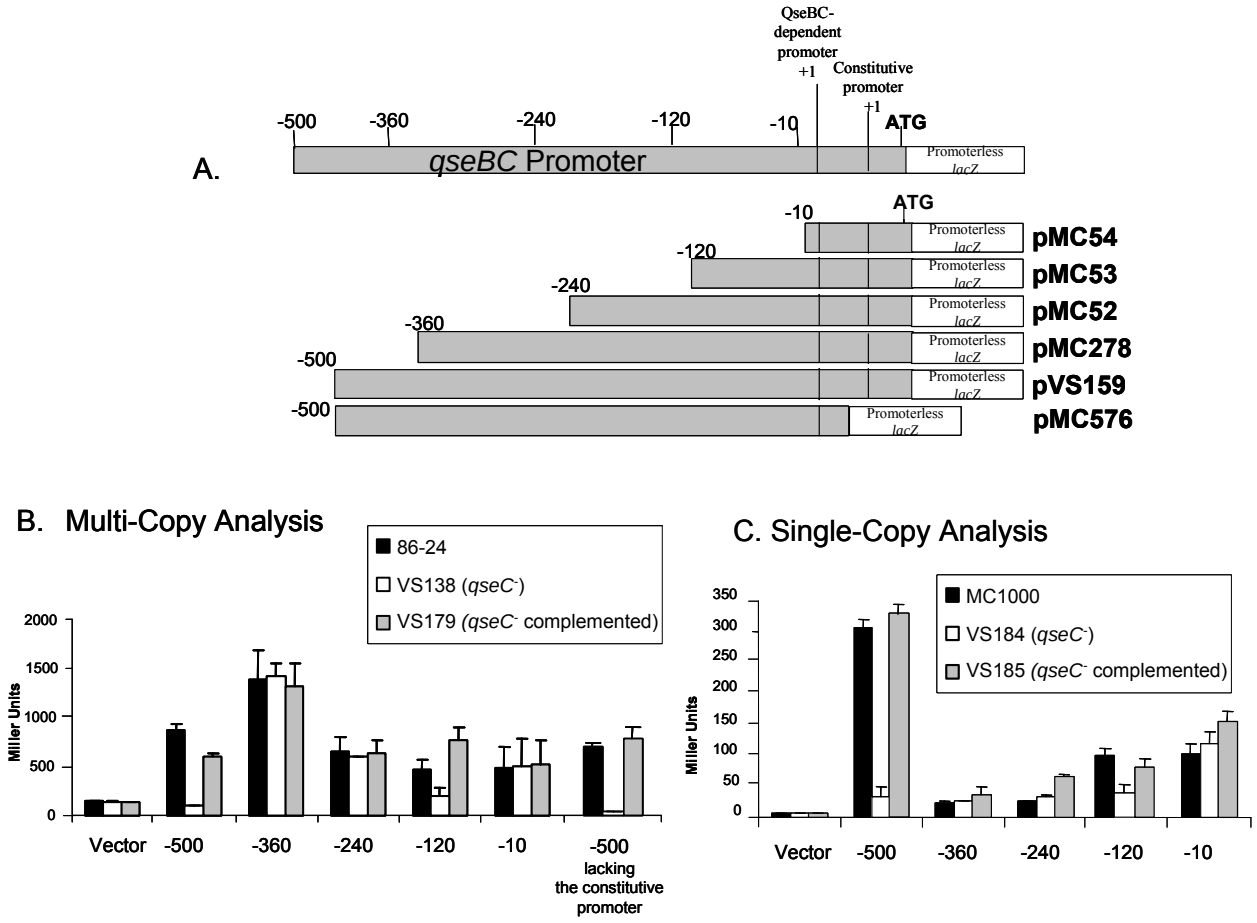


Figure 6.4 - Deletion analysis of *qseBC*. A.) Diagram of *qseBC* promoter fusions in relationship to the transcriptional start site and ATG. B.) β-galactosidase assays were performed in multi-copy on a series of *qseBC::lacZ* nested deletion promoter fusions in wild-type (86-24), *qseC*⁻ (VS138) and complemented (VS179) strains. Vector (pRS551) alone was used as a negative control. C.) β-galactosidase assays were performed on single-copy chromosomal *qseBC::lacZ* transcriptional fusions in wild-type (MC1000), *qseC*⁻ (VS184) and complemented (VS185) strains.

Purification of QseB

Due to the fact that a *qseBC::lacZ* transcriptional fusion showed autoregulation (Fig. 6.2) in a wild-type background as compared to a *qseC* mutant, it is hypothesized that QseB, the predicted response regulator, acts as a transcription factor that directly interacts with its own promoter to initiate transcription. This conclusion is strengthened by the observation that, upon autophosphorylation, QseC transfers its phosphate to QseB (Chapter 4). In order to test this hypothesis, we purified QseB in native conditions to perform DNA-binding assays.

The *qseB* gene was cloned into a pBADmycHis vector (Invitrogen) in order to generate a C-terminal Myc-His protein fusion under the control of the pBAD promoter. QseB protein was then expressed from the resulting vector, pVS154, and purified under native conditions using a nickel-affinity column. Purity of QseB was estimated to be over 90% (Figure 6.5A).

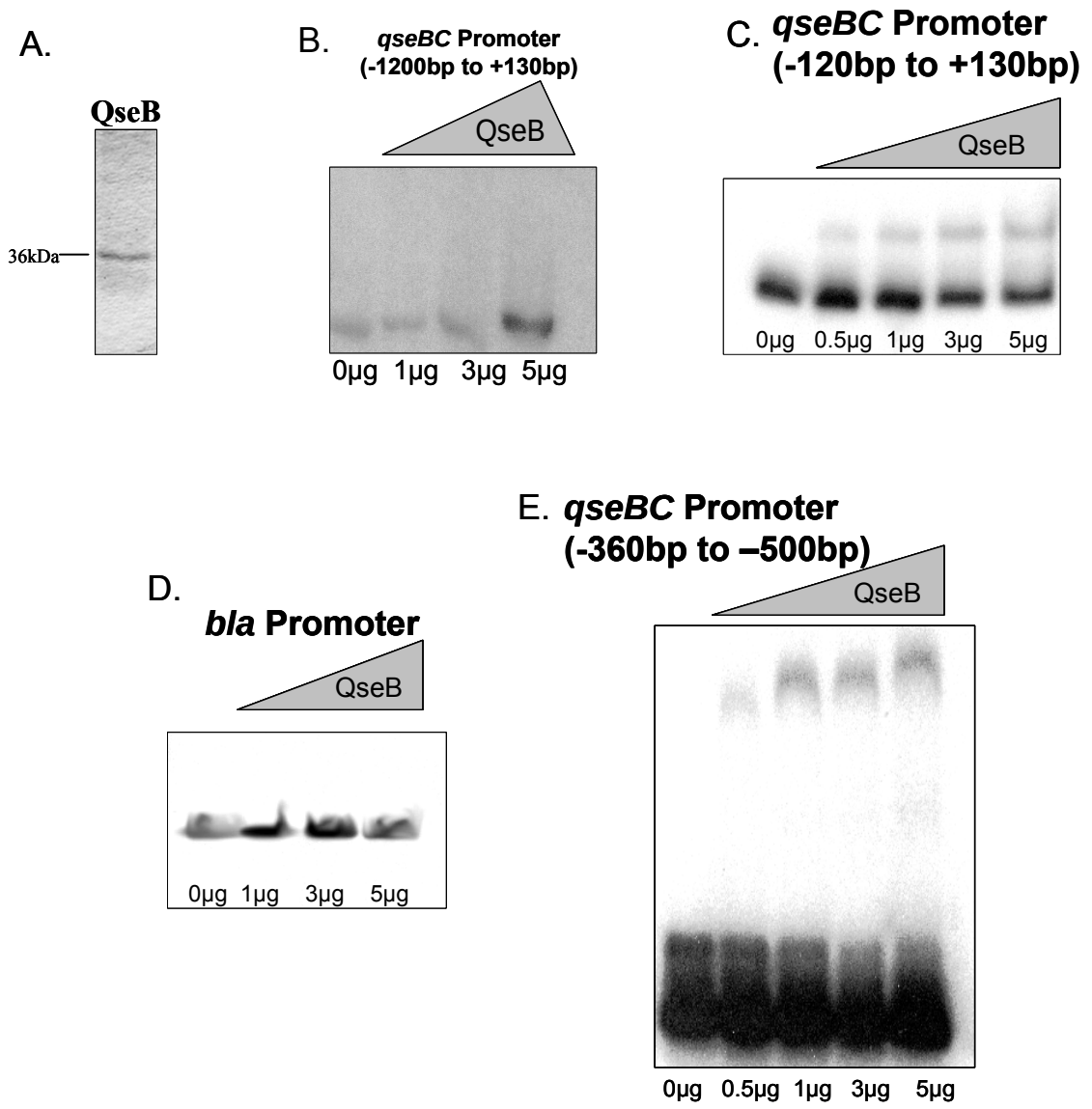


Figure 6.5 – EMSA and competition assays with *qseBC*. A.) Purification of QseB. QseB protein was purified under native conditions and stained with Coomassie. B.) An EMSA was performed using purified, mostly unphosphorylated QseB and the *qseBC* (-120bp to +130bp). C.) An EMSA was performed using purified, phosphorylated QseB and the *qseBC* (-120bp to +130bp) promoter fragment. D.) An EMSA was performed using phosphorylated QseB and a negative control, the *bla* promoter. E.) An EMSA was performed using purified, phosphorylated QseB and the *qseBC* (-360bp to –500bp) promoter fragment.

Electrophoretic Mobility Shift Assays with purified QseB

To demonstrate that QseB was directly binding to its own promoter, electrophoretic mobility shift assays (EMSAs) were performed. The proximal promoter fragment (-120bp to +130bp) and the distal promoter fragment (-360bp to –500bp) that previously showed QseB&C-dependent activation in the deletion analysis (Figure 6.4B and 6.4C) were PCR amplified and end-labeled using γ-^{32}P ATP. Additionally, a *bla* promoter fragment was used as a negative control. Initially, the EMSAs were performed using the mostly unphosphorylated, natively purified QseB protein. This mostly unphosphorylated QseB did not appear to bind or shift the *qseBC* proximal promoter fragment (–120bp to +130bp), a *bla* negative control, or the distal promoter fragment (-360bp to -500bp) (Figure 6.5B and data not shown).

It is known, however, that other response regulators require the addition of a small phosphodonor in order for the protein to be phosphorylated in the absence of its histidine kinase or ATP [196, 203]. We hypothesized that the addition of 0.1M acetyl phosphate, a small phosphodonor, to the EMSA reaction might result in a phosphorylated, active QseB protein. An EMSA using this phosphorylated QseB and the proximal (–120bp to +130bp) *qseBC* promoter fragment showed that QseB bound and shifted this promoter region (Figure 6.5C) and did not bind to or shift the *bla* negative control (Figure 6.5D). Additionally, the distal (-360bp to –500bp) *qseBC* promoter region was also bound and shifted by phosphorylated QseB (Figure 6.5E). These results suggest that phosphorylation of QseB is necessary for it to be able to actively bind its regulatory promoter region.

In order to confirm specificity of binding, competition assays were also performed (Figure 6.6). Upon addition of unlabeled probe, QseB binding was competed out by the cold *qseBC* (-120bp to +130bp) probe at a ratio of about 1:1. The unlabeled probe almost completely competes out QseB binding to radiolabeled probe at a ratio of 2:1 unlabeled probe to hot probe, as can be observed in Figure 6.6. The addition of unlabeled *bla* probe, as a negative control reaction, shows no competition, indicating that QseB specifically binds to two different areas of its own promoter.

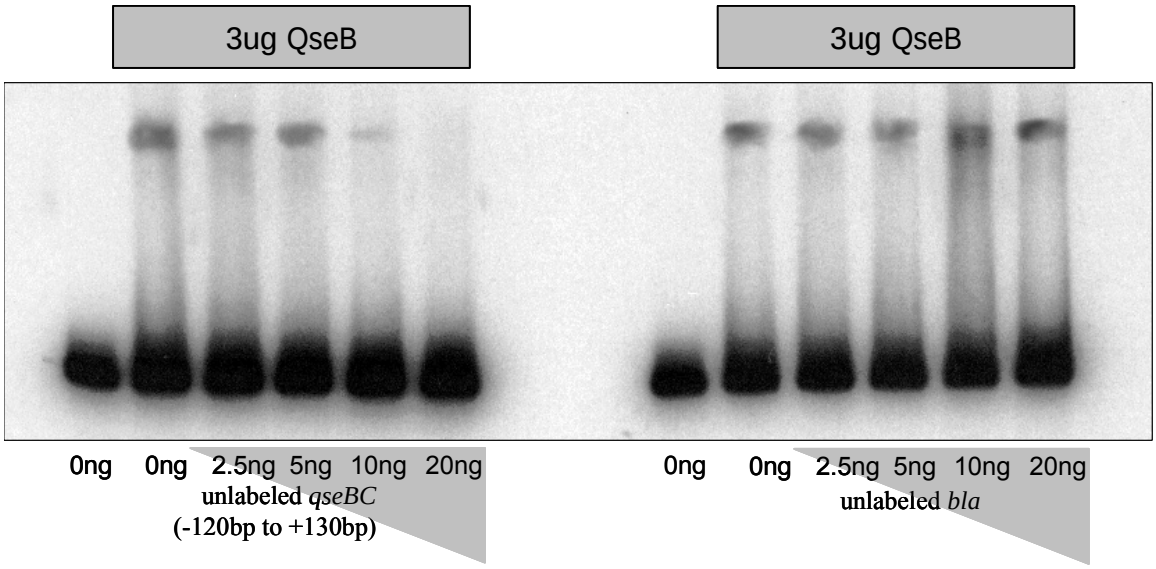


Figure 6.6 - Competition experiments with QseB. The competition assay was performed using 3µg of purified, phosphorylated QseB and adding increasing amounts of unlabeled *qseBC* promoter (-120bp to +130bp) probe. A competition assay was performed using the *bla* promoter as a negative control.

Double EMSA with QseB

In order to determine whether QseB bound cooperatively or in a specific order to the *qseBC* promoter, we performed double-gel shifts (Figure 6.7A). The proximal (-120bp to +130bp) and distal (-360bp to –500bp) *qseBC* promoter fragments to which phosphorylated QseB had previously been shown to bind (Figure 6.5B and 6.5D) were utilized again. End-labeled promoter fragments were incubated at identical concentrations (5ng) together with purified, phosphorylated QseB protein in increasing concentrations (0-16μg). Changes in band intensity are due to differences in labeling, not in the concentration of DNA. These results may indicate that phosphorylated QseB binds first to the proximal promoter fragment (-120bp to +130bp) (Figure 6.7A) and secondly to the smaller, distal (-360bp to –500bp) fragment. These data suggest that the proximal promoter fragment (-120bp to +130bp) may contain a high-affinity QseB binding site, while the distal promoter fragment (-360bp to –500bp) appears to contain a low-affinity QseB binding site. At higher concentrations of QseB (16μg), both binding sites appear to be saturated.

Due to the fact that the binding affinity of QseB to the two *qseBC* promoter regions may play a role in its autoregulation, we designed a competition assay to determine the relative affinity of QseB for the binding sites (Figure 6.7B). 20ng of the end-labeled distal (-360bp to -500bp) *qseBC* promoter fragment was incubated with a constant amount (3μg) of phosphorylated QseB protein. We then added increasing amounts (0ng to 10ng) of unlabeled proximal (-120bp to +130bp) *qseBC* probe. Figure 6.7B represents that QseB binding began to be competed out at a ratio of 1:20 and was

completely competed out at a ratio of 1:2. This data further substantiate the theory that phosphorylated QseB has increased binding affinity for the proximal region (120bp to +130bp) of the *qseBC* promoter.

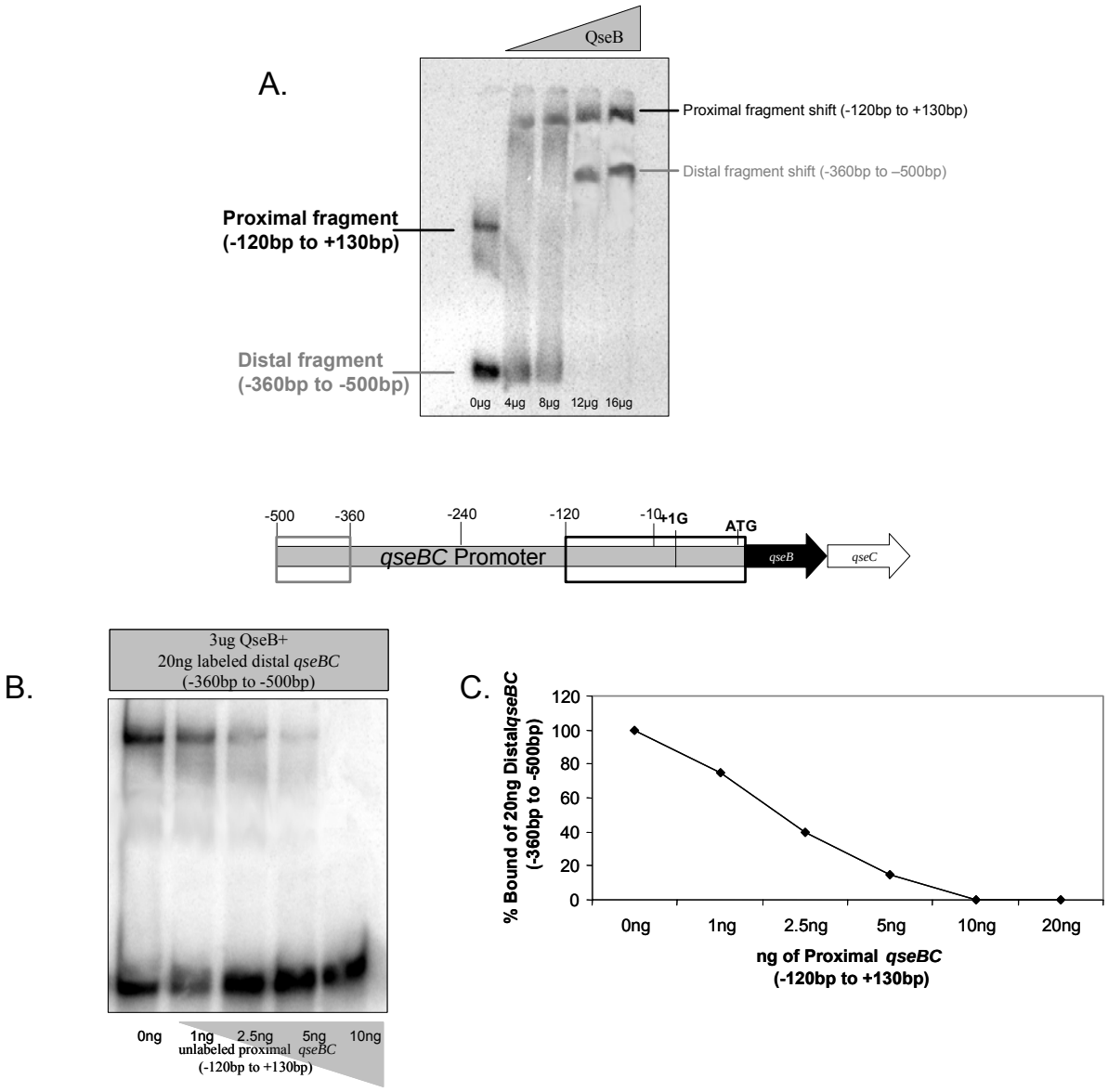


Figure 6.7 - Double EMSA and competitions of *qseBC* fragments using phosphorylated QseB. A.) 5ng of the proximal (-120bp to +130bp) and 5ng of the distal (-360bp to –500bp) *qseBC* promoter fragments were added to increasing amounts of QseB. B.) A competition assay was performed by adding 3µg of purified, phosphorylated QseB to 20ng of the labeled distal (-360bp to -500bp) with increasing amounts of unlabeled proximal (-120bp to +130bp) probe. C.) The graphical representation of this datum is shown here.

DNaseI Footprints with QseB

We utilized a DNaseI footprinting technique to determine the specific binding sites of phosphorylated QseB to its own promoter (Figure 6.8). Footprint analysis of the low-affinity distal (-360bp to -500bp) *qseBC* fragment revealed that phosphorylated QseB protein protects a region of about 14 base pairs from -409bp to -423bp (Figure 6.8A). The size of the protected region did not appear to change with increasing amounts of QseB (0µg to 5µg), nor did we observe any additional regions of protection in this area of the *qseBC* promoter. We also determined the specific binding site of phosphorylated QseB to the high-affinity proximal (-120bp to +130bp) *qseBC* promoter using DNaseI footprinting (Figure 6.8B). Phosphorylated QseB protein, added in increasing concentrations from 0µg/µl to 5µg/µl protects a region of about 14bp from –27bp to -40bp. We did not observe any additional protection of the *qseBC* proximal (-120bp to +130bp) promoter region when phosphorylated QseB was added at higher concentrations. These data allowed us to define a QseB binding consensus sequence for the *qseBC* promoter (Figure 6.8C). Alignment of these *qseBC* sequences with sequences bound by QseB in the *flhDC* promoter [161] yielded a consensus sequence to which phosphorylated QseB binds. This consensus sequence, CAATTACGAATTA, will be useful in the search for additional genes that may be regulated by the QseB&C two-component system in EHEC.

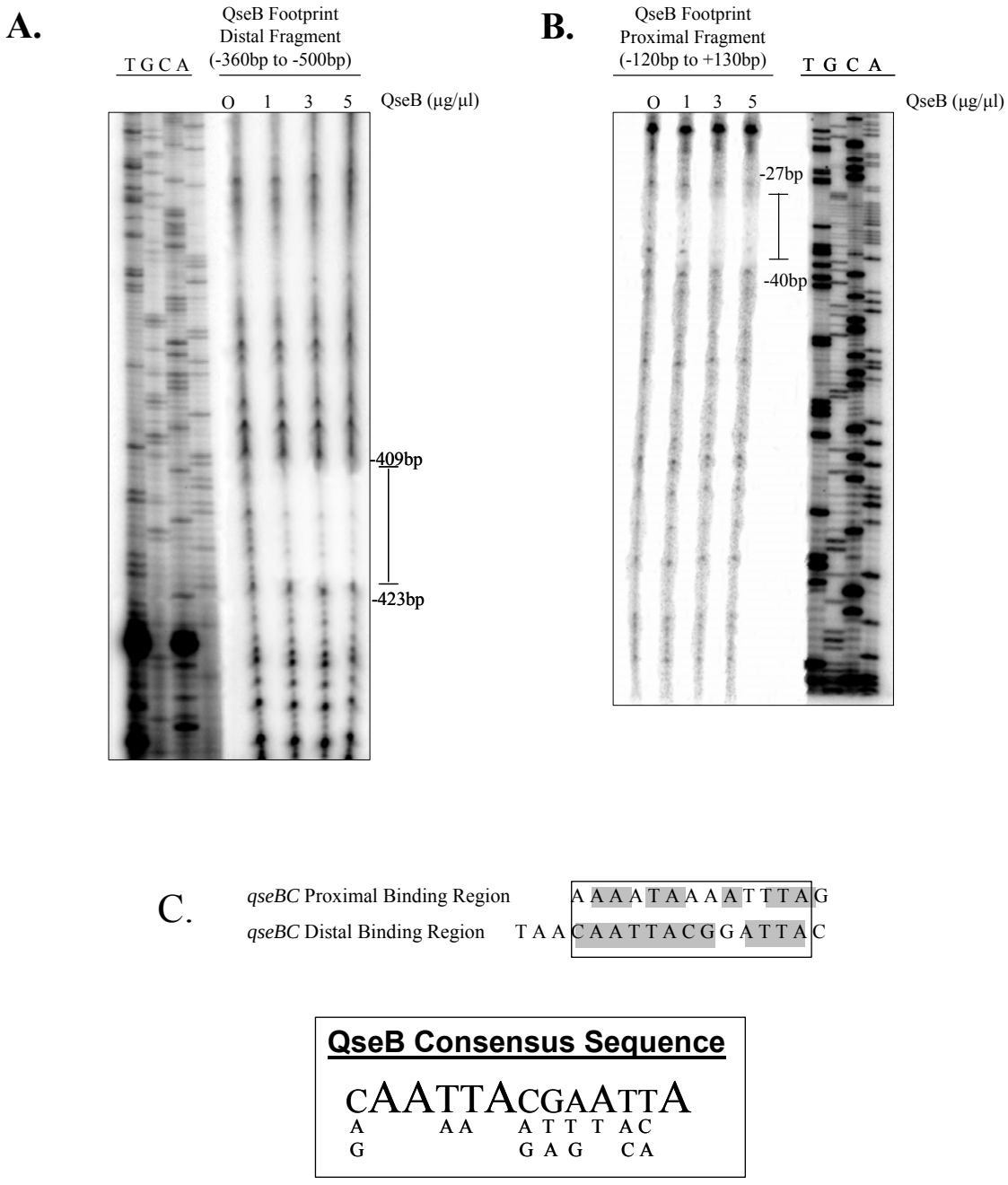


Figure 6.8 - DNaseI footprints of *qseBC*. A.) A DNaseI footprint was performed using the *qseBC* distal fragment (-360bp to -500bp) and increasing amounts of phosphorylated QseB. B.) A DNaseI footprint was performed using the *qseBC* proximal promoter fragment (-120bp to +130bp) and increasing amounts of phosphorylated QseB. C.) QseB binding consensus sequence.

DISCUSSION

Quorum sensing is a process by which bacteria secrete a hormone-like compound called an autoinducer. At a certain threshold density, the autoinducer signals interact with transcription factors to drive bacterial gene expression in response to the presence of these signals in the environment. In EHEC, which colonizes the large intestine, quorum sensing is responsible for the regulation of *flhDC*, the master regulator of the flagella regulon, through the quorum sensing *E. coli* regulators B and C (*qseBC*) two component system [167]. QseB&C are homologous to the PmrA&B two-component system of *S. typhimurium*, with QseB being the predicted response regulator and QseC being the putative sensor kinase. Since many two-component systems also autoregulate their own transcription [158], this study aimed to investigate whether QseB may also act in this fashion. After confirming that *qseBC* are transcribed in an operon (Figure 6.1B) and mapping their transcriptional start site (Figure 6.3), we showed that QseB and QseC regulate their own transcription through direct binding of QseB to proximal (-120bp to +130bp) and distal (-360bp to –500bp) regions of their promoter (Figure 6.7). We were not originally able to obtain an isogenic *qseB* mutant in EHEC, hence this study utilized an isogenic *qseC* mutant for all of the experiments. The genetic organization of *qseBC* comprising an operon was the initial suggestion that they comprise a cognate two-component system. Furthermore, the observation that, upon phosphorylation, QseC will transfer its phosphate to QseB (Chapter 4) and that phosphorylated QseB specifically binds to the *qseBC* promoter further enhances this suggestion.

It is known that the QseB&C two-component system appears to be activated by quorum-sensing through the *luxS* system [1]. Initial gel shifts (data not shown) performed with unphosphorylated QseB-His did not reveal QseB binding. The lack of response regulator binding in an unphosphorylated state is not unusual, and is similar to the results reported with the DcuR response regulator of *E. coli* [169]. Consequently, addition of an exogenous small phosphor-donor such as acetyl phosphate allowed phospho-QseB to specifically shift its promoter region (as compared to a *bla* negative control), indicating that the phosphorylation of QseB is essential for DNA binding and, consequently, transcriptional regulation (Figure 6.6).

Our comprehensive nested deletion analysis indicates that two areas of the *qseBC* regulatory region appeared to be dependent on the presence of QseB&C for activation (Figure 6.4). These results suggested that two binding sites for QseB might exist. Additionally, considering the facts that the -500bp to –360bp fragment showed QseB-binding in an EMSA (Fig. 6.6D) and that there appeared to be copy-number dependent repression on the next two smaller fragments (-360bp to +130bp and –240bp to +130bp), we predict that there is some form of repression that is relieved in the larger *qseBC* promoter fragment. This pattern is identical to QseB&C-dependent deletion analyses observed in the *flhDC* promoter in EHEC [161]. Data obtained during the double gel shifts (Figure 6.8), may indicate that phosphorylated QseB is binding first to a high-affinity site in the proximal promoter fragment (-120bp to +130bp), which does not lead to high levels of transcriptional activation. When QseB is highly phosphorylated by QseC, however, QseB is available to bind to its low-affinity binding site, located in the more distal promoter region (-350bp to -500bp). Occupation of the high- and low-

affinity binding sites may lead to an increase in transcriptional activation, resulting in an additional level of *qseBC* regulation. Additionally, QseB also appears to have high- and low-affinity binding sites in the *flhDC* promoter [161]. However, the binding sites in the *flhDC* promoter may be reversed, with the high-affinity binding site situated in the more distal region of the *flhDC* promoter, while the low-affinity binding site is positioned in the proximal region of the *flhDC* promoter [161]. This suggests that, even though the binding sites may be reversed, QseB is acting in a similar manner to activate transcription of both its own promoter and the *flhDC* promoter.

Another similar model of transcriptional regulation occurs in the OmpR regulation of *ompC* [201]. In the OmpR system, *ompC* low-affinity binding sites are occupied at low concentrations of OmpR, leading to very low levels of transcriptional activation of *ompC*. As the concentration of phosphorylated OmpR increases, however, a low-affinity *ompC* binding site is occupied by OmpR. As a result, transcription of *ompC* is highly activated [204]. According to our data, QseB may be acting in the same manner on its own promoter to regulate transcription. Additionally, the *qseBC* promoter may be regulated by other factors, as suggested by the repression observed in the central region of the deletion analysis (Figure 6.4B and 6.4C). This same pattern of repression is also seen in the QseB&C-dependent *flhDC* promoter deletion analysis [161]. Determining the factor(s) that may be responsible for the repression of the *qseBC* and *flhDC* promoters will not be an easy task, but could be addressed in future studies.

Transcription of the smallest promoter fragment pMC54 (-10bp to +130bp), which does not have the complete QseB&C-dependent promoter, has a higher level of transcription than vector alone (Figure 6.4C). This datum raises the possibility that there

is yet another, unidentified promoter in *qseBC* that is independent of QseB&C. This additional promoter was mapped using primer extension analysis (Figure 6.3). Many two-component systems harbor a constitutive promoter in addition to an autoregulated promoter [158], as is the case with BvgA&S of *B. pertussis* [205] and PhoP&Q of *S. typhimurium* [206, 207]. The presence of a constitutive promoter would ensure a basal level of expression of these genes, which may allow them to respond quickly in the presence of the correct environmental stimuli.

Several two-component systems act to positively autoregulate their own transcription [158]. We have demonstrated that the QseB&C two-component system may also act this way (Figure 6.2). The function of autoregulation could have several purposes, including signal amplification, which has been observed with other two component systems such as PhoP&Q of *Salmonella typhimurium* [206], BvgA&S of *B. pertussis* [208], CpxA&R of *E. coli* [209], and PmrA&B of *Salmonella typhimurium* [135]. Hoffer *et al.* (2001) also suggested that autoregulation of a two-component system could be necessary for a "learning" system in which bacteria respond more effectively to a signal that has been seen in the recent past. This appears to be the case with the PhoP&Q two-component system of *Salmonella*, in which previous exposure to a signal appears to boost reaction during the second exposure [210]. Finally, autoregulation could possibly lead to an additional threshold for gene activation, as seen with the CpxA&R two-component system in *E. coli.* In this case, signal persistence is necessary for autoamplification and accumulation of the CpxR response regulator [209]. This additional level of control could allow the bacterial cell to activate the energetically expensive production of flagella through QseB&C only at appropriate conditions.

These experiments have allowed the development of a model by which QseB&C regulates its own transcription (Figure 6.9). When cell-to-cell signaling systems are activated, QseB is expressed and then phosphorylated by QseC. The high concentration of phosphorylated QseB may then saturate the *qseBC* high-affinity binding site (-120bp to +130bp), followed by binding of the low-affinity site located in the –360bp to –500bp promoter region. When both the high- and low-affinity binding sites are occupied by QseB, autoregulation occurs, and *qseBC* transcription is greatly increased.

This chapter describes, for the first time, the autoregulation of QseB&C and its direct binding sites. An in-depth understanding of how this two-component system regulates gene expression may allow us to gain insight into the regulation of virulence in enterohemorrhagic *E. coli.*

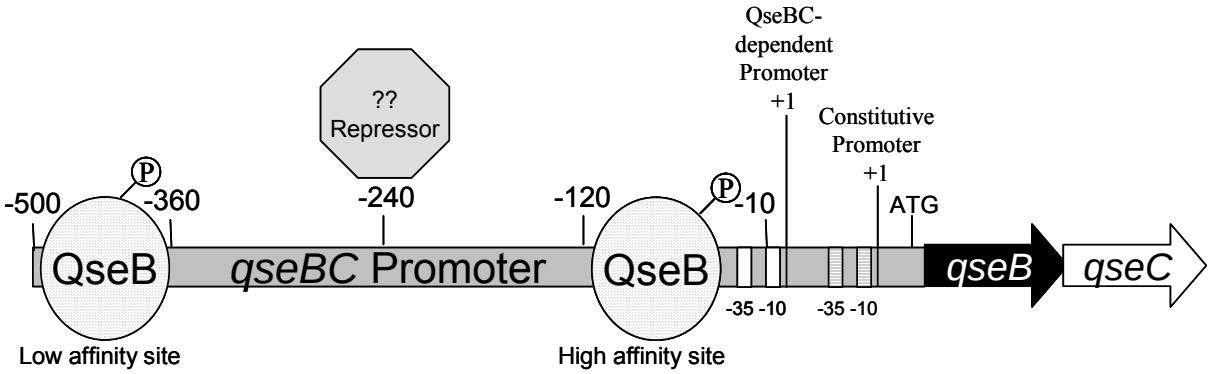


Figure 6.9 - Model of *qseBC* transcriptional regulation in EHEC. When quorum sensing occurs, QseC autophosphorylates and then transfers its phosphate to a conserved aspartate residue on QseB. Phosphorylated QseB is then free to bind both the high-and low-affinity binding sites, and to autoactivate its own transcription. An unknown repressor may bind the central region of the *qseBC* promoter.

CHAPTER SEVEN

UNPHOSPHORYLATED QseB MAY ACT AS A TRANSCRIPTIONAL REPRESSOR

INTRODUCTION

So far, the information presented in this dissertation suggests that QseB&C may be responding to the AI-3/epinephrine/norepinephrine cell-to-cell signaling system in order to activate both its own transcription and that of *flhDC* (Chapters 4-6). The results from these studies have also suggested that an unknown repressor is acting on the central region of the *flhDC* and *qseBC* promoters in EHEC (Figures 5.10 and 6.9).

In this chapter, we report that the QseB&C two-component system may also be responsible for repression of these two promoters. We show that an isogenic *qseB* mutant has no obvious phenotype and explain our hypothesis for this observation. We also demonstrate that unphosphorylated QseB binds to the central region of *flhDC,* and possibly, its own promoter. These studies suggest a mechanism by which the balance of phosphorylated versus unphosphorylated QseB regulates gene transcription.

RESULTS

qseB mutant phenotypes.

The preceding studies described in this dissertation (Chapters 4-6) utilized an isogenic *qseC* mutant for all of the transcriptional analyses. When these studies were initiated, an isogenic *qseB* mutant in EHEC was unavailable and, consequently, proved extremely difficult to create. After three years, however, we finally constructed a *qseB* mutant and performed several phenotypic assays, hoping to observe similar phenotypes to that of the *qseC* mutant. Not surprisingly, a *qseB* mutant shows reduced motility in tryptone as compared to wild-type and complemented strains (Figure 7.1A), although the phenotype is not nearly as striking as the *qseC* mutant [1]. Additionally, a western blot probed with anti-flagellin shows that the *qseB* mutant exhibits reduced levels of flagellin as compared to wild-type and complemented strains (Figure 7.1B).

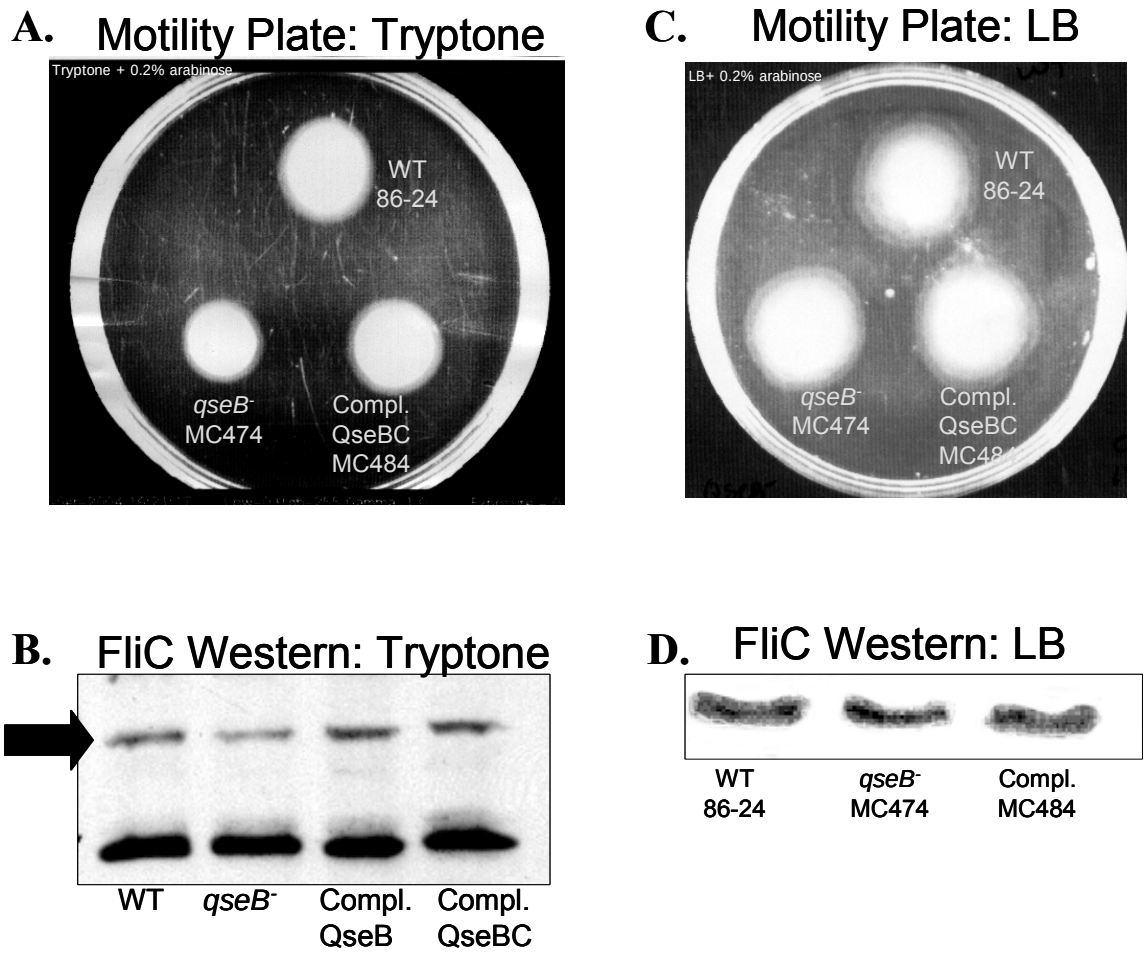


Figure 7.1 – *qseB* mutant phenotypes. A.) Tryptone motility plate with wild-type, *qseB* mutant, and complemented strains. B.) Western blot of wild-type, *qseB*- mutant and complemented strains grown in tryptone media using an anti-flagellin antibody. C.) LB motility plate with wild-type, *qseB* mutant, and complemented strains. D.) Western blot of wild-type, *qseB* mutant, and complemented strains grown in LB media using an anti-flagellin antibody.

Interestingly, when the same phenotypic experiments were performed with the *qseB* mutant grown in LB media, motility and flagellin expression appears to be the same as wild-type and complemented strains (Figure 7.1C and 7.1D). These results were perplexing, as we expected a mutation in *qseB*, the response regulator, to have the same phenotype as the mutation of *qseC*, the sensor kinase. Other data that have been collected, however, possibly provide an explaination. Both the *flhDC* (Figure 5.4) and *qseBC* (Figure 6.4) deletion analyses suggested the presence of an unidentified repressor in the central promoter regions. We hypothesized that this repressor may be unphosphorylated QseB. To test this hypothesis, we performed a motility plate assay with a *qseC* mutant overexpressing the QseB protein. As there is no QseC sensor kinase present in this strain, QseB will be mostly unphosphorylated in the bacterial cell. Figure 7.2A shows that this *qseC*$^-$, QseB++ strain shows an even larger reduction in motility than the *qseC* mutant alone. In the case of a *qseC* mutant, there is no QseC present to phosphorylate QseB. Therefore, all of the QseB present is mostly unphosphorylated. In the situation where the *qseC* mutant is overexpressing QseB, the bacterial cell is loaded with unphosphorylated QseB. It is possible that unphosphorylated QseB is binding to the central *flhDC* and *qseBC* promoter regions in order to inhibit their expression.

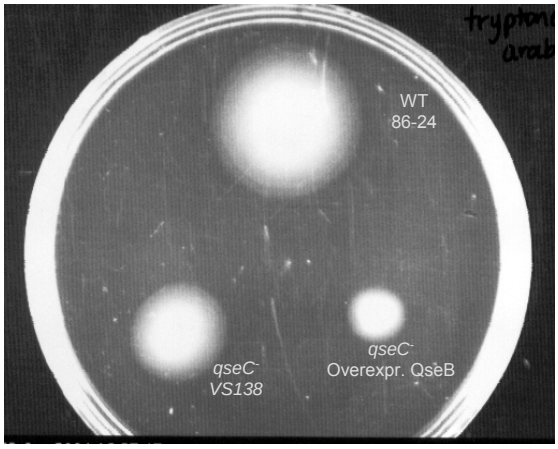


Figure 7.2 – Tryptone motility plate of wild-type, *qseC* mutant, and the *qseC* mutant overexpressing QseB.

EMSAs using the QseB&C-repressed *flhDC* promoter region.

We hypothesized that, when there is no quorum-sensing activation, unphosphorylated QseB may be binding to the central region of the *flhDC* promoter (-650bp to –300bp) and the *qseBC* promoter (-360bp to -120bp) in order to repress transcriptional activation. In order to test this theory, we performed an EMSA using natively purified, mostly unphosphorylated QseB and the central *flhDC* promoter region (-650bp to –300bp). Figure 5.5B shows that unphosphorylated QseB does not bind and shift a negative control, the *bla* promoter. Figure 7.3, however, shows that unphosphorylated QseB does bind and shift the central *flhDC* promoter region. In contrast, unphosphorylated QseB does not bind the -300bp to +130bp or -900bp to

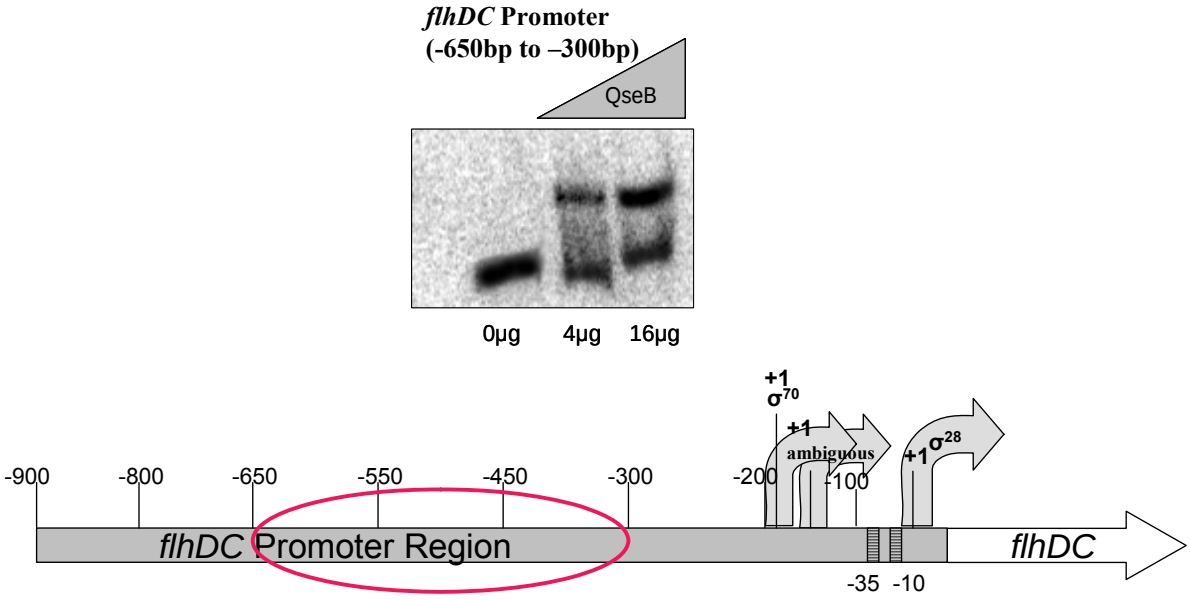


Figure 7.3 - EMSA with unphosphorylated QseB. An EMSA was performed using purified, phosphorylated QseB and the *flhDC* (-650bp to -300bp) promoter fragment.

-650bp regions of *flhDC* (Figure 5.5). This datum further supports the results showing that a *qseC* mutant overexpressing QseB is even less motile than a *qseC* mutant (Figure 7.2). It is possible that unphosphorylated QseB is binding to this central *flhDC* promoter region to inhibit the expression of *flhDC* even more than in the case of a *qseC* mutant. We hypothesize that unphosphorylated QseB may be repressing the *qseBC* central promoter in a similar fashion, although these experiments have not been undertaken. In the future, we would like to perform a transcriptional analysis of the *flhDC* (-900bp to +50bp) and *qseBC* (-500bp to +130bp) full promoters in wild-type, the *qseC* mutant, and the *qseC* mutant overexpressing QseB to further substantiate this hypothesis.

DISCUSSION

Although much more work needs to be performed using the *qseB* isogenic mutant, our preliminary studies suggest a model by which QseB&C act to regulate its own transcription and that of *flhDC* (Figure 7.4). In this model, QseB acts both as a repressor and an activator (or anti-repressor) of *flhDC* and *qseBC* expression. In the absence of signals, we predict that most of the QseB protein within the cell would be unphosphorylated. This unphosphorylated QseB binds to the central region of *flhDC* (Figure 7.3) and may repress its transcription. Upon sensing AI-3/Epi/NE, QseC autophosphorylates (Figure 4.2A) and transfers its phosphate to QseB (Figure 4.6). Hence, there is a shift in the balance of phosphorylated QseB, with most of the QseB in the cell being phosphorylated. Phosphorylated QseB may then bind to the two sites (-900bp to -650bp and -300bp to +50bp) in *flhDC*, displacing unphosphorylated QseB and allowing transcription of *flhDC* to proceed. This model is further reinforced by the phenotypes of a *qseB* mutant. In a *qseB* mutant, one does not observe any difference in *flhDC* regulation and motility from wild-type (data not shown and Figure 7.1). In this mutant, there is no QseB protein, and therefore neither repression nor activation (anti-repression) of *flhDC* transcription by unphosphorylated and phosphorylated QseB, respectively. The observation that overexpression of unphosphorylated QseB in a *qseC* mutant decreases motility even further (Figure 7.2) would be consistent with this model.

We recognize that these data are very preliminary, and extensive future studies (delineated in the next chapter) will be necessary to further define this model.

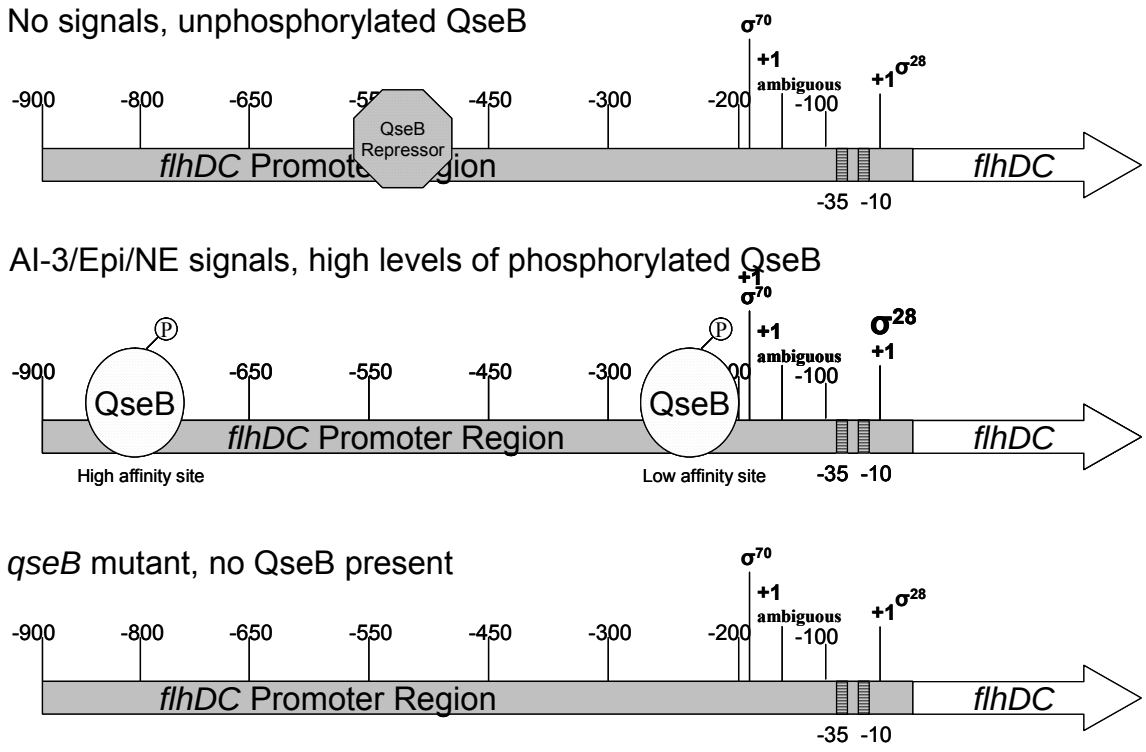


Figure 7.4 – Model of QseB&C autoregulation based upon the phosphorylation state of QseB. When no QseB is present, as in the case of the *qseB* mutant, transcription occurs through the basally-transcribed promoter, and no obvious phenotype is seen. However, in the case of the *qseC* mutant, most of the QseB protein is unphosphorylated and may bind the central promoter region to repress transcription. During QS activation in a wild-type situation, however, phosphorylated QseB may bind to high- and low-affinity sites in the *qseBC* promoter and act to derepress qseBC transcription. Transcription may then occur through the QseB&C-dependent promoter.

CHAPTER EIGHT

DISCUSSION AND FUTURE DIRECTIONS

The underlying tenet to this dissertation was to develop an in-depth understanding of the QseB&C two-component system in EHEC. Current research has suggested that microbes and mammals may communicate with each other through an array of hormone and hormone-like compounds [119]. The signals, however, may be "hijacked" by bacterial pathogens such as EHEC in order to activate its virulence genes and infect the host.

Armed with the information gathered previously, we hypothesized that QseC may be directly binding and responding to the AI-3 and epinephrine/norepinephrine signaling compounds during EHEC infection. This would allow EHEC to sense that it is in the large intestine and activate its flagella and motility genes in order to swim closer to the intestinal epithelium. EHEC may then activate type III secretion in order to form the classic attaching and effacing lesions.

In order to study the signaling and phosphorylation of the QseB&C two-component system, QseC-His was purified under native conditions and used to perform

several *in vitro* phosphorylation experiments, either in the absence or presence of the AI-3 and epi/NE signaling compounds. In these first experiments, however, we observed no consistent auto-phosphorylation. We then used a new approach to the *in vitro* phosphorylation experiment. Previous reports had reconstituted sensor kinase proteins into membrane fractions, allowing the protein to adopt its normal, membrane-bound conformation. After reconstituting QseC-His into these liposome fractions, we were able to successfully observe an increase in QseC autophosphorylation (in response to AI-3 and epi/NE), as well as transfer of phosphate to QseB (Chapter 4).

Interestingly, we observed that QseC autophosphorylation could be blocked by the addition of the α -adrenergic antagonist, phentolamine (PE), but not a β-adrenergic antagonist, propranolol (PO). This may suggest that QseC's recognition of signaling compounds may more resemble that of an α - than a β-adrenergic receptor.

Although we endeavored to find sequence homology between the complete bacterial QseC sensor kinase protein and adrenergic receptors, we were unable to identify any alignment. However, an *in silico* search using only the periplasmic domain of QseC reveals that its structure may be conserved far beyond that of bacteria (Figure 8.1) and is found in *Shigella flexneri, Salmonella enterica, Salmonella typhimurium, Erwinia carotovora, Haemophilus influenzae, Pasteurella multocida, Actinobacillus pleuropneumoniae, Chromobacterium violaceium, Rubrivivax gelatinosus, Thiobacillus denitrificans, Ralstonia eutropa, Ralstonia metallidurans,* and *Psychrobacter* sp. In fact, the search revealed homology to a fungal protein from *Aspergillus nidulans* of unknown function. We believe that this sensor protein may have a long evolutionary history that reaches past that of just *Escherichia coli*.

Future studies will be necessary to understand QseC signal recognition. Site-directed mutagenesis of amino acid residues in the periplasmic recognition domain would allow us to define important amino acid residues involved in QseC signal recognition. As can be observed in Figure 8.1, residues highlighted in green indicate amino acids that are identical in the majority of the proteins aligned (Figure 8.1). With this in mind, future experiments may include the site-directed mutagenesis of several of the highly conserved residues in the QseC periplasmic domain. This would allow the study of these mutants in the liposome system in order to determine which residues are important for signal binding. Ultimately, crystallization to determine the structure of QseC would prove very useful in understanding the specifics of signal binding. Given that mammalian intestinal enterocytes harbor α-adrenergic receptors, it would be consistent that a microbial adrenergic sensor (QseC) would mostly resemble (in an orthologous, and not a homologous fashion) an α- and not a β-adrenergic receptor. Taken together, these exciting and technically demanding results suggest that QseC may be a microbial adrenergic receptor conserved amongst different bacterial and fungal species.

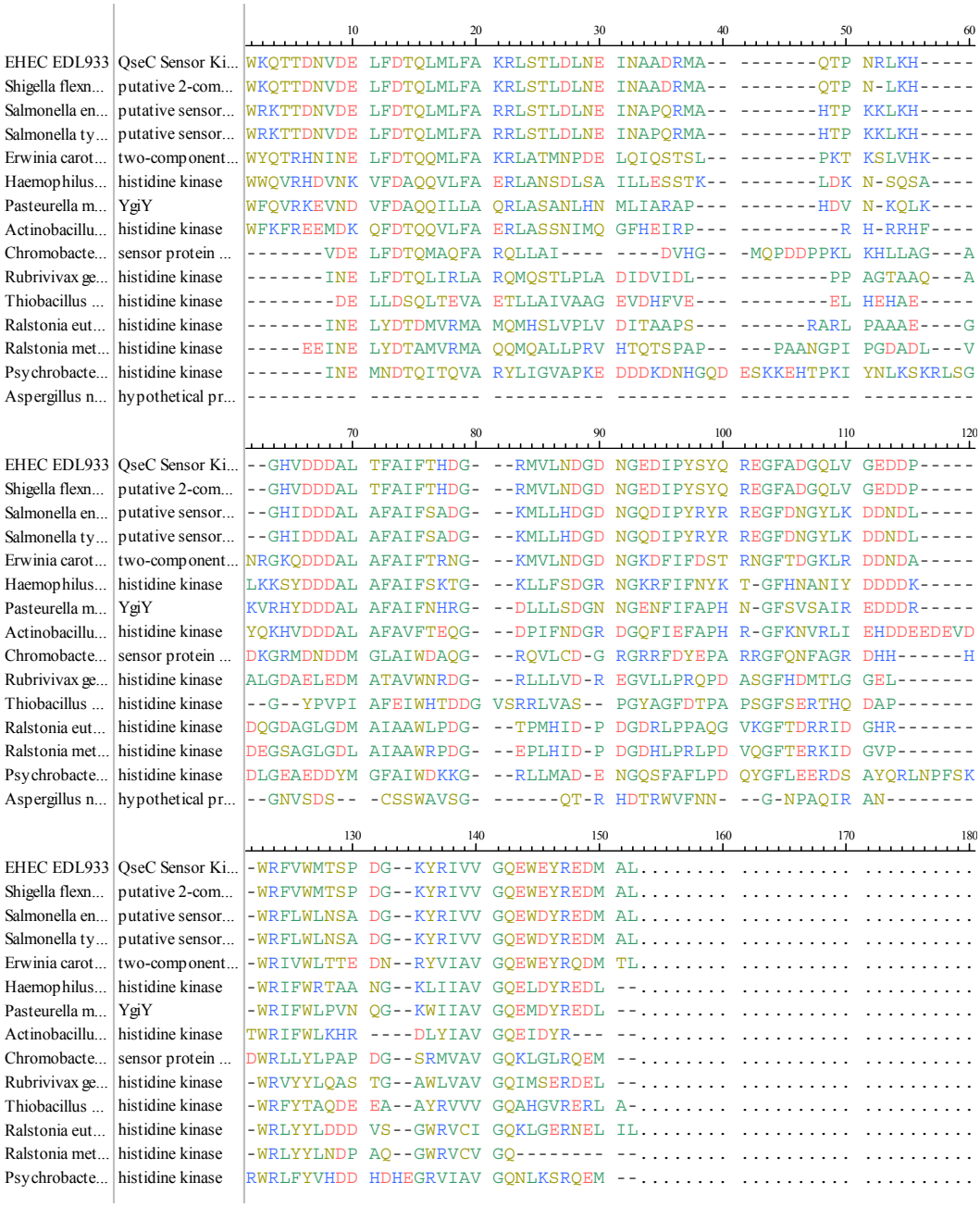


Figure 8.1 – Sequence alignment of the periplasmic (sensing) domain of QseC.

In our current model of EHEC virulence, QseC senses and responds to a signal (AI-3, epi, or NE), autophosphorylates, and transfers its phosphate to its cognate response regulator, QseB. We cannot rule out the possibility that QseC phosphorylates other proteins or that QseB is phosphorylated by other sensor kinases. However, the chances of any cross-phosphorylation are low, as a study by Yamamoto *et al.* (2005) showed that phosphorylation of non-cognate response regulators by histidine kinases is rare and occurs in only 22 of 692 combinations *in vitro* [197]. QseB and QseC were not used in the Yamamoto study due to technical problems with protein purification [197]. However, future studies should endeavor to find additional signaling partners for both QseC and QseB. This could be performed using the liposome system. Briefly, QseC-liposomes could be incubated with other non-cognate response regulator proteins, including QseF, to assess phospho-transfer. QseF is part of a predicted two-component system that may regulate the formation of the attaching and effacing lesions, the second step in the pathogenesis of EHEC infection (N. Reading and V. Sperandio, unpublished data). Another way of identifying signaling partners for QseC would be to perform genomic array analysis using our isogenic *qseC* and *qseB* mutants and comparing them with wild-type EHEC strains. These data would prove extremely beneficial, as we would be able to identify genes that may also be regulation targets and could gain much insight into this initial signaling step.

Interestingly, the flagella and motility genes do not seem to be expressed at the same time as the LEE genes. However, it is known that QseB&C activates *flhDC* transcription [161], and that FlhD&C are known to activate *csgD* transcription [211]. CsgD is an AraC homolog that has been shown to repress transcription of *tir/LEE5* (A.

Torres, unpublished data). Additionally, we have identified phosphorylated QseB binding sites in the *sdiA* and *cgsD* promoters. SdiA is a LuxR homolog and has a repressive role on the expression of the LEE genes [212](Hughes and Sperandio, unpublished).

We have identified QseC as a sensor for AI-3, Epi, and NE. The role of QseC in flagella regulation was also defined, and we have recognized a possible link for QseC regulation in *LEE* gene expression. To define the role of QseC in EHEC virulence *in vivo*, we utilized a natural animal model, the rabbit, infected with rabbit enteropathogenic *E. coli* (REPEC) in order to study intestinal pathogenesis (manuscript in preparation). Similar to EHEC, REPEC causes attaching and effacing lesions in intestinal cells and possesses the same virulence factors as human EHEC strains [213]. Wild-type REPEC caused severe weight loss and diarrhea in 7/8 rabbits by day 6. The *qseC* mutant showed attenuated virulence in rabbits, with only 2/8 developing diarrhea by day 7, and the rabbits gained weight like the PBS controls. Thus, these studies suggest that QseC may play an important role in EHEC virulence.

These results suggest an exciting possible alternative for treatment of EHEC infections by using adrenergic antagonists, such as phentolamine, which we have shown to inhibit the signaling cascade in EHEC through QseC. These studies may aide in the development of a new class of antimicrobials that can block AI-3 and Epi/NE signaling to pathogens. Additionally, these antimicrobials may be useful against a broad spectrum of other pathogens that contain homologs of QseC, including enteropathogenic *E. coli* (EPEC), *Salmonella, Shigella, Yersinia pestis, Fransicella tularensis,* and *Haemophilus influenzae*. Thus, the knowledge gained by studying QseC may ultimately lead to the

development of therapeutic drugs that may not only aide in the therapy against EHEC infection, but may also lead to a greater understanding of the communication between microbes and their host.

The other half of this dissertation endeavored to understand the molecular mechanism of the transcriptional regulation of *flhDC* and *qseBC* by the QseB&C two-component system. During EHEC infection, QseC senses the AI-3 produced by the normal flora and the Epi/NE produced naturally by the host and autophosphorylates. QseC then transfers its phosphate to QseB. Through comprehensive deletion analyses, electrophoretic mobility shift assays, and DNaseI footprinting, we were able to identify the specific binding sites to which phosphorylated QseB binds to regulate its own transcription and that of *flhDC*, the master regulator of flagella and motility genes. This consensus sequence (Figure 8.2) was utilized in an *in silico* search to identify other genes that may be regulated by QseB&C.

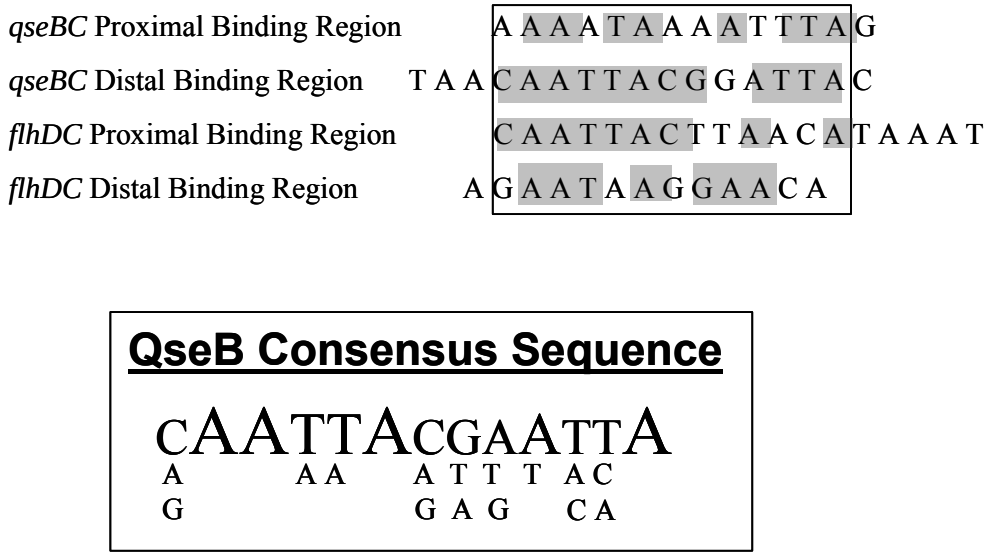


Figure 8.2 – Consensus sequence for phosphorylated QseB.

The results of the search yielded some interesting results (Appendix 1). The *yecG* gene, which is directly upstream from *flhDC* but divergently transcribed, also contained this consensus sequence. This suggests that QseB may also play a role in the regulation of *yecG* expression. However, preliminary studies using a *yecG::lacZ* transcriptional fusion suggest that there is no QseB&C-dependent regulation of *yecG* (B. Habdas and V. Sperandio, unpublished results). The presence of the consensus in the *yecG* promoter region may be due that fact that its regulatory region and the *flhDC* regulatory region overlap.

The *fliA* promoter also contained a QseB consensus sequence. Interestingly, previously performed transcriptional analyses demonstrated that the transcription from *flhDC* was reduced two-fold in a *qseC* mutant, while transcription from *fliA* was reduced 17-fold in this same mutant [1]. At the time, the difference in activation was attributed to the positive autoregulation of FliA [214]. However, the presence of a QseB consensus sequence alerts us to the possibility that QseB may also act to regulate transcription of *fliA* directly. It will be necessary to perform a comprehensive study of the *fliA* promoter, including electrophoretic mobility shift assays and DNaseI footprints to address this hypothesis.

Additional genes that may be regulated by QseB&C *in vivo* include *nleA* (Z6024), the non-LEE encoded effector A, which has recently been shown to be secreted through the LEE-encoded type III secretion system in EHEC [73]. A *Citrobacter rodentium nleA* mutant has also shown to be attenuated for virulence in mice [73]. Other genes include *yadM*, a putative fimbrial protein, several genes encoded in prophage CP-933, and *sdiA*. SdiA has recently been shown to be a receptor in *Escherichia coli* and *Salmonella*

typhimurium that detects the N-acyl homoserine lactone (AHL) quorum sensing signals exclusively from other species [108]. A signature tagged mutagenesis study identified SdiA in EHEC as being important for disease in cattle [215]. Furthermore, as previously mentioned, there is mounting evidence that SdiA is involved in *LEE* gene regulation [212]. Taken together, our *in silico* search for QseB consensus sites has led to the identification of several other interesting virulence genes that may be regulated through the QseB&C two-component system in EHEC.

The studies from chapter 7 suggest that QseB may be acting to repress transcription of both its own promoter and *flhDC* when in an unphosphorylated state. This hypothesis will have to be extensively addressed in the future. This scenario may allow QseB&C to regulate transcription of virulence genes in a very intricate fashion. The overall model of the QseB&C two-component system is depicted in Figure 8.3. Before EHEC enters the intestine, QseC is not sensing AI-3, epinephrine or norepinephrine. Thus, QseC is not autophosphorylating, and most of the QseB present in the cell is unphosphorylated (Figure 8.3). Unphosphorylated QseB may be binding to the central region of its own promoter to inhibit transcription. A basal level of transcription occurs from *qseBC* through a basally transcribed, QseB&C-independent σ^{70} promoter. Additionally, unphosphorylated QseB binds the central region of the *flhDC* promoter. Transcription of *flhDC*, however, is complex and basal-level transcription may still be occurring mostly through the σ^{28} promoter in addition to the previously mapped σ^{70} and ambiguous promoters (Figure 8.3).

During infection, however, EHEC enters the intestinal lumen and senses the AI-3 produced by the normal flora and the epinephrine/norepinephrine produced naturally by

the host. These compounds interact with the periplasmic domain of QseC. QseC then autophosphorylates on a conserved histidine residue and transfers this phosphate to QseB. Phosphorylated QseB then binds to high- and low-affinity sites in its own promoter, leading to positive amplification of its own transcription through its σ^{70} QseB&C-dependent transcriptional start site. Phosphorylated QseB also interacts with high- and low-affinity sites in the *flhDC* promoter, possibly saturating both sites. Binding at both promoter sites may expose the FliA (σ^{28}) promoter. FliA (σ^{28}) is subsequently able to interact with RNA polymerase in order to increase *flhDC* transcription through the FliA (σ^{28}) promoter. These interactions may displace the unphosphorylated QseB. Hence, we hypothesize that the binding of phosphorylated QseB may have more of an anti-repressive role by overcoming repression of unphosphorylated QseB, than an activating role. Thus, only after sensing AI-3 and epi/NE, EHEC activates the energetically expensive production of flagella in order to swim proficiently through the intestinal mucus layer and attain close contact with the enteric epithelia. Finally, EHEC is able to express its type III secretion system and form its characteristic attaching and effacing lesions.

Future experiments should be able to better define these proposed models. One could generate QseB site-directed mutants in the conserved Asp residue by changing it to Glu, which has been shown to mimic the phosphorylated conformation of these response regulators, and to Ala, which has been shown to mimic the unphosphorylated state. These mutant proteins could harbor different tags, so double EMSAs with these proteins and *qseBC* and *flhDC* could establish the affinity of each one and the rate at which phosphorylated QseB could, in theory, displace unphosphorylated QseB. Additionally,

plasmids harboring these proteins could be used to complement a *qseB* mutant to assess *qseBC* and *flhDC* transcription in the presence of just phosphorylated QseB (QseB-Glu), just unphosphorylated QseB (QseB-Ala) or in varied concentrations of both.

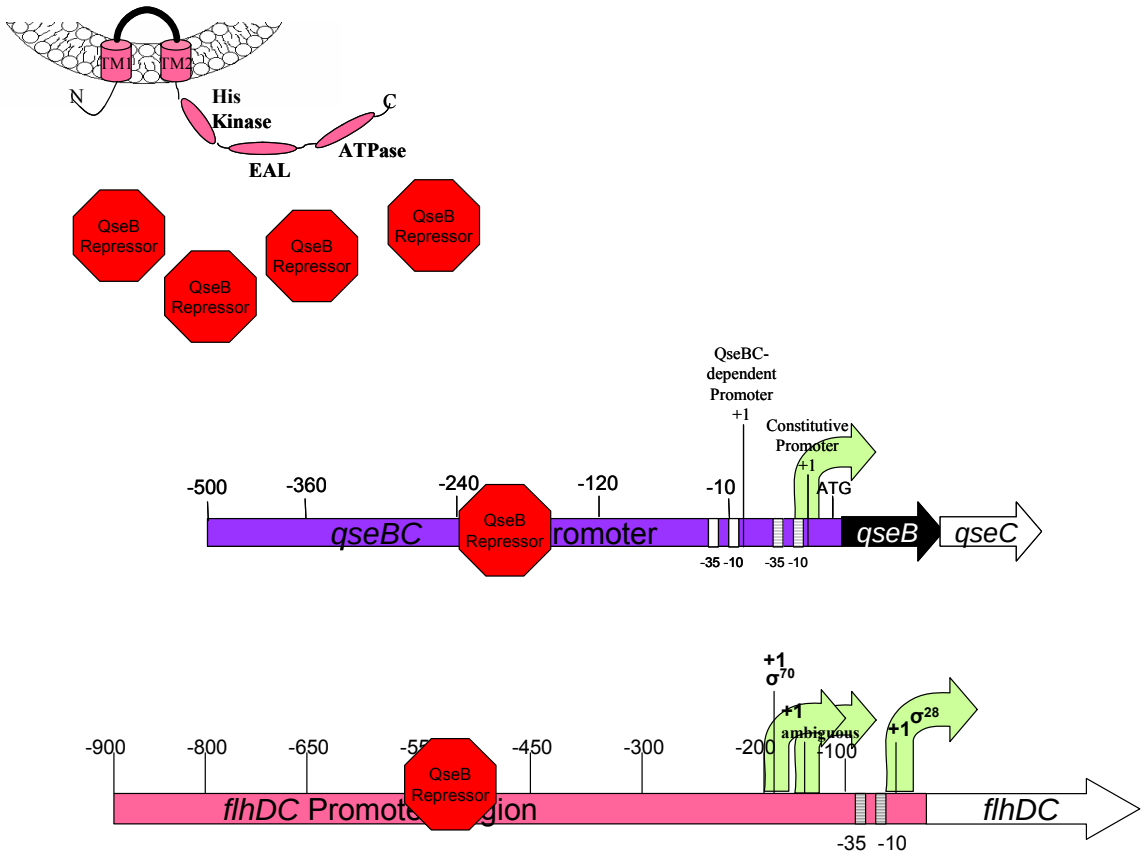


Figure 8.3 – QseB&C Model: No signals present. When EHEC is not in the presence of AI-3, epinephrine or norepinephrine, QseC does not increase its autophosphorylation. Thus, most of the QseB is unphosphorylated. Unphosphorylated QseB may bind to a central region of its own promoter to repress transcription. Thus, *qseBC* transcription only occurs through a basally transcribed, QseB&C-independent constitutive promoter. Unphosphorylated QseB also binds to a central region of the *flhDC* promoter to repress transcription. *flhDC* transcriptional regulation is complex. We believe that *flhDC* transcription may occur at a basal level through previously mapped σ^{70} and ambiguous promoters, but still occurs mostly through the σ^{28} promoter.

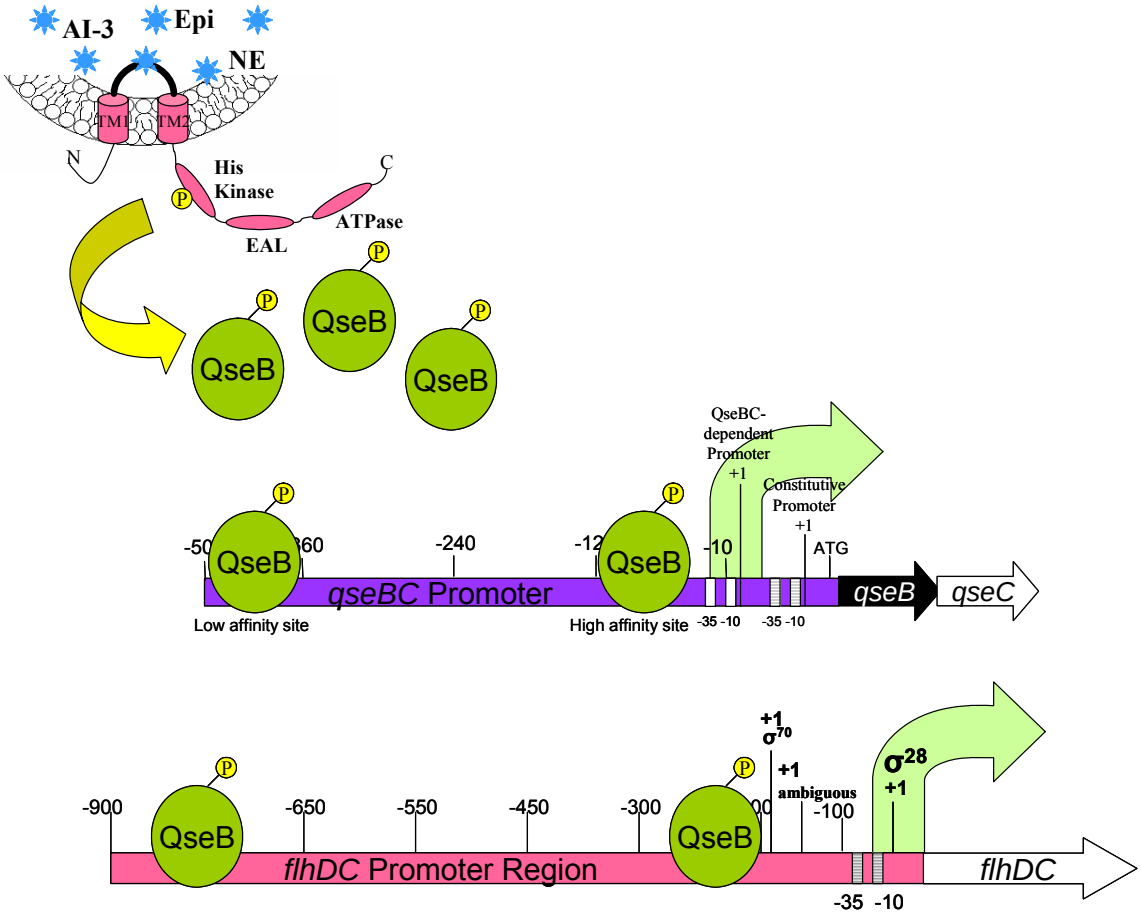


Figure 8.4 – QseB&C Model: During infection, signals present. During infection, QseC interacts with the AI-3 produced by the normal flora and the epinephrine/norepinephrine produced naturally by the host. QseC increases its autophosphorylation on a conserved histidine residue in response to signal binding. This phosphate is transferred to a conserved aspartate residue on QseB. Phosphorylated QseB binds to high- and low-affinity binding sites in its own promoter in order to increase its own transcription through the QseB&C-dependent promoter. Phosphorylated QseB also binds to high- and low-affinity binding sites in the *flhDC* promoter in order to increase *flhDC* transcription through the σ^{28} promoter.

In conclusion, the QseB&C two-component system may play an important role in the regulation of virulence factors in EHEC. We were able to create an *in vitro* liposome system, which can be "loaded" with signaling compounds and inhibitors in order to study the signaling of the QseB&C two-component system (Chapter 4). Additionally, our studies were the first to show a direct interaction between signaling compounds and the QseC sensor kinase (Chapter 4) and may represent a new frontier for antimicrobial drug targets. Finally, our studies have allowed us to gain a better understanding of the mechanism by which phosphorylated QseB regulates gene transcription (Chapters 5, 6, and 7). The results of these analyses have allowed us to determine the potential consensus sequence to which QseB binds, which we have utilized in an *in silico* search to identify potential novel targets of QseB. Through the use of both genetics and biochemistry, we have performed a comprehensive functional analysis of the mechanism by which the QseB&C two-component system signals and regulates the transcription of both itself and the flagellar master regulator, *flhDC*, in EHEC.

APPENDIX

APPENDIX 1 - in silico search for QseB consensus sequences

GeneID and annotation
Z2948: yecG putative regulator
Z2945: flhC regulator of flagellar biosynthesis acting on class 2 operons; transcription initiation factor?
Z2946: flhD regulator of flagellar biosynthesis, acting on class 2 operons; transcriptional initiation factor?
Z0321: - putative AraC-type regulatory protein encoded in prophage CP-933H
Z0333: psuI putative polarity suppression protein encoded in CP-933I
Z0334: sidI putative capsid morphogenesis protein encoded in CP-933I
Z0469: - putative structural protein (partial)
Z2195: ydeK orf, hypothetical protein
Z2780: cstC acetylornithine delta-aminotransferase
Z2780: cstC acetylornithine delta-aminotransferase
Z2781: xthA exonuclease III
Z2926: yecK putative cytochrome C-type protein
Z3004: sdiA transcriptional regulator of ftsQAZ gene cluster
Z3012: fliA flagellar biosynthesis; alternative sigma factor 28; regulation of flagellar operons
Z3737: dapA dihydrodipicolinate synthase
Z5732: lysU lysine tRNA synthetase, inducible; heat shock protein
Z5923: yjiC orf, hypothetical protein
Z5932: - putative invasin
Z6024: - unknown protein encoded by cryptic prophage CP-933P
Z0047: fixA probable flavoprotein subunit, carnitine metabolism
Z0086: leuO probable transcriptional activator for leuABCD operon
Z0144: panC pantothenate synthetase
Z0145: panB 3-methyl-2-oxobutanoate hydroxymethyltransferase
Z0149: yadM putative fimbrial protein
Z0175: yaeH putative structural protein
Z0212: yaeD putative phosphatase
Z0259: - -
Z0263: - -
Z0264: - -
Z0311: - partial O replication protein for prophage CP-933H
Z0312: - partial O replication protein for prophage CP-933H
Z0313: - partial replication protein P for prophage CP-933H
Z0318: pinH DNA invertase from prophage CP-933H
Z0319: - unknown protein from prophage CP-933H
Z0319: - unknown protein from prophage CP-933H
Z0328: - unknown protein encoded in prophage CP-933I
Z0330: - unknown protein encoded in prophage CP-933I
Z0335: - unknown protein encoded in prophage CP-933I
Z0336: - putative regulatory protein encoded in prophage CP-933I
Z0409: yahF putative oxidoreductase subunit
Z0468: hemB 5-aminolevulinate dehydratase = porphobilinogen synthase
Z0487: yaiE orf, hypothetical protein
Z0609: - -
Z0764: citA putative sensor-type protein
Z0766: dcuC transport of dicarboxylates
Z0845: kdpA ATPase of high-affinity potassium transport system, A chain
Z0851: - -
Z0898: - -
Z0899: - -
Z0928: galT galactose-1-phosphate uridylyltransferase
Z0932: - orf, hypothetical protein
Z0933: modA molybdate-binding periplasmic protein; permease
Z0955: - unknown protein encoded by prophage CP-933K
Z1035: ybiF putative transmembrane subunit
Z1072: - putative DEOR-type transcriptional regulator
Z1094: artP ATP-binding component of 3rd arginine transport system
Z1095: ybjP putative enzyme
Z1129: - putative helicase
Z1185: - -
Z1190: - putative glucosyltransferase
Z1308: sulA suppressor of lon; inhibits cell division and ftsZ ring formation
Z1319: - putative oxidoreductase
Z1333: - similar to DicA, regulator of DicB; encoded within cryptic prophage CP-933M
Z1337: - unknown protein encoded by cryptic prophage CP-933M

Z4362: yghB orf, hypothetical protein
Z4364: yqhD putative oxidoreductase
Z4369: yqhH orf, hypothetical protein
Z4372: plsC 1-acyl-sn-glycerol-3-phosphate acyltransferase
Z4373: parC DNA topoisomerase IV subunit A
Z4373: parC DNA topoisomerase IV subunit A
Z4374: - putative transport periplasmic protein
Z4376: ygiW orf, hypothetical protein
Z4376: ygiW orf, hypothetical protein
Z4377: qseB quorum sensing Escherichia coli regulator B
Z4378: qseC quorum sensing Escherichia coli regulator C
Z4379: mdaB modulator of drug activity B
Z4380: ygiN orf, hypothetical protein
Z4380: ygiN orf, hypothetical protein
Z4382: - putative iron compound-binding protein of ABC transporter family
Z4383: - putative iron compound permease protein of ABC transporter family
Z4384: - putative iron compound permease protein of ABC transporter family
Z4384: - putative iron compound permease protein of ABC transporter family
Z4385: - putative ATP-binding protein of ABC transporter family
Z4385: - putative ATP-binding protein of ABC transporter family
Z4385: - putative ATP-binding protein of ABC transporter family
Z4385: - putative ATP-binding protein of ABC transporter family
Z4385: - putative ATP-binding protein of ABC transporter family
Z4386: - putative iron compound receptor
Z4386: - putative iron compound receptor
Z4386: - putative iron compound receptor
Z4386: - putative iron compound receptor
Z4386: - putative iron compound receptor
Z4390: yqiB putative enzyme
Z4391: yqiE orf, hypothetical protein
Z4391: yqiE orf, hypothetical protein
Z4396: ygiD orf, hypothetical protein
Z4396: ygiD orf, hypothetical protein
Z4397: ygiE orf, hypothetical protein
Z4397: ygiE orf, hypothetical protein
Z4398: - -
Z4399: ribB 3,4 dihydroxy-2-butanone-4-phosphate synthase
Z4401: glgS glycogen biosynthesis, rpoS dependent
Z4402: - putative oxidoreductase
Z4403: - putative membrane protein
Z4403: - putative membrane protein
Z4405: - putative kinase
Z4407: ygiF orf, hypothetical protein
Z4408: ygiM orf, hypothetical protein
Z4408: ygiM orf, hypothetical protein
Z4408: ygiM orf, hypothetical protein
Z4408: ygiM orf, hypothetical protein
Z4409: cca tRNA nucleotidyl transferase
Z4409: cca tRNA nucleotidyl transferase
Z4409: cca tRNA nucleotidyl transferase
Z4409: cca tRNA nucleotidyl transferase
Z4410: bacA bacitracin resistance; possibly phosphorylates undecaprenol
Z4412: ygiH orf, hypothetical protein
Z4414: ttdA L-tartrate dehydratase, subunit A
Z4415: ttdB L-tartrate dehydratase, subunit B
Z4416: ygjE orf, hypothetical protein
Z4418: rpsU 30S ribosomal subunit protein S21
Z4419: dnaG DNA biosynthesis; DNA primase
Z4423: yqjH orf, hypothetical protein
Z4423: yqjH orf, hypothetical protein
Z4424: yqjI orf, hypothetical protein
Z4424: yqjI orf, hypothetical protein
Z4424: yqjI orf, hypothetical protein
Z4425: aer aerotaxis sensor receptor, flavoprotein
Z4426: ygjG probable ornithine aminotransferase
Z4428: ebgR regulator of ebg operon
Z4428: ebgR regulator of ebg operon

ACKNOWLEDGEMENTS

I would first like to thank my mentor, Dr. Vanessa Sperandio, for her guidance, support, and patience during my graduate training. I couldn't have done it without you! I know that the time and effort spent mentoring me and responding to relentless questions was tremendous, and I thank you for your efforts. I also thank my co-workers from the Sperandio lab: Ben Habdas, David Hughes, Nicola Reading, Regan Russell, Faith Sharp, and Matthew Walters. Without you all, I would not have had such a pleasant experience in graduate school. You have become wonderful friends.

I thank the Division of Basic Science and the Molecular Microbiology program, and I especially thank my committee members Dr. Melanie Cobb, Dr. Eric Hansen, and Dr. Kevin McIver. I was also supported by the NIH Training Grant 5-T32-AI007520-07.

I thank my parents Frederick F. Cunningham, Jr. and M. Beth Cunningham and my brother, Brian Cunningham for their love, support, and encouragement while I was growing up and during my graduate studies. I would not be the person that I am today without you. I also thank my grandparents, Cecelia Cunningham, Robert Gelczer, and Marjorie Gelczer. I thank my mother- and father-in-law, Christine and William Clarke, and my sister- and brother-in-law, Kathryn and Douglas Clarke, for their support.

Thanks to all of my friends, who know who they are, for their friendship and advice during my graduate studies. Finally, I would especially like to thank my husband, Daniel W. Clarke, for his encouragement, support, hugs, and advice. You are my best friend, and I could not have done it without you!

REFERENCES

1. Sperandio, V., A.G. Torres, and J.B. Kaper, *Quorum sensing Escherichia coli regulators B and C (QseBC): a novel two-component regulatory system involved in the regulation of flagella and motility by quorum sensing in E. coli.* Mol. Microbiol., 2002. **43**(3): p. 809-821.
2. Riley, L.W., et al., *Hemorrhagic colitis associated with a rare Escherichia coli serotype.* N Engl J Med, 1983. **308**(12): p. 681-5.
3. Nataro, J.P. and J.B. Kaper, *Diarrheagenic Escherichia coli.* Clin Microbiol Rev, 1998. **11**(1): p. 142-201.
4. Kimmitt, P.T., C.R. Harwood, and M.R. Barer, *Induction of type 2 Shiga toxin synthesis in Escherichia coli O157 by 4-quinolones.* Lancet, 1999. **353**(9164): p. 1588-9.
5. Kimmitt, P.T., C.R. Harwood, and M.R. Barer, *Toxin gene expression by shiga toxin-producing Escherichia coli: the role of antibiotics and the bacterial SOS response.* Emerg Infect Dis, 2000. **6**(5): p. 458-65.
6. Banatvala, N., et al., *The United States National Prospective Hemolytic Uremic Syndrome Study: microbiologic, serologic, clinical, and epidemiologic findings.* J Infect Dis, 2001. **183**(7): p. 1063-70.
7. Karmali, M.A., et al., *Sporadic cases of haemolytic-uraemic syndrome associated with faecal cytotoxin and cytotoxin-producing Escherichia coli in stools.* Lancet, 1983. **1**(8325): p. 619-20.
8. Rangel, J.M., et al., *Epidemiology of Escherichia coli O157:H7 outbreaks, United States, 1982-2002.* Emerg Infect Dis, 2005. **11**(4): p. 603-9.

9. Bell, B.P., et al., *A multistate outbreak of Escherichia coli O157:H7-associated bloody diarrhea and hemolytic uremic syndrome from hamburgers. The Washington experience.* Jama, 1994. **272**(17): p. 1349-53.

10. Administration, U.S.F.a.D., *Food Code: 1993 Recommendations of the United States Publich Health Service, Food and Drug Administration.* 1993. **Pub. no. PB94-11394**.

11. Prevention, C.f.D.C.a., *Escherichia coli O157:h7 infections associated with eating a nationally distributed commercial brand of frozen ground beef patties and burgers- Colorado, 1997.* MMWR Morb Mortal Wkly Rep., 1997. **46**: p. 777-778.

12. Prevention, C.f.D.C.a., *Reducing the risk for transmission of enteric pathogens at petting zoos, open farms, animal exhibits, and other venues where the public has contact with farm animals.* MMWR Morb Mortal Wkly Rep, 2001. **50**: p. 297.

13. Tarr, P.I., C.A. Gordon, and W.L. Chandler, *Shiga-toxin-producing Escherichia coli and haemolytic uraemic syndrome.* Lancet, 2005. **365**(9464): p. 1073-86.

14. Kaper, J.B. and A.D. O'Brien, *Escherichia coli O157:H7 and other Shiga toxin-producing E. coli strains*. first ed. 1998, Washington, D.C.: ASM Press.

15. Bell, B.P., et al., *Predictors of hemolytic uremic syndrome in children during a large outbreak of Escherichia coli O157:H7 infections.* Pediatrics, 1997. **100**(1): p. E12.

16. Carter, A.O., et al., *A severe outbreak of Escherichia coli O157:H7--associated hemorrhagic colitis in a nursing home.* N Engl J Med, 1987. **317**(24): p. 1496-500.

17. Griffin, P.M., et al., *Illnesses associated with Escherichia coli 0157:H7 infections. A broad clinical spectrum.* Ann Intern Med, 1988. **109**(9): p. 705-12.

18. Knutton, S., D.R. Lloyd, and A.S. McNeish, *Adhesion of enteropathogenic Escherichia coli to human intestinal enterocytes and cultured human intestinal mucosa.* Infect Immun, 1987. **55**(1): p. 69-77.

19. Moon, H.W., et al., *Attaching and effacing activities of rabbit and human enteropathogenic Escherichia coli in pig and rabbit intestines.* Infect Immun, 1983. **41**(3): p. 1340-51.

20. Baldwin, T.J., et al., *Elevation of intracellular free calcium levels in HEp-2 cells infected with enteropathogenic Escherichia coli.* Infect Immun, 1991. **59**(5): p. 1599-604.

21. Savkovic, S.D., A. Koutsouris, and G. Hecht, *Attachment of a noninvasive enteric pathogen, enteropathogenic Escherichia coli, to cultured human intestinal epithelial monolayers induces transmigration of neutrophils.* Infect Immun, 1996. **64**(11): p. 4480-7.

22. Elliott, E., et al., *Modulation of host response to Escherichia coli o157:H7 infection by anti-CD18 antibody in rabbits.* Gastroenterology, 1994. **106**(6): p. 1554-61.

23. Li, Y., et al., *Human response to Escherichia coli O157:H7 infection: antibodies to secreted virulence factors.* Infect Immun, 2000. **68**(9): p. 5090-5.

24. Dean-Nystrom, E.A., *Bovine Escherichia coli O157:H7 infection model.* Methods Mol Med, 2003. **73**: p. 329-38.

25. Acheson, D.W., et al., *Protective immunity to Shiga-like toxin I following oral immunization with Shiga-like toxin I B-subunit-producing Vibrio cholerae CVD 103-HgR.* Infect Immun, 1996. **64**(1): p. 355-7.

26. Hayashi, T., et al., *Complete genome sequence of enterohemorrhagic Escherichia coli O157:H7 and genomic comparison with a laboratory strain K-12.* DNA Res, 2001. **8**(1): p. 11-22.

27. Perna, N.T., et al., *Genome sequence of enterohaemorrhagic Escherichia coli O157:H7.* Nature, 2001. **409**(6819): p. 529-33.

28. Wadolkowski, E.A., J.A. Burris, and A.D. O'Brien, *Mouse model for colonization and disease caused by enterohemorrhagic Escherichia coli O157:H7.* Infect Immun, 1990. **58**(8): p. 2438-45.

29. Wadolkowski, E.A., et al., *Acute renal tubular necrosis and death of mice orally infected with Escherichia coli strains that produce Shiga-like toxin type II.* Infect Immun, 1990. **58**(12): p. 3959-65.

30. Woods, J.B., et al., *Ferrets as a model system for renal disease secondary to intestinal infection with Escherichia coli O157:H7 and other Shiga toxin-producing E. coli.* J Infect Dis, 2002. **185**(4): p. 550-4.

31. Dean-Nystrom, E.A., et al., *Escherichia coli O157:H7 causes more-severe systemic disease in suckling piglets than in colostrum-deprived neonatal piglets.* Infect Immun, 2000. **68**(4): p. 2356-8.

32. Dean-Nystrom, E.A., et al., *Pathogenicity of Escherichia coli O157:H7 in the intestines of neonatal calves.* Infect Immun, 1997. **65**(5): p. 1842-8.

33. Ritchie, J.M., et al., *Critical roles for stx2, eae, and tir in enterohemorrhagic Escherichia coli-induced diarrhea and intestinal inflammation in infant rabbits.* Infect Immun, 2003. **71**(12): p. 7129-39.

34. Heczko, U., A. Abe, and B.B. Finlay, *In vivo interactions of rabbit enteropathogenic Escherichia coli O103 with its host: an electron microscopic and histopathologic study.* Microbes Infect, 2000. **2**(1): p. 5-16.

35. Heczko, U., et al., *Decreased apoptosis in the ileum and ileal Peyer's patches: a feature after infection with rabbit enteropathogenic Escherichia coli O103.* Infect Immun, 2001. **69**(7): p. 4580-9.

36. Sjogren, R., et al., *Role of Shiga-like toxin I in bacterial enteritis: comparison between isogenic Escherichia coli strains induced in rabbits.* Gastroenterology, 1994. **106**(2): p. 306-17.

37. Tai, Y.H., et al., *Electrolyte transport in rabbit cecum. I. Effect of RDEC-1 infection.* Am J Physiol, 1989. **256**(4 Pt 1): p. G721-6.

38. Marches, O., et al., *Role of tir and intimin in the virulence of rabbit enteropathogenic Escherichia coli serotype O103:H2.* Infect Immun, 2000. **68**(4): p. 2171-82.

39. Tauschek, M., R.A. Strugnell, and R.M. Robins-Browne, *Characterization and evidence of mobilization of the LEE pathogenicity island of rabbit-specific strains of enteropathogenic Escherichia coli.* Mol Microbiol, 2002. **44**(6): p. 1533-50.

40. Zhu, C., et al., *Complete nucleotide sequence and analysis of the locus of enterocyte Effacement from rabbit diarrheagenic Escherichia coli RDEC-1.* Infect Immun, 2001. **69**(4): p. 2107-15.

41. Snider, T., Fabich, AJ, Washburn, K, Sims, W, Leatham, MP, Cohen, PS, Conway, T, Clinkenbeard KD. *Streptomycin-Treated Cattle Model for Colonization by Escherichia coli O157:H7*. in *General Meeting for the American Society of Microbiology*. 2005. Atlanta, Georgia, USA.

42. McDaniel, T.K., et al., *A genetic locus of enterocyte effacement conserved among diverse enterobacterial pathogens.* Proc Natl Acad Sci U S A, 1995. **92**(5): p. 1664-8.

43. Elliott, S.J., et al., *The complete sequence of the locus of enterocyte effacement (LEE) from enteropathogenic Escherichia coli E2348/69.* Mol Microbiol, 1998. **28**(1): p. 1-4.

44. Mellies, J.L., et al., *The Per regulon of enteropathogenic Escherichia coli : identification of a regulatory cascade and a novel transcriptional activator, the locus of enterocyte effacement (LEE)-encoded regulator (Ler).* Mol Microbiol, 1999. **33**(2): p. 296-306.

45. Elliott, S.J., J. Yu, and J.B. Kaper, *The cloned locus of enterocyte effacement from enterohemorrhagic Escherichia coli O157:H7 is unable to confer the attaching and effacing phenotype upon E. coli K-12.* Infect Immun, 1999. **67**(8): p. 4260-3.

46. Jarvis, K.G., et al., *Enteropathogenic Escherichia coli contains a putative type III secretion system necessary for the export of proteins involved in attaching and effacing lesion formation.* Proc Natl Acad Sci U S A, 1995. **92**(17): p. 7996-8000.

47. Jerse, A.E., et al., *A genetic locus of enteropathogenic Escherichia coli necessary for the production of attaching and effacing lesions on tissue culture cells.* Proc Natl Acad Sci U S A, 1990. **87**(20): p. 7839-43.

48. Kenny, B., et al., *Enteropathogenic E. coli (EPEC) transfers its receptor for intimate adherence into mammalian cells.* Cell, 1997. **91**(4): p. 511-20.

49. Bustamante, V.H., et al., *Transcriptional regulation of type III secretion genes in enteropathogenic Escherichia coli: Ler antagonizes H-NS-dependent repression.* Mol Microbiol, 2001. **39**(3): p. 664-78.

50. Elliott, S.J., et al., *The locus of enterocyte effacement (LEE)-encoded regulator controls expression of both LEE- and non-LEE-encoded virulence factors in enteropathogenic and enterohemorrhagic Escherichia coli.* Infect Immun, 2000. **68**(11): p. 6115-26.

51. Sanchez-SanMartin, C., et al., *Transcriptional regulation of the orf19 gene and the tir-cesT-eae operon of enteropathogenic Escherichia coli.* J Bacteriol, 2001. **183**(9): p. 2823-33.

52. Sperandio, V., et al., *Activation of enteropathogenic Escherichia coli (EPEC) LEE2 and LEE3 operons by Ler.* Mol Microbiol, 2000. **38**(4): p. 781-93.

53. Roe, A.J., D.E. Hoey, and D.L. Gally, *Regulation, secretion and activity of type III-secreted proteins of enterohaemorrhagic Escherichia coli O157.* Biochem Soc Trans, 2003. **31**(Pt 1): p. 98-103.

54. Wilson, R.K., et al., *Role of EscF, a putative needle complex protein, in the type III protein translocation system of enteropathogenic Escherichia coli.* Cellular Microbiology, 2001. **3**(11): p. 753-62.

55. Ide, T., et al., *Characterization of translocation pores inserted into plasma membranes by type III-secreted Esp proteins of enteropathogenic Escherichia coli.* Cell Microbiol, 2001. **3**(10): p. 669-79.

56. Kenny, B. and B.B. Finlay, *Protein secretion by enteropathogenic Escherichia coli is essential for transducing signals to epithelial cells.* Proc Natl Acad Sci U S A, 1995. **92**(17): p. 7991-5.

57. Rosenshine, I., et al., *A pathogenic bacterium triggers epithelial signals to form a functional bacterial receptor that mediates actin pseudopod formation.* Embo J, 1996. **15**(11): p. 2613-24.

58. DeVinney, R., et al., *Enterohemorrhagic Escherichia coli O157:H7 produces Tir, which is translocated to the host cell membrane but is not tyrosine phosphorylated.* Infect Immun, 1999. **67**(5): p. 2389-98.

59. Deibel, C., et al., *EspE, a novel secreted protein of attaching and effacing bacteria, is directly translocated into infected host cells, where it appears as a tyrosine-phosphorylated 90 kDa protein.* Mol Microbiol, 1998. **28**(3): p. 463-74.

60. Campellone, K.G., et al., *Clustering of Nck by a 12-residue Tir phosphopeptide is sufficient to trigger localized actin assembly.* J Cell Biol, 2004. **164**(3): p. 407-16.

61. Gruenheid, S., et al., *Enteropathogenic E. coli Tir binds Nck to initiate actin pedestal formation in host cells.* Nat Cell Biol, 2001. **3**(9): p. 856-9.

62. Goosney, D.L., R. DeVinney, and B.B. Finlay, *Recruitment of cytoskeletal and signaling proteins to enteropathogenic and enterohemorrhagic Escherichia coli pedestals.* Infect Immun, 2001. **69**(5): p. 3315-22.

63. Campellone, K.G., D. Robbins, and J.M. Leong, *EspFU is a translocated EHEC effector that interacts with Tir and N-WASP and promotes Nck-independent actin assembly.* Dev Cell, 2004. **7**(2): p. 217-28.

64. Wainwright, L.A. and J.B. Kaper, *EspB and EspD require a specific chaperone for proper secretion from enteropathogenic Escherichia coli.* Mol Microbiol, 1998. **27**(6): p. 1247-60.

65. Kenny, B., R. Haigh, and I.B. Holland, *Analysis of the haemolysin transport process through the secretion from Escherichia coli of PCM, CAT or beta-galactosidase fused to the Hly C-terminal signal domain.* Mol Microbiol, 1991. **5**(10): p. 2557-68.

66. Kenny, B. and M. Jepson, *Targeting of an enteropathogenic Escherichia coli (EPEC) effector protein to host mitochondria.* Cell Microbiol, 2000. **2**(6): p. 579-90.

67. Creasey, E.A., et al., *CesT is a bivalent enteropathogenic Escherichia coli chaperone required for translocation of both Tir and Map.* Molecular Microbiology, 2003. **47**(1): p. 209-21.

68. McNamara, B.P. and M.S. Donnenberg, *A novel proline-rich protein, EspF, is secreted from enteropathogenic Escherichia coli via the type III export pathway.* FEMS Microbiol Lett, 1998. **166**(1): p. 71-8.

69. McNamara, B.P., et al., *Translocated EspF protein from enteropathogenic Escherichia coli disrupts host intestinal barrier function.* J Clin Invest, 2001. **107**(5): p. 621-9.

70. Tomson, F.L., et al., *Enteropathogenic Escherichia coli EspG disrupts microtubules and in conjunction with Orf3 enhances perturbation of the tight junction barrier.* Mol Microbiol, 2005. **56**(2): p. 447-64.

71. Tu, X., et al., *EspH, a new cytoskeleton-modulating effector of enterohaemorrhagic and enteropathogenic Escherichia coli.* Mol Microbiol, 2003. **47**(3): p. 595-606.

72. Charpentier, X. and E. Oswald, *Identification of the secretion and translocation domain of the enteropathogenic and enterohemorrhagic Escherichia coli effector Cif, using TEM-1 beta-lactamase as a new fluorescence-based reporter.* J Bacteriol, 2004. **186**(16): p. 5486-95.

73. Gruenheid, S., et al., *Identification and characterization of NleA, a non-LEE-encoded type III translocated virulence factor of enterohaemorrhagic Escherichia coli O157:H7.* Mol Microbiol, 2004. **51**(5): p. 1233-49.

74. Iyoda, S. and H. Watanabe, *Positive effects of multiple pch genes on expression of the locus of enterocyte effacement genes and adherence of enterohaemorrhagic Escherichia coli O157 : H7 to HEp-2 cells.* Microbiology, 2004. **150**(Pt 7): p. 2357-571.

75. Friedberg, D., et al., *Hierarchy in the expression of the locus of enterocyte effacement genes of enteropathogenic Escherichia coli.* Mol Microbiol, 1999. **34**(5): p. 941-52.

76. Zhang, L., et al., *Regulators encoded in the Escherichia coli type III secretion system 2 gene cluster influence expression of genes within the locus for enterocyte effacement in enterohemorrhagic E. coli O157:H7.* Infect Immun, 2004. **72**(12): p. 7282-93.

77. Haack, K.R., et al., *Interaction of Ler at the LEE5 (tir) operon of enteropathogenic Escherichia coli.* Infect Immun, 2003. **71**(1): p. 384-92.

78. Sperandio, V., et al., *Quorum sensing controls expression of the type III secretion gene transcription and protein secretion in enterohemorrhagic and enteropathogenic Escherichia coli.* Proc Natl Acad Sci U S A, 1999. **96**(26): p. 15196-201.

79. Sharma, V.K. and R.L. Zuerner, *Role of hha and ler in transcriptional regulation of the esp operon of enterohemorrhagic Escherichia coli O157:H7.* J Bacteriol, 2004. **186**(21): p. 7290-301.

80. Deng, W., et al., *Dissecting virulence: systematic and functional analyses of a pathogenicity island.* Proc Natl Acad Sci U S A, 2004. **101**(10): p. 3597-602.

81. Russell, R., Sharp, FC, Sperandio, Vanessa, *GrlR/A transcriptional regulation of the locus of enterocyte effacement (LEE) genes in enterohemorrhatic E. coli (EHEC).* Infection and Immunity, 2005. **Submitted**.

82. Zhang, W., et al., *Identification, characterization, and distribution of a Shiga toxin 1 gene variant (stx(1c)) in Escherichia coli strains isolated from humans.* J Clin Microbiol, 2002. **40**(4): p. 1441-6.

83. Perera, L.P., L.R. Marques, and A.D. O'Brien, *Isolation and characterization of monoclonal antibodies to Shiga-like toxin II of enterohemorrhagic Escherichia coli and use of the monoclonal antibodies in a colony enzyme-linked immunosorbent assay.* J Clin Microbiol, 1988. **26**(10): p. 2127-31.

84. Schmitt, C.K., M.L. McKee, and A.D. O'Brien, *Two copies of Shiga-like toxin II-related genes common in enterohemorrhagic Escherichia coli strains are responsible for the antigenic heterogeneity of the O157:H- strain E32511.* Infect Immun, 1991. **59**(3): p. 1065-73.

85. Boerlin, P., et al., *Associations between virulence factors of Shiga toxin-producing Escherichia coli and disease in humans.* J Clin Microbiol, 1999. **37**(3): p. 497-503.

86. Friedrich, A.W., et al., *Escherichia coli harboring Shiga toxin 2 gene variants: frequency and association with clinical symptoms.* J Infect Dis, 2002. **185**(1): p. 74-84.

87. Neely, M.N. and D.I. Friedman, *Functional and genetic analysis of regulatory regions of coliphage H-19B: location of shiga-like toxin and lysis genes suggest a role for phage functions in toxin release.* Mol Microbiol, 1998. **28**(6): p. 1255-67.

88. Donohue-Rolfe, A., et al., *Pathogenesis of Shigella diarrhea. IX. Simplified high yield purification of Shigella toxin and characterization of subunit composition and function by the use of subunit-specific monoclonal and polyclonal antibodies.* J Exp Med, 1984. **160**(6): p. 1767-81.

89. Jacewicz, M., et al., *Pathogenesis of shigella diarrhea. XI. Isolation of a shigella toxin-binding glycolipid from rabbit jejunum and HeLa cells and its identification as globotriaosylceramide.* J Exp Med, 1986. **163**(6): p. 1391-404.

90. Lindberg, A.A., et al., *Identification of the carbohydrate receptor for Shiga toxin produced by Shigella dysenteriae type 1.* J Biol Chem, 1987. **262**(4): p. 1779-85.

91. Waddell, T., et al., *Globotriosyl ceramide is specifically recognized by the Escherichia coli verocytotoxin 2.* Biochem Biophys Res Commun, 1988. **152**(2): p. 674-9.

92. Endo, Y., et al., *Site of action of a Vero toxin (VT2) from Escherichia coli O157:H7 and of Shiga toxin on eukaryotic ribosomes. RNA N-glycosidase activity of the toxins.* Eur J Biochem, 1988. **171**(1-2): p. 45-50.

93. Toth, I., et al., *Influence of the 60-megadalton plasmid on adherence of Escherichia coli O157:H7 and genetic derivatives.* Infect Immun, 1990. **58**(5): p. 1223-31.

94. Bauer, M.E. and R.A. Welch, *Characterization of an RTX toxin from enterohemorrhagic Escherichia coli O157:H7.* Infect Immun, 1996. **64**(1): p. 167-75.

95. Fratamico, P.M., S. Bhaduri, and R.L. Buchanan, *Studies on Escherichia coli serotype O157:H7 strains containing a 60-MDa plasmid and on 60-MDa plasmid-cured derivatives.* J Med Microbiol, 1993. **39**(5): p. 371-81.

96. Schmidt, H., L. Beutin, and H. Karch, *Molecular analysis of the plasmid-encoded hemolysin of Escherichia coli O157:H7 strain EDL 933.* Infect Immun, 1995. **63**(3): p. 1055-61.

97. Schmidt, H., B. Henkel, and H. Karch, *A gene cluster closely related to type II secretion pathway operons of gram-negative bacteria is located on the large plasmid of enterohemorrhagic Escherichia coli O157 strains.* FEMS Microbiol Lett, 1997. **148**(2): p. 265-72.

98. Brunder, W., H. Schmidt, and H. Karch, *EspP, a novel extracellular serine protease of enterohaemorrhagic Escherichia coli O157:H7 cleaves human coagulation factor V.* Mol Microbiol, 1997. **24**(4): p. 767-78.

99. Nealson, K.H., T. Platt, and J.W. Hastings, *Cellular control of the synthesis and activity of the bacterial luminescent system.* J Bacteriol, 1970. **104**(1): p. 313-22.

100. Nealson, K.H. and J.W. Hastings, *Bacterial bioluminescence: its control and ecological significance.* Microbiol Rev, 1979. **43**(4): p. 496-518.

101. Engebrecht, J., K. Nealson, and M. Silverman, *Bacterial bioluminescence: isolation and genetic analysis of functions from Vibrio fischeri.* Cell, 1983. **32**(3): p. 773-81.

102. Engebrecht, J. and M. Silverman, *Identification of genes and gene products necessary for bacterial bioluminescence.* Proc Natl Acad Sci U S A, 1984. **81**(13): p. 4154-8.

103. Davies, D.G., et al., *The involvement of cell-to-cell signals in the development of a bacterial biofilm.* Science, 1998. **280**(5361): p. 295-8.

104. de Kievit, T.R. and B.H. Iglewski, *Bacterial quorum sensing in pathogenic relationships.* Infect Immun, 2000. **68**(9): p. 4839-49.

105. Parsek, M.R. and E.P. Greenberg, *Acyl-homoserine lactone quorum sensing in gram-negative bacteria: a signaling mechanism involved in associations with higher organisms.* Proc Natl Acad Sci U S A, 2000. **97**(16): p. 8789-93.

106. Wang, X.D., P.A. de Boer, and L.I. Rothfield, *A factor that positively regulates cell division by activating transcription of the major cluster of essential cell division genes of Escherichia coli.* Embo J, 1991. **10**(11): p. 3363-72.

107. Swift, S., et al., *Quorum sensing-dependent regulation and blockade of exoprotease production in Aeromonas hydrophila.* Infect Immun, 1999. **67**(10): p. 5192-9.

108. Michael, B., et al., *SdiA of Salmonella enterica is a LuxR homolog that detects mixed microbial communities.* J Bacteriol, 2001. **183**(19): p. 5733-42.

109. Surette, M.G. and B.L. Bassler, *Quorum sensing in Escherichia coli and Salmonella typhimurium.* Proc Natl Acad Sci U S A, 1998. **95**(12): p. 7046-50.

110. Surette, M.G., M.B. Miller, and B.L. Bassler, *Quorum sensing in Escherichia coli, Salmonella typhimurium, and Vibrio harveyi: a new family of genes responsible for autoinducer production.* Proc Natl Acad Sci U S A, 1999. **96**(4): p. 1639-44.

111. Bassler, B.L., et al., *Intercellular signalling in Vibrio harveyi: sequence and function of genes regulating expression of luminescence.* Mol Microbiol, 1993. **9**(4): p. 773-86.

112. Schauder, S., et al., *The LuxS family of bacterial autoinducers: biosynthesis of a novel quorum-sensing signal molecule.* Mol Microbiol, 2001. **41**(2): p. 463-76.

113. Miller, S.T., et al., *Salmonella typhimurium recognizes a chemically distinct form of the bacterial quorum-sensing signal AI-2.* Mol Cell, 2004. **15**(5): p. 677-87.

114. Chen, X., et al., *Structural identification of a bacterial quorum-sensing signal containing boron.* Nature, 2002. **415**(6871): p. 545-9.

115. Henke, J.M. and B.L. Bassler, *Quorum sensing regulates type III secretion in Vibrio harveyi and Vibrio parahaemolyticus.* J Bacteriol, 2004. **186**(12): p. 3794-805.

116. Day, W.A., Jr. and A.T. Maurelli, *Shigella flexneri LuxS quorum-sensing system modulates virB expression but is not essential for virulence.* Infect Immun, 2001. **69**(1): p. 15-23.

117. Sperandio, V., et al., *Quorum sensing is a global regulatory mechanism in enterohemorrhagic Escherichia coli O157:H7.* J Bacteriol, 2001. **183**(17): p. 5187-97.

118. Sperandio, V., C.C. Li, and J.B. Kaper, *Quorum-sensing Escherichia coli regulator A (QseA): a regulator of the LysR family involved in the regulation of the LEE pathogenicity island in enterrohemorrhagic Escherichia coli.* Infect. Immun., 2002. **70**(6): p. 3085-3093.

119. Sperandio, V., et al., *Bacteria-host communication: the language of hormones.* Proc. Natl. Acad. Sci. USA, 2003. **100**(15): p. 8951-8956.

120. Henderson, B., et al., *Prokaryotic and eukaryotic signaling mechanisms*, in *Cellular Microbiology: bacteria-host interactions in health and disease*, B. Henderson, et al., Editors. 2000, John Wiley and Sons, Ltd.: West Sussex, England. p. 89-162.

121. Schauder, S. and B.L. Bassler, *The languages of bacteria.* Genes Dev, 2001. **15**(12): p. 1468-80.

122. Lyte, M., C.D. Frank, and B.T. Green, *Production of an autoinducer of growth by norepinephrine cultured Escherichia coli O157:H7.* FEMS Microbiol Lett, 1996. **139**(2-3): p. 155-9.

123. Kinney, K.S., et al., *Norepinephrine as a growth stimulating factor in bacteria--mechanistic studies.* Life Sci, 2000. **67**(25): p. 3075-85.

124. Eisenhofer, G., et al., *Substantial production of dopamine in the human gastrointestinal tract.* J Clin Endocrinol Metab, 1997. **82**(11): p. 3864-71.

125. Horger, S., G. Schultheiss, and M. Diener, *Segment-specific effects of epinephrine on ion transport in the colon of the rat.* Am J Physiol, 1998. **275**(6 Pt 1): p. G1367-76.

126. Furness, J.B., *Types of neurons in the enteric nervous system.* J Auton Nerv Syst, 2000. **81**(1-3): p. 87-96.

127. Purves D, F.D., Williams SM, McNamara JO, Augustine GJ, Katz LC, and LaMantia A., *Neuroscience*. 2001: Sinauer Associates, Inc.

128. Freddolino, P.L., et al., *Predicted 3D structure for the human beta 2 adrenergic receptor and its binding site for agonists and antagonists.* Proc Natl Acad Sci U S A, 2004. **101**(9): p. 2736-41.

129. Kaper, J.B. and V. Sperandio, *Bacterial cell-to-cell signaling in the gastrointestinal tract.* Infect Immun, 2005. **73**(6): p. 3197-209.

130. Telford, G., et al., *The Pseudomonas aeruginosa quorum-sensing signal molecule N-(3-oxododecanoyl)-L-homoserine lactone has immunomodulatory activity.* Infect Immun, 1998. **66**(1): p. 36-42.

131. Smith, R.S., et al., *The Pseudomonas autoinducer N-(3-oxododecanoyl) homoserine lactone induces cyclooxygenase-2 and prostaglandin E2 production in human lung fibroblasts: implications for inflammation.* J Immunol, 2002. **169**(5): p. 2636-42.

132. Coburn, P.S., et al., *Enterococcus faecalis senses target cells and in response expresses cytolysin.* Science, 2004. **306**(5705): p. 2270-2.

133. Chun, C.K., et al., *Inactivation of a Pseudomonas aeruginosa quorum-sensing signal by human airway epithelia.* Proc Natl Acad Sci U S A, 2004. **101**(10): p. 3587-90.

134. Wosten, M.M., et al., *A signal transduction system that responds to extracellular iron.* Cell, 2000. **103**(1): p. 113-25.

135. Gunn, J.S. and S.I. Miller, *PhoP-PhoQ activates transcription of pmrAB, encoding a two-component regulatory system involved in Salmonella typhimurium antimicrobial peptide resistance.* J Bacteriol, 1996. **178**(23): p. 6857-64.

136. Soncini, F.C. and E.A. Groisman, *Two-component regulatory systems can interact to process multiple environmental signals.* J Bacteriol, 1996. **178**(23): p. 6796-801.

137. Merkel, T.J., C. Barros, and S. Stibitz, *Characterization of the bvgR locus of Bordetella pertussis.* J Bacteriol, 1998. **180**(7): p. 1682-90.

138. Tischler, A.D. and A. Camilli. *VieA Represses Vibrio cholerae Biofilm Formation by Controllin Cyclic Diguanylate Concentration*. in *ASM General Meeting*. 2004. New Orleans, LA.

139. McNab, R.M., *Flagella and Motility*, in *Escherichia coli and Salmonella*, F.C. Neidhardt, Editor. 1996, ASM Press: Washington, D.C. p. 123-145.

140. Kutsukake, K., Y. Ohya, and T. Iino, *Transcriptional analysis of the flagellar regulon of Salmonella typhimurium.* J Bacteriol, 1990. **172**(2): p. 741-7.

141. Yanagihara, S., et al., *Structure and transcriptional control of the flagellar master operon of Salmonella typhimurium.* Genes Genet Syst, 1999. **74**(3): p. 105-11.

142. Liu, X. and P. Matsumura, *The FlhD/FlhC complex, a transcriptional activator of the Escherichia coli flagellar class II operons.* J Bacteriol, 1994. **176**(23): p. 7345-51.

143. Claret, L. and C. Hughes, *Interaction of the atypical prokaryotic transcription activator FlhD2C2 with early promoters of the flagellar gene hierarchy.* J Mol Biol, 2002. **321**(2): p. 185-99.

144. Jones, C.J. and R.M. Macnab, *Flagellar assembly in Salmonella typhimurium: analysis with temperature-sensitive mutants.* J Bacteriol, 1990. **172**(3): p. 1327-39.

145. Ohnishi, K., et al., *Gene fliA encodes an alternative sigma factor specific for flagellar operons in Salmonella typhimurium.* Mol Gen Genet, 1990. **221**(2): p. 139-47.

146. Gillen, K.L. and K.T. Hughes, *Negative regulatory loci coupling flagellin synthesis to flagellar assembly in Salmonella typhimurium.* J Bacteriol, 1991. **173**(7): p. 2301-10.

147. Ohnishi, K., et al., *A novel transcriptional regulation mechanism in the flagellar regulon of Salmonella typhimurium: an antisigma factor inhibits the activity of the flagellum-specific sigma factor, sigma F.* Mol Microbiol, 1992. **6**(21): p. 3149-57.

148. Schaubach, O.L. and A.J. Dombroski, *Transcription initiation at the flagellin promoter by RNA polymerase carrying sigma28 from Salmonella typhimurium.* J Biol Chem, 1999. **274**(13): p. 8757-63.

149. Chilcott, G.S. and K.T. Hughes, *Coupling of flagellar gene expression to flagellar assembly in Salmonella enterica serovar typhimurium and Escherichia coli.* Microbiol Mol Biol Rev, 2000. **64**(4): p. 694-708.

150. Adler, J. and B. Templeton, *The effect of environmental conditions on the motility of Escherichia coli.* J Gen Microbiol, 1967. **46**(2): p. 175-84.

151. Shin, S. and C. Park, *Modulation of flagellar expression in Escherichia coli by acetyl phosphate and the osmoregulator OmpR.* J Bacteriol, 1995. **177**(16): p. 4696-702.

152. Silverman, M. and M. Simon, *Characterization of Escherichia coli flagellar mutants that are insensitive to catabolite repression.* J Bacteriol, 1974. **120**(3): p. 1196-203.

153. Yokota, T. and J.S. Gots, *Requirement of adenosine 3', 5'-cyclic phosphate for flagella formation in Escherichia coli and Salmonella typhimurium.* J Bacteriol, 1970. **103**(2): p. 513-6.

154. Nishimura, A. and Y. Hirota, *A cell division regulatory mechanism controls the flagellar regulon in Escherichia coli.* Mol Gen Genet, 1989. **216**(2-3): p. 340-6.

155. Francez-Charlot, A., et al., *RcsCDB His-Asp phosphorelay system negatively regulates the flhDC operon in Escherichia coli.* Mol Microbiol, 2003. **49**(3): p. 823-32.

156. Bertin, P., et al., *The H-NS protein is involved in the biogenesis of flagella in Escherichia coli.* J Bacteriol, 1994. **176**(17): p. 5537-40.

157. Yona-Nadler, C., et al., *Integration host factor (IHF) mediates repression of flagella in enteropathogenic and enterohaemorrhagic Escherichia coli.* Microbiology, 2003. **149**(Pt 4): p. 877-84.

158. Bijlsma, J.J. and E.A. Groisman, *Making informed decisions: regulatory interactions between two-component systems.* Trends Microbiol, 2003. **11**(8): p. 359-66.

159. Silhavy, T.J. and J.R. Beckwith, *Uses of lac fusions for the study of biological problems.* Microbiol Rev, 1985. **49**(4): p. 398-418.

160. Silhavy, T.J., Berman, M.L. and Enquist, L.W., *Experi-ments with Gene Fusions*. 1984, Cold Spring Haror, NY: Cold Spring Harbor Laboratory Pres.

161. Clarke, M.B. and V. Sperandio, *Transcriptional Regulation of flhDC by QseBC and sigma28 (FliA) in EHEC.* Molecular Microbiology, 2005. **In Press**.

162. Clarke, M.B. and V. Sperandio, *Transcriptional Autoregulation by Quorum Sensing E. coli Regulators B and C (QseBC) in Enterohemorrhagic E. coli (EHEC).* Molecular MIcrobiology, 2005. **In Press**.

163. Simons, R.W., F. Houman, and N. Kleckner, *Improved single and multicopy lac-based cloning vectors for protein and operon fusions.* Gene, 1987. **53**: p. 85-96.

164. Guzman, L.M., et al., *Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter.* J Bacteriol, 1995. **177**(14): p. 4121-30.

165. Sambrook, J., E.F. Fritsch, and T. Maniatis, *Molecular cloning: a laboratory manual*. second ed. 1989: Cold Spring Harbor Laboratory Press.

166. Datsenko, K.A. and B.L. Wanner, *One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products.* Proc Natl Acad Sci U S A, 2000. **97**(12): p. 6640-5.

167. Sperandio, V., A.G. Torres, and J.B. Kaper, *Quorum sensing Escherichia coli regulators B and C (QseBC): a novel two-component regulatory system involved in the regulation of flagella and motility by quorum sensing in E. coli.* Mol Microbiol, 2002. **43**(3): p. 809-21.

168. Miller, J.H., *Experiments in molecular genetics*. 1972, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

169. Janausch, I.G., et al., *Phosphorylation and DNA binding of the regulator DcuR of the fumarate-responsive two-component system DcuSR of Escherichia coli.* Microbiology, 2004. **150**(Pt 4): p. 877-83.

170. Berg, R.D., *The indigenous gastrointestinal microflora.* Trends Microbiol, 1996. **4**(11): p. 430-5.

171. Hooper, L.V. and J.I. Gordon, *Commensal host-bacterial relationships in the gut.* Science, 2001. **292**(5519): p. 1115-8.

172. Neish, A.S., et al., *Prokaryotic regulation of epithelial responses by inhibition of IkappaB-alpha ubiquitination.* Science, 2000. **289**(5484): p. 1560-3.

173. Smith, R.S., et al., *IL-8 production in human lung fibroblasts and epithelial cells activated by the Pseudomonas autoinducer N-3-oxododecanoyl homoserine lactone is transcriptionally regulated by NF-kappa B and activator protein-2.* J Immunol, 2001. **167**(1): p. 366-74.

174. Gilles-Gonzalez, M.A., G.S. Ditta, and D.R. Helinski, *A haemoprotein with kinase activity encoded by the oxygen sensor of Rhizobium meliloti.* Nature, 1991. **350**(6314): p. 170-2.

175. Lois, A.F., et al., *Autophosphorylation and phosphatase activities of the oxygen-sensing protein FixL of Rhizobium meliloti are coordinately regulated by oxygen.* J Biol Chem, 1993. **268**(6): p. 4370-5.

176. Schroder, I., et al., *Phosphorylation and dephosphorylation of the NarQ, NarX, and NarL proteins of the nitrate-dependent two-component regulatory system of Escherichia coli.* J Bacteriol, 1994. **176**(16): p. 4985-92.

177. Bird, T.H., S. Du, and C.E. Bauer, *Autophosphorylation, phosphotransfer, and DNA-binding properties of the RegB/RegA two-component regulatory system in Rhodobacter capsulatus.* J Biol Chem, 1999. **274**(23): p. 16343-8.

178. Emmerich, R., et al., *Phosphorylation, dephosphorylation and DNA-binding of the Bradyrhizobium japonicum RegSR two-component regulatory proteins.* Eur J Biochem, 1999. **263**(2): p. 455-63.

179. Georgellis, D., O. Kwon, and E.C. Lin, *Amplification of signaling activity of the arc two-component system of Escherichia coli by anaerobic metabolites. An in vitro study with different protein modules.* J Biol Chem, 1999. **274**(50): p. 35950-4.

180. Roberts, D.L., D.W. Bennett, and S.A. Forst, *Identification of the site of phosphorylation on the osmosensor, EnvZ, of Escherichia coli.* J Biol Chem, 1994. **269**(12): p. 8728-33.

181. Janausch, I.G., I. Garcia-Moreno, and G. Unden, *Function of DcuS from Escherichia coli as a fumarate-stimulated histidine protein kinase in vitro.* J Biol Chem, 2002. **277**(42): p. 39809-14.

182. Rigaud, J.L., B. Pitard, and D. Levy, *Reconstitution of membrane proteins into liposomes: application to energy-transducing membrane proteins.* Biochim Biophys Acta, 1995. **1231**(3): p. 223-46.

183. Mizuno, T., E.T. Wurtzel, and M. Inouye, *Osmoregulation of gene expression. II. DNA sequence of the envZ gene of the ompB operon of Escherichia coli and characterization of its gene product.* J Biol Chem, 1982. **257**(22): p. 13692-8.

184. Grebe, T.W. and J.B. Stock, *The histidine protein kinase superfamily.* Adv Microb Physiol, 1999. **41**: p. 139-227.

185. Koretke, K.K., et al., *Evolution of two-component signal transduction.* Mol Biol Evol, 2000. **17**(12): p. 1956-70.

186. Wolanin, P.M., P.A. Thomason, and J.B. Stock, *Histidine protein kinases: key signal transducers outside the animal kingdom.* Genome Biol, 2002. **3**(10): p. REVIEWS3013.

187. Roychoudhury, S., et al., *Inhibitors of two-component signal transduction systems: inhibition of alginate gene activation in Pseudomonas aeruginosa.* Proc Natl Acad Sci U S A, 1993. **90**(3): p. 965-9.

188. Kato, J. and A.M. Chakrabarty, *Purification of the regulatory protein AlgR1 and its binding in the far upstream region of the algD promoter in Pseudomonas aeruginosa.* Proc Natl Acad Sci U S A, 1991. **88**(5): p. 1760-4.

189. Barrett, J.F., et al., *Antibacterial agents that inhibit two-component signal transduction systems.* Proc Natl Acad Sci U S A, 1998. **95**(9): p. 5317-22.

190. Macielag, M.J., et al., *Substituted salicylanilides as inhibitors of two-component regulatory systems in bacteria.* J Med Chem, 1998. **41**(16): p. 2939-45.

191. Matsushita, M. and K.D. Janda, *Histidine kinases as targets for new antimicrobial agents.* Bioorg Med Chem, 2002. **10**(4): p. 855-67.

192. Zientz, E., J. Bongaerts, and G. Unden, *Fumarate regulation of gene expression in Escherichia coli by the DcuSR (dcuSR genes) two-component regulatory system.* J Bacteriol, 1998. **180**(20): p. 5421-5.

193. Park, K., et al., *Novel sigmaF-dependent genes of Escherichia coli found using a specified promoter consensus.* FEMS Microbiol Lett, 2001. **202**(2): p. 243-50.

194. Liu, X. and P. Matsumura, *An alternative sigma factor controls transcription of flagellar class-III operons in Escherichia coli: gene sequence, overproduction, purification and characterization.* Gene, 1995. **164**(1): p. 81-4.

195. Soutourina, O., et al., *Multiple control of flagellum biosynthesis in Escherichia coli: role of H-NS protein and the cyclic AMP-catabolite activator protein complex in transcription of the flhDC master operon.* J Bacteriol, 1999. **181**(24): p. 7500-8.

196. Kenney, L.J., M.D. Bauer, and T.J. Silhavy, *Phosphorylation-dependent conformational changes in OmpR, an osmoregulatory DNA-binding protein of Escherichia coli.* Proc Natl Acad Sci U S A, 1995. **92**(19): p. 8866-70.

197. Yamamoto, K., et al., *Functional characterization in vitro of all two-component signal transduction systems from Escherichia coli.* J Biol Chem, 2005. **280**(2): p. 1448-56.

198. Kutsukake, K., *Autogenous and global control of the flagellar master operon, flhD, in Salmonella typhimurium.* Mol Gen Genet, 1997. **254**(4): p. 440-8.

199. Barker, C.S., B.M. Pruss, and P. Matsumura, *Increased motility of Escherichia coli by insertion sequence element integration into the regulatory region of the flhD operon.* J Bacteriol, 2004. **186**(22): p. 7529-37.

200. Lehnen, D., et al., *LrhA as a new transcriptional key regulator of flagella, motility and chemotaxis genes in Escherichia coli.* Mol Microbiol, 2002. **45**(2): p. 521-32.

201. Bergstrom, L.C., et al., *Hierarchical and co-operative binding of OmpR to a fusion construct containing the ompC and ompF upstream regulatory sequences of Escherichia coli.* Genes Cells, 1998. **3**(12): p. 777-88.

202. Sperandio, V., et al., *Bacteria-host communication: the language of hormones.* Proc Natl Acad Sci U S A, 2003. **100**(15): p. 8951-6.

203. Yamamoto, K., et al., *Negative regulation of the bolA1p of Escherichia coli K-12 by the transcription factor OmpR for osmolarity response genes.* FEMS Microbiol Lett, 2000. **186**(2): p. 257-62.

204. Rampersaud, A., S.L. Harlocker, and M. Inouye, *The OmpR protein of Escherichia coli binds to sites in the ompF promoter region in a hierarchical manner determined by its degree of phosphorylation.* J Biol Chem, 1994. **269**(17): p. 12559-66.

205. Scarlato, V., et al., *Positive transcriptional feedback at the bvg locus controls expression of virulence factors in Bordetella pertussis.* Proc Natl Acad Sci U S A, 1990. **87**(24): p. 10067.

206. Soncini, F.C., E.G. Vescovi, and E.A. Groisman, *Transcriptional autoregulation of the Salmonella typhimurium phoPQ operon.* J Bacteriol, 1995. **177**(15): p. 4364-71.

207. Kato, A., H. Tanabe, and R. Utsumi, *Molecular characterization of the PhoP-PhoQ two-component system in Escherichia coli K-12: identification of extracellular Mg2+-responsive promoters.* J Bacteriol, 1999. **181**(17): p. 5516-20.

208. Roy, C.R., J.F. Miller, and S. Falkow, *Autogenous regulation of the Bordetella pertussis bvgABC operon.* Proc Natl Acad Sci U S A, 1990. **87**(10): p. 3763-7.

209. Raivio, T.L., D.L. Popkin, and T.J. Silhavy, *The Cpx envelope stress response is controlled by amplification and feedback inhibition.* J Bacteriol, 1999. **181**(17): p. 5263-72.

210. Hoffer, S.M., et al., *Autoamplification of a two-component regulatory system results in "learning" behavior.* J Bacteriol, 2001. **183**(16): p. 4914-7.

211. Pruss, B.M. and P. Matsumura, *Cell cycle regulation of flagellar genes.* J Bacteriol, 1997. **179**(17): p. 5602-4.

212. Kanamaru, K., et al., *SdiA, an Escherichia coli homologue of quorum-sensing regulators, controls the expression of virulence factors in enterohaemorrhagic Escherichia coli O157:H7.* Mol Microbiol, 2000. **38**(4): p. 805-16.

213. Sperandio, V., Zhu, C., Rios, H., Davis, K., Clarke, M.B., Boedeker, E.C., *Quorum sensing qseC and qseA rabbit enteropathogenic E. coli (REPEC)*

mutants have altered virulence in rabbits. Infection and Immunity, 2005. **Submitted**.

214. Gillen, K.L. and K.T. Hughes, *Transcription from two promoters and autoregulation contribute to the control of expression of the Salmonella typhimurium flagellar regulatory gene flgM.* J Bacteriol, 1993. **175**(21): p. 7006-15.

215. Dziva, F., et al., *Identification of Escherichia coli O157 : H7 genes influencing colonization of the bovine gastrointestinal tract using signature-tagged mutagenesis.* Microbiology, 2004. **150**(Pt 11): p. 3631-45.

VITAE

Marcie Beth Cunningham Clarke was born in Canton, Ohio, on January 16, 1981, the daughter of M. Beth Gelczer Cunningham and Frederick Franklin Cunningham, Jr. She has one brother, Brian D. Cunningham, who is four years younger. Marcie attended high school at The Texas Academy of Mathematics and Science in Denton, Texas. During the summer of 1998, she studied with Texas A&M University on the Texas Clipper II, a marine research vessel which traveled to South America. In 1999, she entered The University of Texas at Austin, Texas. In May 2001 she graduated, *Magna Cum Laude,* with honors, with a Bachelor of Science in Molecular Biology. In June of 2001, she entered the Graduate School of Biomedical Sciences at The University of Texas Southwestern Medical Center at Dallas where she joined the Molecular Microbiology program. During her time at UT Southwestern, she has been awarded the Ruth Kirschstein Pre-Doctoral Fellowship, from the National Institute of Health, the 2005 Richard and Mary Finkelstein Student Travel Grant from the American Society of Microbiology, a travel grant from the American Society of Microbiology for the 2005 Meeting on Beneficial Microbes, and a Microbiology journal-club award. Additionally, Marcie was an invited student speaker at the General Meeting for the American Society of Microbiology in 2005. In June of 2003, she married Daniel William Clarke.

Permanent Address: 4417 Misty Meadow Drive
Fort Worth, TX 76133