CHARACTERIZATION OF QSEA AND QSED
IN THE QUORUM SENSING CASCADE
OF ENTEROHEMORRHAGIC *ESCHERICHIA COLI*

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DEDICATION

I dedicate this thesis to
Mom, Dad, Julie, Matt, Maria,
Brianna, Kaitlyn, Madison, and Zachary
for their advice, support, and love.

CHARACTERIZATION OF QSEA AND QSED IN THE QUORUM SENSING CASCADE OF ENTEROHEMORRHAGIC *ESCHERICHIA COLI*

by

Faith Christine Sharp

THESIS

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Enterohemorrhagic *E. coli* O157:H7 (EHEC) is an enteric pathogen that has been implicated in many outbreaks of bloody diarrhea worldwide. EHEC senses its environment through quorum sensing, a mechanism by which bacteria use chemical signals, termed autoinducers, to regulate key genes. In the gastrointestinal tract, EHEC responds to AI-3 produced by the endogenous gastrointestinal microbial flora and epinephrine/norepinephrine produced by the host to regulate expression of virulence genes. In particular, EHEC utilizes quorum sensing to regulate virulence processes, including motility and chemotaxis and the production of attaching and effacing lesions. Motility and chemotaxis processes are controlled under the complex flagella regulon in EHEC. The expression of genes within the locus of enterocyte effacement (LEE) results in the production of the characteristic attaching and effacing lesions created as a result of production of a type III secretion apparatus. The *LEE1* operon encodes for a

transcriptional activator, Ler, which is responsible for the activation of other genes within the pathogenicity island. The virulence mechanisms that enable EHEC to circumvent the host defenses and compete for essential nutrients for survival are controlled by several transcriptional regulators, many of which are controlled in response to quorum sensing in EHEC.

Quorum sensing *E. coli* regulator A, QseA, recently was described as a transcription factor that is activated via quorum sensing in EHEC. QseA, which belongs to the family of LysR transcription factors, activates the transcription of *LEE1/ler* directly; therefore, QseA indirectly activates the expression of other genes within the LEE pathogenicity island. The work in the first specific aim of this thesis examines the specific regulation of the *LEE1/ler* promoter by QseA through the use of genetic and biochemical methods.

Quorum sensing *E. coli* regulator D, QseD, is a previously uncharacterized transcription factor that is repressed through quorum sensing in EHEC. QseD appears to play a significant role in the overall quorum sensing cascade, as it is involved in the modulation of both motility and type III secretion in EHEC. The second aim of this thesis is to study the role of QseD modulation in quorum sensing signaling in EHEC.

LIST OF PUBLICATIONS

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AE	attaching and effacing
AI	autoinducer
bp	base pair
DEPC	diethyl pyrocarbonate
DMEM	Dulbecos modified Eagle's medium
DNA	deoxyribonucleic acid
dNTP	dideoxy-nucleotide triphosphate
DPD	4,5-dihydrody-2,3-pentanedione
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylenediamine-tetraacetic acid
EHEC	enterohemorragic *E. coli*
EivF	Ecs3734 of EHEC, encoding a homologue of the Spi-1 regulator InvF
EMSA	electro-mobility shift assay
EPEC	enteropathogenic *E. coli*
Esps	*E. coli* secreted proteins
EtrA	ETT2 regulator A
GI	Gastrointestinal Tract
HTH	helix-turn-helix
H-NS	histone-like nucleodie-structuring protein
HUS	hemolytic uremic syndrome
IHF	integration host factor

IPTG	β-D-thiogalactopyranoside
kDa	kilodalton
LB	Luria-Bertani broth
LEE	Locus of Enterocyte Effacement
Ler	LEE-encoded regulator
OD	Optical density
PAGE	polyacrylamide gel electrophoresis
PAI	pathogenicity island
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
Pch	PerC homologs
PE	phentolamine
PNK	polynucleotide kinase
PO	propanolol
QS	quorum sensing
QseA	Quorum sensing *E. coli* regulator A
QseB	Quorum sensing *E. coli* regulator B
QseC	Quorum sensing *E. coli* regulator C
QseD	Quorum sensing *E. coli* regulator D
QseE	Quorum sensing *E. coli* regulator E
QseF	Quorum sensing *E. coli* regulator F
RNA	ribonucleic acid
RpoS	stationary-phase sigma factor
RT-PCR	reverse-transcriptase PCR

SAM	S-adenosyl-methionine
SDS	sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel
Stx	Shiga toxin
T3SS	type III secretion system
Tir	translocated intimin receptor
X-gal	5-bromo-4-chloro-3-indolyl β-galactopyranoside

CHAPTER I. LITERATURE REVIEW

A. Enteric Bacteria and Pathogens

Escherichia coli is the predominant facultative anaerobe of the human gastrointestinal (GI) flora. Although *E. coli* is usually regarded as a member of the normal flora, many pathogenic strains have emerged due to extensive horizontal DNA transfer. These important human pathogens include, but are not limited to, enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC) enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), and diffusely adherent *E. coli* (DEAC). Considering constant challenges by the natural host defenses and severe competition from the normal flora, these pathogens must be able to colonize the mucosa successfully. ETEC is responsible for traveler's diarrhea in adults. EPEC is the leading causative agent of protracted and chronic diarrhea in infants in developing countries, where infections can last for months at a time. EAEC is associated with outbreaks of persistent diarrhea and vomiting, typically in developing countries. Outbreaks of watery diarrhea closely resembling infections of *Shigella*, are associated with infections by EIEC, typically a food or waterborne infectious agent. DAEC has been associated with cases of diarrhea in children. EHEC causes severe bloody diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome [1-4]**.** The genome of two O157:H7 EHEC strains were sequenced in 2001 [5-7]. Among all the serotypes of EHEC, serotype O157:H7 is associated with most outbreaks of severe disease. Comparing the genome of *E. coli* K-12

to those of enterohemorrhagic *E. coli*, EHEC has 1.34 Mb of DNA that is not present in *E. coli* K-12, and is missing 0.53 Mb contained in the genome of *E. coli* K-12 [5]. Much of this extra DNA encoded within the EHEC genome is believed to be important for virulence in EHEC [5].

B. Epidemiology and Clinical Features of EHEC O157:H7

Enterohemorrhagic *E. coli* O157:H7 was first identified as the causative agent of an outbreak of bloody diarrhea due to contaminated food from a fast food restaurant in 1983, and since then has been implicated in numerous outbreaks of hemorrhagic colitis in the United States and elsewhere in the world, which clinically is diagnosed by severe abdominal cramping and bloody diarrhea [1-4]. The low infectious dose, which is as few as 100 bacteria, allows EHEC to be easily transmitted through contaminated water and food [1, 2, 4]. Additionally, a recent outbreak at a petting zoo at the North Carolina State Fair contributed to severe disease in multiple children [8]. EHEC was first identified as a causative agent of bloody diarrhea and hemorrhagic colitis when an association was established between cases of hemolytic uremic syndrome, or HUS, and a fecal cytotoxin producing *E. coli* [9, 10]. *E. coli* O157:H7, the cytotoxin producing bacteria, is particularly dangerous to children and the elderly as they are more susceptible to the adverse effects of the disease and more prone to develop HUS which results in dramatic organ failure.

C. Virulence Factors of EHEC

EHEC colonizes the large intestine where it forms attaching and effacing lesions, or AE lesions. These lesions are caused by the attachment of the bacteria to the epithelial

cells and the rearrangement of the cytoskeleton, resulting in the characteristic pedestal formation [1-4]. The AE lesions are characteristic of EHEC, EPEC, and *Citrobacter rodentium* infections. EHEC and EPEC, described above, are responsible for outbreaks of diarrhea worldwide in humans. *Citrobacter rodentium* (CR) is a natural mouse pathogen that constitutes a mouse model for AE pathogens [11]. Rabbit enteropathogenic *E. coli* is another commonly used animal pathogen that forms similar AE lesions [12]. The genes which are involved in pedestal formation are encoded within a pathogenicity island, named the locus of enterocyte effacement (LEE) [13, 14]. There are distinct differences between the formation of attaching and effacing lesions between EHEC and EPEC. Briefly, there are specific molecular mechanisms, discussed in more detail below, that are different between the formation of the AE lesions in EHEC and EPEC [1-4]. In addition to the formation of AE lesions in the gastrointestinal tract, EHEC uses flagella to swim to the site of infection. The expression of over 50 genes is responsible for the formation of these molecular motors [15, 16].

Finally, enterohemorrhagic *E. coli* encodes a potent toxin, Shiga toxin, responsible for the cytotoxicity of EHEC on Vero cells [10]. The production of Shiga toxin was linked to severe disease and the establishment of bloody diarrhea and HUS by Karmali et al [9]. The family of Shiga toxins contains two groups, Stx1 and Stx2, which are immunologically non-cross-reactive. EHEC strains can produce Stx1 or Stx2 alone, or a combination of the two toxins. The genes encoding Stx1 are within a lysogenic bacteriphage, while Stx2 can be found in lysogenic bacteriophage or within the chromosome [4, 17]. The SOS response, caused by disturbances in bacterial cell membrane, protein synthesis, or DNA replication, signals the bacteriophage to enter into

the lytic cycle, resulting in the replication of the phage. Replication of the bacteriophage causes the production of Shiga toxin and the eventual release into the host [18, 19].

Shiga toxin has an A-B toxin structure, with one 32-kDa A subunit and five identical 7.7-kDa B subunits, and acts as a *N*-glycosidase. The B subunits bind to specific Gb_3 and Gb_4 glycolipid receptors on the host cells, transporting the A subunit to the cytoplasm of the host epithelial cell, where the A subunit acts on 60S ribosomal subunit. The A subunit is proteolytically cleaved, resulting in a 28-kDa A_1 peptide and a 4-kDa A_2 peptide. The A_1 peptide is responsible for the enzymatic activity of the toxin by removing a single adenine residue from the 28S rRNA of ribosomes, resulting in inhibition of protein synthesis [4].

D. Locus of Enterocyte Effacement

The Locus of Enterocyte Effacement (LEE), which was first described in 1995, is present in enterohemorrhagic and enteropathogenic *E. coli* (Figure 1) [13]. This locus is absent in normal flora *E. coli*, and *E. coli* K-12 strains. The genes within the LEE are responsible for the distinctive attaching and effacing lesion in EHEC. In EPEC, the LEE is necessary and sufficient to produce AE lesions, while proteins encoded outside the LEE are required to produce complete AE lesions in EHEC [13, 20].

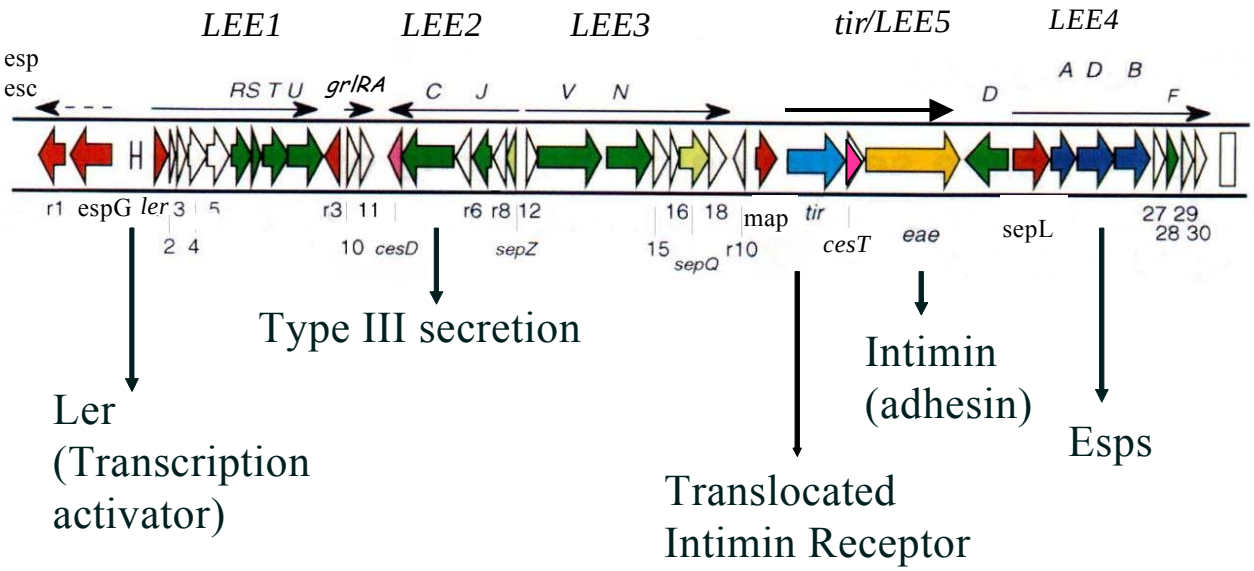


Figure 1. *Locus of Enterocyte Effacement (LEE). The LEE is a pathogenicity island found in EHEC and EPEC that encodes for factors responsible for type III secretion and pedestal formation. LEE1 encodes for Ler, the LEE-encoded regulator. LEE1, 2, and 3 encoded for factors involved in type III secretion. LEE4 encodes for EspA, EspB and EspD. The LEE5/tir operon encodes for Intimin and Tir.*

The LEE encodes 41 genes, the majority of which are organized into five operons (Figure 1). *LEE1* encodes for the LEE-encoded regulator, Ler, which has been shown in EHEC and EPEC to directly activate the expression of other LEE-encoded genes, as well as several non-LEE encoded virulence factors [14, 21]. *LEE1*, *2*, and *3* encode several proteins comprising the type III secretion apparatus (T3SS) [21]. The *LEE5/tir* operon encodes for Intimin and the translocated intimin receptor, Tir [21, 22]. Intimin is an intestinal adherence factor that is inserted into the outer membrane of the bacterial cell [23]. Tir, a novel factor that is translocated into the host cell, serves as a receptor for the adhesin, Intimin [22]. Upon formation of the modified T3SS, the Tir protein is translocated through the T3SS, into the host cell, where it then inserts into the membrane of the host cell. This allows Intimin to attach to Tir, resulting in close attachment of the bacteria to the host cell. *LEE5/tir* encodes for the chaperone, CesT, which is responsible for the translocation of Tir [22, 24]. The *E. coli* secreted proteins, EspA, EspB, and EspD are encoded within the *LEE4* operon. Also encoded in *LEE4* is EscF, which makes up the needle complex of the type III secretion system [25]. The Esp proteins comprise the translocon of the type III secretion apparatus. In particular, EspA makes up the filament of the type III secretion apparatus which forms a shealth around EscF, and EspB and EspD make the pore that inserts into the host epithelial cell (Figure 2) [21, 25].

The LEE also encodes effectors that are translocated into the host cell. The mitochondrial associated protein, Map, which is encoded in the LEE pathogenicity island immediately upstream of *tir*, is also chaperoned by CesT [26]. Map affects the integrity of the host mitochondrial membrane [27]. EspF, responsible for disruption of intestinal barrier function and induction of host cell death by methods currently unknown, is

encoded downstream of *espD* in *LEE4* [28, 29]. EspG, another secreted factor, disrupts microtubule formation and plays a role in virulence in the REPEC mouse model [30, 31]. EspH, encoded in *LEE3*, modulates host cell cytoskeleton through inhibition of cell cycle signals [32]. Cif is encoded within a lambdoid prophage in many EHEC and EPEC strains. Although Cif is encoded outside the LEE pathogenicity island, it is an effector protein secreted by the LEE type III secretion apparatus that induces host cell cycle arrest and significant reorganization of host actin cytoskeleton [33].

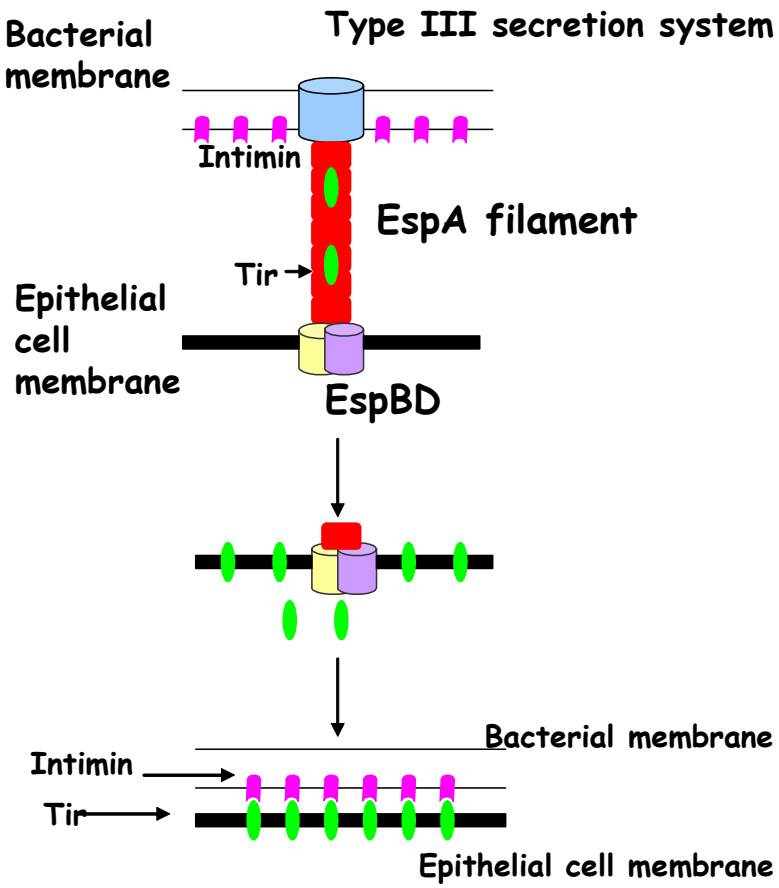


Figure 2. *Type III secretion system. EspA makes up the filament and EspB and EspD make up the pore of the type III secretion system. Tir, the translocated intimin receptor, is transferred from EHEC into the epithelial cell through the T3SS. Once inside the host cell, Tir inserts into the cell membrane and serves as the receptor for Intimin, which is on EHEC. This allows the close, intimate contact between the EHEC and host epithelial cells. Down-stream events lead to the characteristic pedestal formation.*

As mentioned previously, EHEC and EPEC utilize different mechanisms in the formation of attaching and effacing lesions. In EPEC, Tir is phosphorylated on the tyrosine 474 residue by the Src-family kinase c-Fyn [34]. Nck, a host adaptor protein, is recruited by Tir, which then activates an important regulator of actin polymerization, N-WASP, enabling recruitment of the Arp2/3 complex. The recruitment of Nck and N-WASP results in actin polymerization and the formation of the distinct pedestal [35]. EHEC utilizes another mechanism for actin polymerization. The EHEC Tir is unable to undergo phosphorylation, as it contains a cysteine residue instead of tyrosine at position 474; therefore, Tir is unable to recruit Nck in EHEC. In fact, in Nck-deficient cell lines, EHEC is still able to form attaching and effacing lesions [35]. Recently, Campellone et al, reported that an ORF encoded within prophage-U, renamed *espF*$_u$, encodes for a homolog of the LEE-encoded EspF [20]. EspF$_u$ is the unique factor in EHEC that is involved in stimulating actin pedestal formation [20]. EspF$_u$ directly interacts with Tir and directly binds to N-WASP. By binding N-WASP directly, EspF$_u$ activates it; therefore, there is no need for Nck [20].

E. Genetic Regulation of the Locus of Enterocyte Effacement

The LEE pathogenicity island undergoes complex regulation in EHEC and EPEC (Figure 3). Although the regulation of the LEE between EHEC and EPEC carry many parallels, there are some distinct differences in their regulation of this pathogenicity island. EPEC contains three genes encoded on a 70-kb virulence plasmid, *perA, perB,* and *perC* (plasmid-encoded regulator), which encode a regulatory complex that activates genes within EPEC [14, 36]. These genes are involved in full activation of LEE genes. In particular, Per activates the expression of *ler*; therefore, indirectly activating the

expression of *LEE2, LEE3, tir/LEE5* and *LEE4* [14, 36-38]. GadX, a positive regulator of the glutamate decarboxylase genes in EPEC, plays a repressive role in the regulation of the transcription of *per* [39]. In contrast, Per is not present in EHEC; therefore, Per does not regulate the expression of the LEE in this system. Recently, Iyoba et al observed that EHEC encodes for five *perC* homologs, three of which, renamed PchA, B, and C (PerC homologs), positively activate the expression of LEE genes [40].

LEE1 encodes Orf1, renamed Ler, for LEE-encoded regulator. *LEE1/ler* was shown to be required for the expression of other operons within the LEE. Ler shares 24% identity and 44% similarity to H-NS [14]. Using *lacZ* operon fusions, Ler was shown to activate the transcription of *LEE2, LEE3, LEE5,* and *LEE4* in EPEC and *LEE2, LEE3,* and *LEE5* in EHEC [14]. Although there is one report of conflicting evidence suggesting that Ler is involved in the autoregulation of its promoter, there is substantially more evidence showing that Ler is not involved in autoregulation of its own promoter [14, 41]. These studies show that Ler is essential in the regulation of the entire pathogenicity island by directly binding to *LEE2*, *LEE3*, and *LEE5* in EHEC. Sperandio et al observed that *LEE1/ler* contains two transcriptional start sites. The P1 (distal) transcriptional start site, 163 base pairs upstream of the translational start site, is common between EHEC and EPEC, while the P2 (proximal) transcriptional start site, 32 base pairs upstream of the translational start site, is only present in EHEC [38, 42]. IHF, integration-host factor, is another factor that is important in the regulation of the LEE pathogenicity island. IHF is required for expression of the entire LEE, but through the direct binding and activation of the transcription of *ler* [37]. EtrA and EivF, two regulators encoded within the

Escherichia coli type III secretion system 2 (ETT2) gene cluster in EHEC, negatively regulate the expression of LEE genes, possibly through *ler* [43].

LEE2, which encodes for factors involved in type III secretion, contains one promoter common between EHEC and EPEC [38]. H-NS, histone-like nucleoid-structuring protein, represses the transcription of *LEE2* in the absence of Ler [44]. According to these studies, Ler, encoded within *LEE1* of the LEE pathogenicity island acts by antagonizing H-NS, and hence activating transcription of this operon [44]. The *LEE3* operon has one transcriptional start site that is common between EHEC and EPEC [38]. This operon, like *LEE2*, is repressed transcriptionally by H-NS in the absence of Ler [44]. Ler antagonizes H-NS, resulting in the activation of the transcription *LEE3* operon [44]. RpoS, a stationary-phase sigma factor, also activates the transcription of the *LEE3* operon in EHEC [38]. *LEE5*, which encodes for CesT, Tir, and Eae, has one reported promoter [38]. Evidence exists suggesting that RpoS is also involved in the activation of the transcription of *LEE5* [38]. Ler activates the transcription of *LEE5* in both EHEC and EPEC [45]. Hha has been reported in the transcriptional repression of the *LEE4* operon in EHEC [46]. In this study, HHA was shown to repress *esp* genes indirectly, via repression of the transcription of *ler* [46].

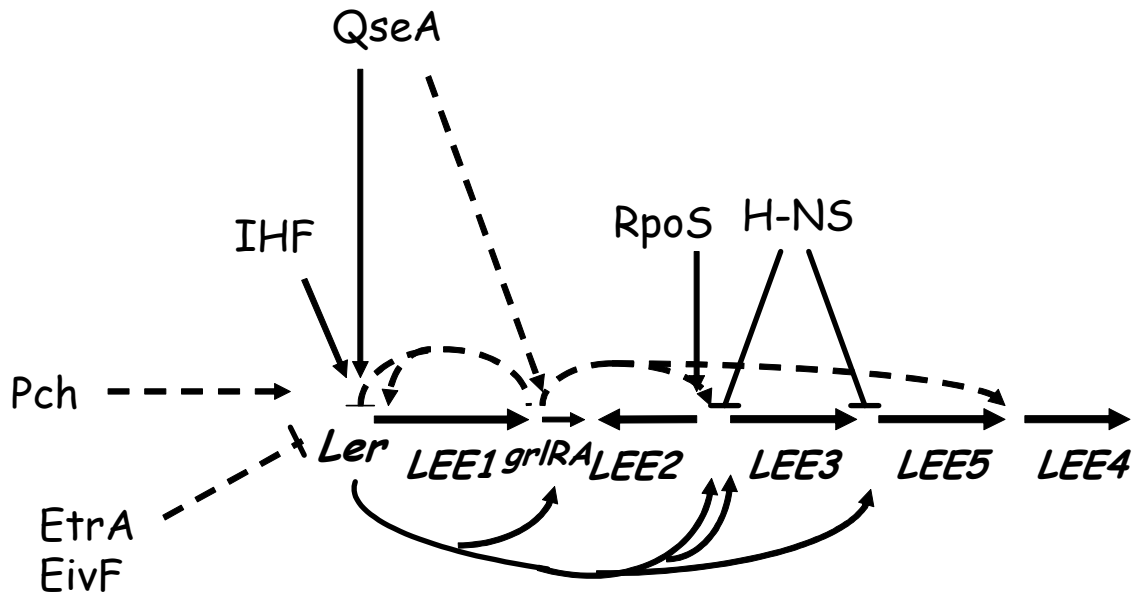


Figure 3. *Regulation of the Locus of Enterocyte Effacement.*

In *Citrobacter rodentium*, a systematic mutagenesis approach was utilized to further understand the complexity of the LEE genes in this system [47]. Deletion mutants in the LEE genes were analyzed for type III secretion, LEE gene expression, changes in actin polymerization, and in virulence in the mouse model. In this thorough analysis, two important regulators were found, *orf10* and *orf11*. Orf10 was renamed GrlR, for global regulator of LEE repressor, while Orf11 was renamed GrlA, for global regulator of LEE activator. This study suggests that GrlA is involved in the activation of the transcription of *ler*, while GrlR represses the transcription of *ler* [47]. Additionally, Ler activates the transcription of *grlRA* [21]. GrlRA form hetero- and homo-dimers *in vitro* [47]. Recently, Iyoda et al observed that the ClpXP protease is involved in the positive regulation of the LEE and may be involved in the stability of GrlR [48]. In EHEC, GrlRA positively activates *LEE2* and *LEE4* transcription, independent of Ler [49].

F. Flagella and Motility

The coordination of the flagellar hiearchy in *E. coli* requires the expression of over 50 genes divided into three classes. At least 17 operons constitute the overall flagella regulon in *E. coli* [15, 16]. Flagella, motility, and chemotaxis genes are controlled by *flhDC*, the master regulator of the flagella regulon. A number of global regulatory signals, including temperature and growth phase, affect the expression of the Class 1 genes (*flhDC*). The expression of *flhDC* is a critical step in the decision to form flagella, as it controls the downstream genes in the flagella regulon [15, 16]. FlhDC activate the transcription of Class 2 genes, including *fliA* encoding for the σ^{28} sigma factor, and genes important in the formation of the basal body and hook of the flagella. The alternative sigma factor, σ^{28}, is required for RNAP recognition of Class 3 promoters. FlgM, the anti-

sigma factor, associates with σ^{28} while the basal body and hook of the flagella are forming. The FlgM is secreted out the cell in response to the formation of the basal body and hook, releasing σ^{28} to activate the expression of Class 3 genes. Class 3 genes encode for flagellin, FliC, and motility and chemotaxis proteins (Figure 4) [15, 16].

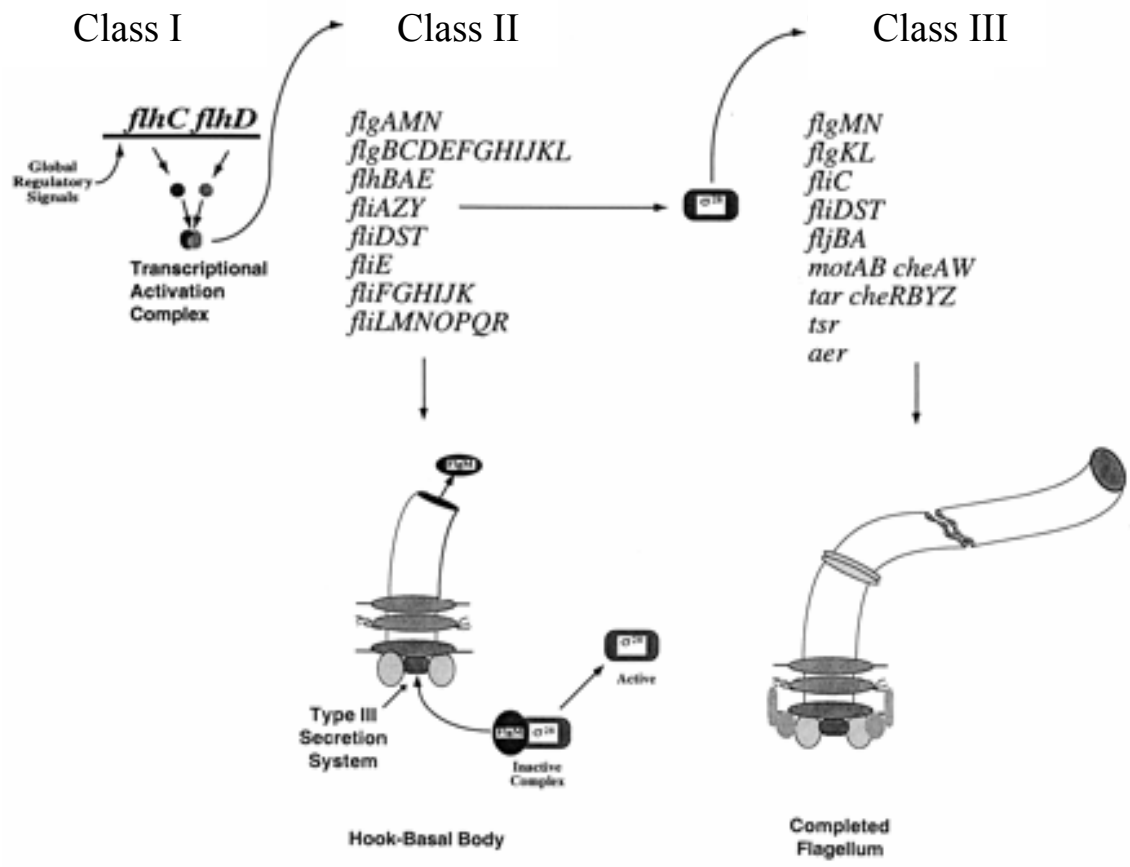


Figure 4. *Flagella and Motility Hierarchy in E. coli [16].*

G. LysR Family of Regulators

Regulation of the LEE and flagella genes also requires transcription factors of the LysR family of regulators. LrhA, a recently described member of the LysR family of proteins, has been shown to repress flagella expression in *E. coli* [50]. Quorum sensing *E. coli* regulator A (QseA), a member of the LysR family of regulators, has been shown to activate transcription of *LEE1/ler*, and consequently the other LEE operons [42]. Members of the LysR family of regulators contain a characteristic helix-turn-helix (HTH) DNA-binding domain at the amino-terminus, typically residues 1-65. They have been shown to repress their own transcription 3- to 10-fold. These proteins also regulate the expression of linked genes from divergent promoters, but this is not always the case as many activate the expression of unlinked virulence genes [51]. LysR proteins have been shown to bind the promoter in proximity to the bacterial RNAP [51]. Many members of the LysR family of regulators have also been identified as regulators of virulence factors in pathogenic bacteria. For example, PtxR positively regulates the production of exotoxin A in *Pseudomonas aeruginosa* [52]. AphA and AphB, of *Vibrio cholerae*, are involved in the quorum sensing cascade and regulation of the ToxR regulon [53-55].

H. Cell-cell Signaling in Bacteria and the Host

The mechanism by which bacterial species respond to chemical molecules (autoinducers, AI) produced by other bacterial cells in a given environment, has been named quorum sensing. Autoinducers (AI) are hormone-like molecules produced by bacteria of the same or different species. When a threshold concentration of autoinducer is reached in the environment, the bacteria detect the signal and respond by altering gene expression, resulting in a collective population response [56-61].

Quorum sensing in bacteria, or cell-to-cell signaling, was first associated with the regulation of bioluminescence in the marine bacterium, *Vibrio fischeri* [62]. The luciferase operon, which controls bioluminescence, is regulated by LuxI and LuxR. LuxI is responsible for the synthesis of the acyl-homoserine lactone (AHL) autoinducer and LuxR binds to the AHL to then activate transcription of the luciferase operon [63, 64]. Homologs to the LuxI-LuxR system have been identified in many bacteria. These AHL LuxI-LuxR systems are typically used for intra-species communication in Gram-negative bacteria. In *E. coli* and *Salmonella* species, the LuxR homolog is SdiA, but no LuxI homolog exists and no AHL is produced [65, 66]. SdiA in *Salmonella* senses the AHLs produced by other bacteria in the environment [65].

Bacteria also use an inter-species cell-to-cell signaling mechanism, present in both Gram-positive and Gram-negative bacteria, called the LuxS system, that has been identified in over 55 bacterial species [67]. The LuxS enzyme metabolizes S-adenosyl-methionine (SAM) by converting ribose-homocysteine into homocysteine and 4,5-dihydrody-2,3-pentanedione (DPD). DPD reacts with water and cyclizes forming several furanones [68]. The structure of AI-2 was identified in crystallization studies with its receptor LuxP in *V. harveyi*. In this study, AI-2 was described as a furanosyl-borate-diester [69]. In *Salmonella* and *E. coli* there is no LuxP homolog, but the AI-2 receptor has been identified as LsrB [70, 71]. The crystallization of LsrB with ligand revealed that the AI-2 had a 2R, 4S-2-methyl-2,3,3,4-tetrahydrofuran (R-THMF) structure, not the previously described furanosyl-borate-diester structure [72]. LsrB binds AI-2, which is transported into the cell where is believed to interact with other factors in repressing the

expression of the *lsr* operon [70, 71]. These studies reveal that there is a fundamental difference in the AI-2 detection in *E. coli* and *Salmonella* compared to the detection by *V. harveyi*, suggesting that AI-2 may be used as a metabolite by some bacteria rather than a signaling compound [73, 74].

The synthesis of another key autoinducer, AI-3, is dependent on the presence of the LuxS enzyme. Purified and *in vitro* synthesized AI-2 are unable to activate type III secretion and the flagella regulon in EHEC. AI-2 is a polar furanone compound which is unable to bind to C-18 columns. On the other hand, AI-3 binds to C-18 columns and elutes with methanol [75]. At present, the chemical structure of AI-3 is unknown, although evidence shows that it may be an aromatic compound without a sugar skeleton (V. Sperandio, unpublished data). The activities of both these signals can be determined through biological tests specific to AI-2 and AI-3. Phenotypes associated with AI-2 signaling dependence include using purified or *in vitro* synthesized AI-2 to observe bioluminescence in *V. harveyi* and the expression of the *lsr* operon in *Salmonella* [68, 71]. Sperandio et al observed no apparent AI-2 bioassay activity with AI-3 [75]. Separately, AI-3 activity can be assessed via the transcription of the LEE genes in EHEC. AI-2 has no effect on the activity of this assay [75]. The activities of both these autoinducers were identified in cultured stools from healthy human volunteers [75]. AI-2 and AI-3 activity was also observed in spent supernatants of other pathogenic and commensal bacteria, suggesting that AI-3 may be involved in inter-species communication like AI-2 (M. Sircili and V. Sperandio, unpublished data).

I. Genetic Regulation of Quorum Sensing in EHEC O157:H7

Quorum sensing has become an important feature of pathogenic bacteria, including EHEC. Quorum sensing was determined to be important in the regulation of essential virulence and basic physiological mechanisms in EHEC [76]. In EHEC, autoinducer-3 (AI-3) is the signal responsible for regulating the transcription of key virulence genes, including flagella and LEE genes [38, 76]. In these studies, it was also determined that the transcription of *LEE1* was activated by normal flora supernatants [75]. Additionally, Sperandio et al showed that the purified human hormones epinephrine and norepinephrine are able to activate the transcription of *LEE1* and type III secretion in the *luxS* mutant in EHEC [75]. These host hormones cross-signal with AI-3, suggesting that there is a level of communication between the bacteria and the host [75]. These studies also determined that α– or β-adrenergic receptor antagonists, phentolamine (PE) and propanolol (PO), respectively, antagonize recognition of AI-3 and epinephrine, preventing activation of *LEE1*, T3SS, and AE lesion formation [75].

Using an *E. coli* gene array comparing wild-type 86-24 to the *luxS* mutant, over 400 genes were found to be differentially regulated between wild-type and the *luxS* mutant [77]. Several up-regulated genes were involved in flagella, motility and chemotaxis. In particular, QseBC, a two-component system was identified in this array study and shown to activate the transcription of *flhDC*, the master regulator of flagella and motility genes in EHEC [77]. The transcription of *fliC, motA,* and *fliA*, three genes within the flagellar hierarchy, were decreased in the *qseC* mutant, as seen in the transcriptional fusion β-galactosidase activity assays [77]. Additionally, QseB, the response regulator, autoactivates its own transcription [78]. Using genetic and biochemical methods, QseB

was shown to directly bind to two binding sites in the promoter of *flhDC* [78]. There is evidence that QseC, the sensor kinase of the two component system, responds to epinephrine and purified AI-3 (M. C. Clarke and V. Sperandio, unpublished data).

As well as regulating the complex flagellar hierarchy in EHEC, quorum sensing was shown to regulate the expression of the LEE genes, and consequently, type III secretion system involved in the formation of AE lesions [38]. Sperandio et al observed that the transcription of *LEE1* and *LEE2* operons were directly activated through quorum sensing [38]. Quorum sensing indirectly activates transcription of *LEE2, 3,* and *5* by influencing the transcription of *LEE1* and production of Ler [38].

From the array studies described above, 19 putative transcription regulators were identified. The activation of transcription of the locus of enterocyte effacement, or LEE, through quorum sensing in EHEC occurs through the transcriptional regulator, quorum sensing *E. coli* regulator A (QseA) [42]. The transcription of *qseA* was up-regulated 23-fold in wild-type EHEC as compared to the *luxS* mutant in array studies [42]. QseA is homologous to members of the LysR-family of regulators, including AphB and PtxR regulators of *V. cholerae* and *P. aeruginosa*, respectively [42]. QseA is present in EHEC, EPEC, UPEC, K-12, *Shigella felxneri, Salmonella enterica Typhi, Salmonella enterica Typhimurium, Yersinia pestis, Yersinia enterocolitica, Erwinia carotovora,* and *Serratia marcescens.* Specifically, QseA was reported to activate transcription of *LEE1*, which encodes for the *LEE*-encoded regulator, Ler. As previously mentioned, *LEE1* in EHEC, has two promoters, a distal (P1) promoter common between EPEC and EHEC, and a proximal promoter (P2) present only in EHEC [42]. Using primer extension analysis,

QseA was shown to act on the distal (P1) promoter of *ler* (Figure 5) [42]. This promoter is located 163 base pairs upstream from the *ler* translational start site [42]. QseA also represses its own transcription [79]. Recent data suggests that QseA may be involved in the control of the virulence of REPEC, rabbit enteropathogenic *E. coli*. The *qseA* mutant of REPEC causes more severe disease in rabbits than the wild-type REPEC (V. Sperandio and E. Boedecker, unpublished data). Important to virulence in humans, John et al found that *qseA* is highly expressed during infection [80]. The regulation by QseA in the quorum sensing cascade has recently become more complex in that QseA may be involved in the regulation of *grlRA* in EHEC [49]. Russell et al recently observed that the transcription of *grlRA* was activated indirectly by QseA [49].

Other factors have been identified that are involved in this quorum sensing cascade. Genes encoding a putative two-component system, *qseE* and *qseF*, are down-regulated by quorum sensing. Evidence shows that QseEF may be involved in the intricate regulation of the type III secretion system, as the *qseF* mutant has a significant loss in activation of the transcription of $espF_u$ promoter (N. C. Reading and V. Sperandio, unpublished data). Additionally, with data presented in this thesis, another putative regulator, *qseD*, may be involved in the regulation of both the LEE genes and flagella and motility in enterohemorrhagic *E. coli*.

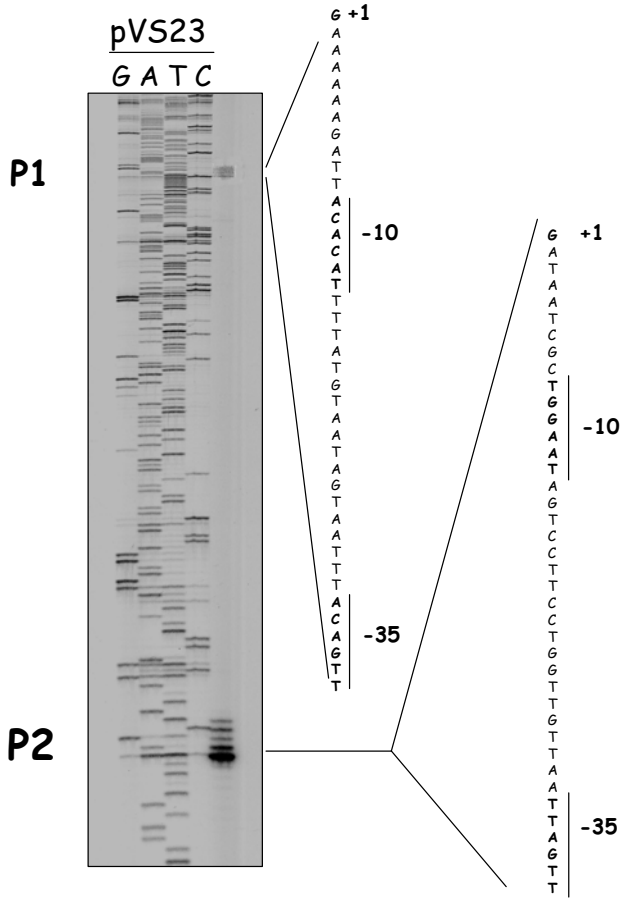


Figure 5. *Primer extension analysis of the LEE1/ler promoter in EHEC [42].*

J. Pathogenesis of EHEC O157:H7

The pathogenesis of EHEC is essential for successful establishment of disease within the host. The bacteria are able to sense the epinephrine and norepinephrine produced by the host, as well as the Autoinducer-3 (AI-3)produced by the normal flora, to activate key virulence genes, including flagella and motility, and the LEE genes. Through quorum sensing signals, genes within the flagella hierarchy are activated, allowing EHEC to swim to the epithelial cell layer. When in close proximity with the host epithelial cells, genes within the locus of enterocyte effacement (LEE) are activated resulting in the intimate attachment of EHEC to the host epithelial cells. This intimate attachment leads to a distinct feature of disease, the attaching and effacing (AE) lesion. Progression of the disease leads to significant host cell damage. Additionally, upon cell lysis, Shiga toxin is released and travels through the blood stream where it can then proceed to essential organs, including the kidneys, resulting in hemolytic uremic syndrome, which can progress to organ failure in many young patients (Figure 6) [1-4].

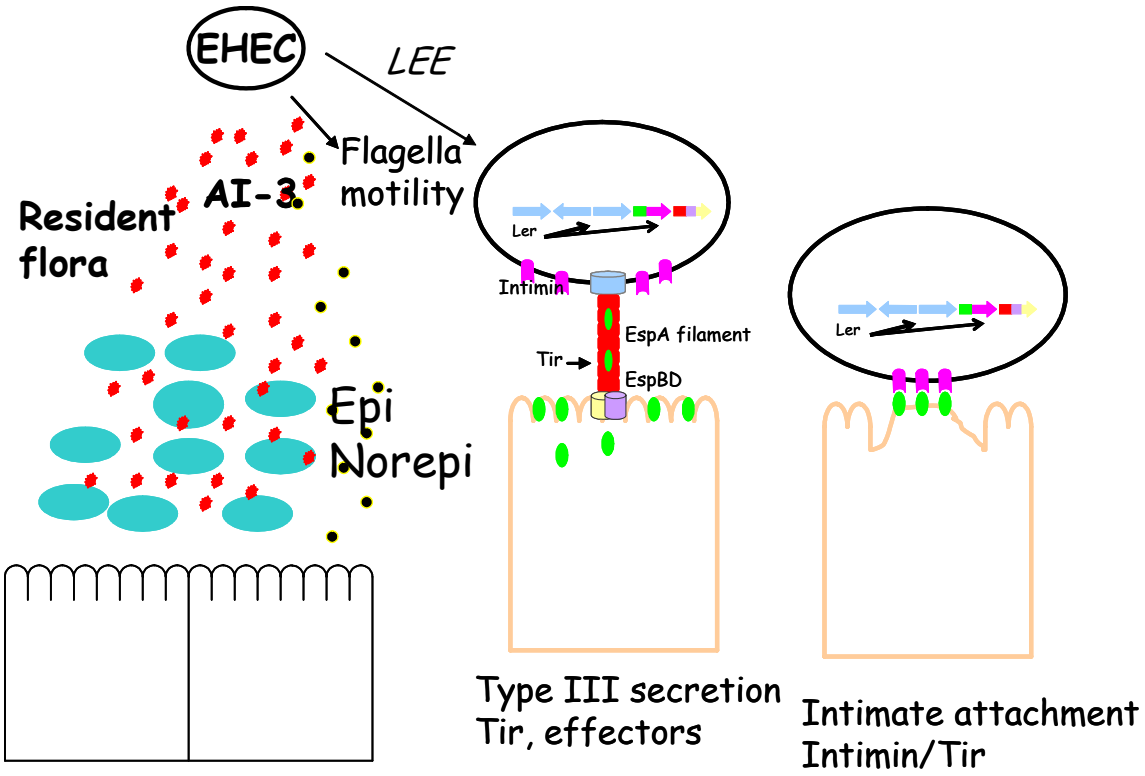


Figure 6. *Schematic of pathogenesis of EHEC.*

K. Statement of Project Overview

The work in this thesis focused on the further characterization of quorum sensing *E. coli* regulator A and the initial characterization of quorum sensing *E. coli* regulator D. Using the knowledge that QseA is involved in the activation of the *ler* promoter, nested deletion analyses and biochemical methods were used to determine the minimal region of the *ler* promoter necessary for activation via QseA. Other initial work performed on QseD tested the hypothesis that QseD is involved in the regulation of key virulence mechanisms in enterohemorrhagic *E. coli*, including flagella and type III secretion.

CHAPTER II. MATERIALS AND METHODS

A. Bacterial strains and culture conditions

The strains and plasmids used in this study are listed in Table 1. Strain FS02 is a *qseA* isogenic mutant of MC4100 and FS76 is FS02 complemented with pVS150, which is previously described [42, 49]. Strain JS5 is a *qseD* isogenic mutant of EHEC and JS5pJS4 is complemented with pJS4, FS55 is complemented with pFS09, and MW82 is complemented with pMW81. All *E. coli* strains were grown aerobically in LB medium or Dulbecco modified Eagle medium (DMEM) at 37^{o}C. Selective antibiotics were added at the following concentrations: 100 μg ml^{-1} ampicillin, 50 μg ml^{-1} kanamycin, 100 μg ml^{-1} streptamycin, 30 μg ml^{-1} tetracycline, and 30 μg ml^{-1} chloramphenicol.

B. Recombinant DNA techniques

Standard methods were used to perform plasmid purification, PCR, ligation, restriction digestion, transformation, and DNA gel electrophoresis [81]. All oligonucleotide primers are listed in Table 2.

The *qseA* gene was amplified by using primers qseAF (5' CTCGAGGGAACGACTAAAACGCATGTCGG 3') and qseAR (5' AAGCTTCTTCTCTTTCCCGCGCCCGT 3') containing *Xho* I and *Hin*d III linkers respectively, using *Pfx* DNA polymerase (Invitrogen). The PCR product was gel purified using the Qiaquick DNA purification kit (Qiagen) according to manufacturer's instructions, digested with *Xho* I / *Hin*d III, and then cloned into pBADMycHisA

(digested by *Xho* I / *Hin*d III) to generate plasmid pVS241 containing a QseAMycHis C-terminal fusion.

The *qseD* gene from EHEC (750 bp) was amplified by using primers QseD 1 F (5' GGATCCACGCCGCTGCAACTCTCAGA 3') and QseD 1 R (5' CCCGGGTCAGCTAAGCACAATCTCCAGCTC 3') containing *BamHI* and *SmaI* linkers respectively, using *Pfx* DNA polymerase (Invitrogen). The PCR product was gel purified using the Qiaquick DNA purification kit (Qiagen) according to manufacturer's instructions, digested with BamHI / SmaI, and then cloned into pQE30 (digested by BamHI / SmaI) to generate plasmid pFS09 containing a QseD His N-terminal fusion.

The *qseD* gene from EHEC (1.02 kb) was amplified by using primers qseDiadF (5' CAAAGACGGCAAAGCCTGCGTG 3') and QseD 1 R (5' CCCGGGTCAGCTAAGCACAATCTCCAGCTC 3') containing *BamHI* and *SmaI* linkers respectively, using *Pfx* DNA polymerase (Invitrogen). The PCR product was gel purified using the Qiaquick DNA purification kit (Qiagen) according to manufacturer's instructions, digested with *BamHI* / *SmaI*, and then cloned into pACYC184 (digested by BamHI / SmaI) (M. Walters and V. Sperandio, unpublished data).

The *qseD* gene from K-12 was amplified by using primers yjiEB Rev (5' ATGGATGACTGTGGTGCGA 3') and yjiEC Rev (5' ATCTCCAGCTCGCGCAGTTC 3') containing *BamHI* and *SmaI* linkers respectively, using *Pfx* DNA polymerase (Invitrogen). The PCR product was gel purified using the Qiaquick DNA purification kit (Qiagen) according to manufacturer's instructions, digested with *BamHI* / *SmaI*, and then cloned into pBadMycHisA (digested by *BamHI* / *SmaI*) to generate plasmid pJS4

containing a QseD MycHis C-terminal fusion (J. Smart and V. Sperandio, unpublished data).

Plasmid pJS2 was constructed by amplifying *yjiE* in two sections. YjiEAB (1.0 kb) was amplified by using primers yjiEA (5' ACGGAAGCGGGGCGTCACGTC 3') and yjiEB (5' CGCGAATTCTCGCACCACAGTCATCCATT 3') containing *EcoRI* and *KpnI* linkers respectively, using *Pfx* DNA polymerase (Invitrogen). The PCR product was gel purified using the Qiaquick DNA purification kit (Qiagen) according to manufacturer's instructions, digested with *EcoRI* / *KpnI*, and then cloned into pBlueScript (digested by *EcoRI* / *KpnI*) to generate plasmid pVS147. pVS147 (digested with *EcoRI* and *KpnI*) was ligated to Tc, tetracycline cassette from pACYC184, (digested with *EcoRI* and *KpnI*) to generate pVS148. YjiECD (1.3 kb) was amplified by using primers yjiEC (5' CGGGGTACCGAACTGCGCGAGCTGGAGAT 3') and yjiED (5' CCGCTCGAGGTTCCGATAGAGCATACGTC 3') containing *KpnI* and *XhoI* linkers respectively, using *Pfx* DNA polymerase (Invitrogen). The PCR product was gel purified using the Qiaquick DNA purification kit (Qiagen) according to manufacturer's instructions, digested with *KpnI* / *XhoI*, and then cloned into Topo (digested by *KpnI* / *XhoI*) to generate plasmid pVS146. pVS148 (digested with *EcoRI* and *KpnI*) and pVS146 (digested with *KpnI* and *XhoI*) were ligated to generate pJS2 (*qseD::tet* cloned into the R6K plasmid pCVD442) (Figure 7A). The pJS2 plasmid was verified using primer sets yjiEA / Tc2 and yjiED / Tc1 (Figure 7B) (J. Smart and V. Sperandio, unpublished data).

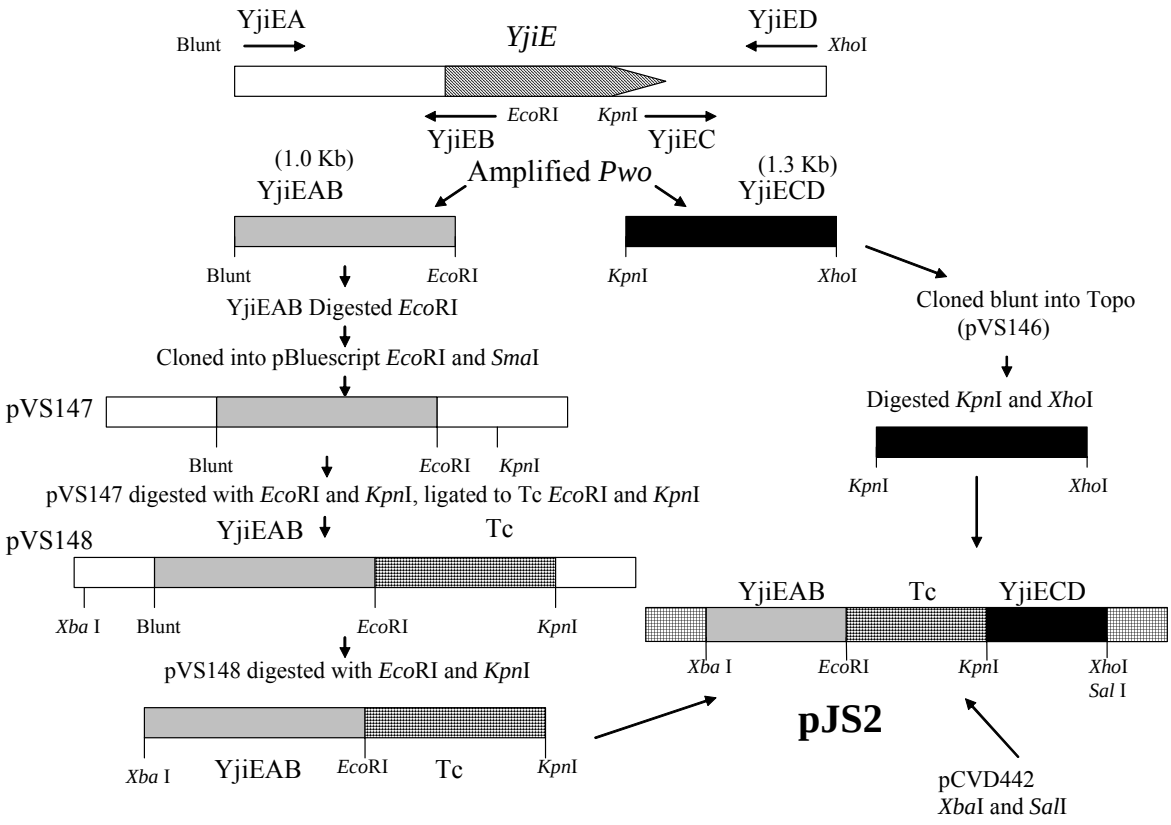


Figure 7A. *Construction of pJS2 (qseD::tet cloned into the R6K plasmid pCVD442). (J. Smart and V. Sperandio, unpublished data)*

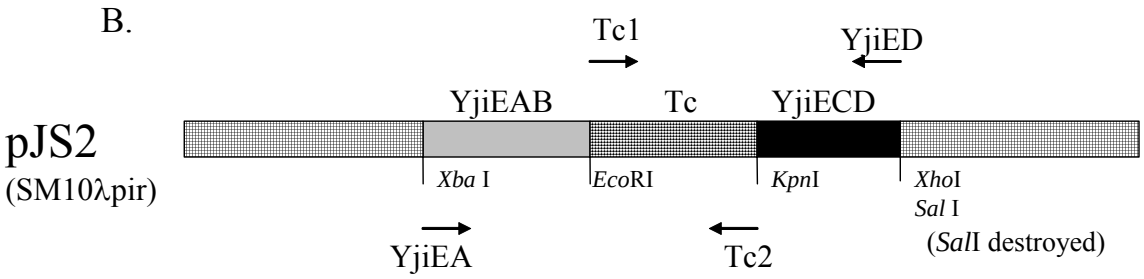


Figure 7B. *Diagram of vector pJS2. Construction checked by PCR using primer sets yjiEA / Tc2 and yjiED / Tc1 (J. Smart and V. Sperandio, unpublished data).*

C. Construction of *E. coli* O157:H7 mutants

The EHEC *qseD* mutant (named JS5) was generated by allelic exchange using vector pJS2 (*qseD::tet* cloned into the R6K plasmid pCVD442), and the mutants were selected in plates containing tetracycline and streptomycin and 5% sucrose as previously described [42] (Figure 8A). The *qseD* mutant was complemented with pJS4 (generating strain JS5pJS4), pMW81 (generating strain MW82), and pFS09 (generating strain FS55). The *qseD* mutant was verified using primers yjiEB Rev and yjiEC Rev (Figure 8B).

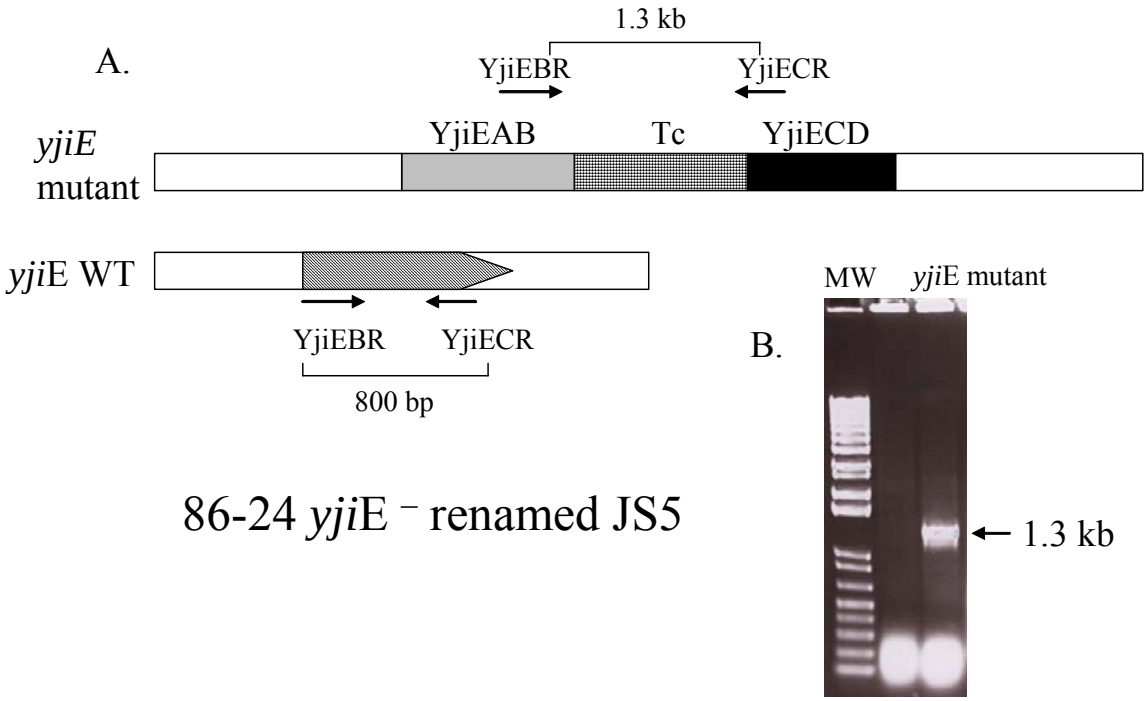


Figure 8. ***A.*** *Diagram of construction of EHEC qseD mutant. The qseD gene was insertionally inactivated in EHEC using allelic exchange using vector pJS2.* ***8B.*** *Construction checked by PCR using primers yjiEBR and yjiECR (J. Smart and V. Sperandio, unpublished data)*

D. Construction of *E. coli* K-12 mutants

The MC4100 *qseA* mutant (named FS02) was generated by allelic exchange using vector pVS143 (*qseA::cat* cloned into the R6K plasmid pCVD442), and the mutants were selected on media containing choramphenicol and 5% sucrose as previously described [42] (Figure 9). The *qseA* mutant (FS02) was complemented with plasmid pVS150, generating strain FS76 [49].

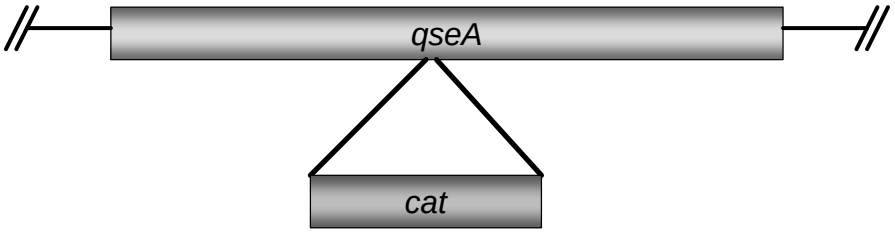


Figure 9. *Diagram of construction of MC4100 qseA mutant. The qseA gene of MC4100 was insertionally inactivated in E. coli K-12 strain MC4100 using allelic exchange using vector pVS143.*

The MC4100 *qseD* mutant (named FS08) was generated by allelic exchange using vector pJS2 (*qseD::tet* cloned into the R6K plasmid pCVD442), and the mutants were selected on media containing tetracycline and 5% sucrose as previously described [42]. The *qseD* mutant was verified using primers yjiEBR and yjiECR.

E. Construction of *LEE1/ler* deletion *lacZ* operon fusions

Transcriptional fusions with the promoterless *lacZ* were constructed by amplifying regions of the *ler* promoter region using *Pfx* DNA polymerase, using primers listed in Table 2, and cloning into the *EcoRI-BamHI* (Invitrogen) restriction sites of plasmid pRS551, which contains a promoterless *lacZ* cassette. This generated plasmids

pVS232Z, pVS204, pVS205, pVS206, pVS224, pVS225, pVS200, and pVS226 listed in Table 1.

Plasmid pVS232Z was constructed by amplifying the regulatory region upstream of *qseA* -393 to +323 bp using primers orf1 F and ler R3. Plasmid pVS204 was constructed by amplifying the regulatory region upstream of *qseA* -343 to +86 bp using primers ler 2F and ler R2. Plasmid pVS205 was constructed by amplifying the regulatory region upstream of *qseA* -300 to +86 bp using primers ler 3F and ler R2. Plasmid pVS206 was constructed by amplifying the regulatory region upstream of *qseA* -218 to +86 bp using primers ler 4F and ler R2. Plasmid pVS224 was constructed by amplifying the regulatory region upstream of *qseA* -173 to +86 bp using primers ler 5F and ler R2. Plasmid pVS225 was constructed by amplifying the regulatory region upstream of *qseA* -123 to +86 bp using primers ler 6F and ler R2. Plasmid pVS200 was constructed by amplifying – 393 to -42 bp using primers orf1 F and ler R1. Plasmid pVS226 was constructed by amplifying the regulatory region upstream of *qseA* -123 to +323 bp using primers ler 6F and ler R3 (Figure 6) [82].

These transcriptional fusions were each electroporated into 86-24 (wild-type EHEC), VS145 (*qseA* isogenic mutant in 86-24), and VS151 (VS145 with pVS151) for the multicopy EHEC deletion analysis. The transcriptional fusions were separately transformed into MC4100 (wild-type K-12), FS02 (*qseA* isogenic mutant in MC4100), and FS76 (FS02 with pVS150) generating strains FS10, FS11, FS12, FS13, FS14, FS15, FS16, FS17, FS18, FS86, FS19, FS20, FS21, FS22, FS23, FS24, FS25, FS87, FS77,

FS78, FS79, FS80, FS81, FS82, FS83, FS84, and FS88 for multicopy *E. coli* K-12 deletion analysis (Table 1) [82].

F. Construction of single-copy chromosomal *lacZ* operon fusions

In order to study the regulation of specific regions of the promoter of *LEE1* in single copy, the deletion constructs in pRS551 were transferred to the *recA*+ chromosome of MC4100 (*E. coli* K-12) using the specialized transducing phage λRS45 as described previously with slight modification [14, 83, 84]. Overnight cultures of donor strains were resuspended in 0.1 M $MgSO_4$ and combined with 1 x 10^7 pfu:phage particles were allowed to absorb for 15 minutes at 37°C, mixed with 3 mls of 0.7% agarose and overlaid on LB plates. After overnight incubation, phage particles were harvested with SM buffer and stored at 16°C for up to 6 months with chloroform. The phage particles were then transferred to single copy into the chromosome of MC4100 by combining an equal volume of recipient strain in 0.01 M $MgSO_4$, allowing to absorb for 15 minutes at room temperature, adding 2 ml LB supplemented with 2 mg/ml maltose, shaking at 37°C for 2 – 4 hrs, and plated on LB agar containing kanamycin. Transductants were replica plated for ampicillin sensitivity, then assayed for β-galactosidase activity. These single-copy fusions were constructed in MC4100, FS02 (*qseA*-), and FS76 (*qseA* complemented). The resulting transductants generated are FS106, FS108, FS109, FS110, FS111, FS112, FS122, FS114, FS116, FS117, FS118, FS119, FS120, FS123, FS125, FS127, FS128, FS129, FS130, FS131, and FS134 (Table 1).

G. β-galactosidase activity assay

The strains containing the transcriptional *lacZ* fusions were grown in LB in the appropriate selective antibiotic at 37°C to an OD_{600} of 1.0. These cultures were diluted 1:10 in Z buffer (60 mM $Na_2HP04*7H_2O$, 40 mM NaH_2PO4*H_2O, 10 mM KCl, 1 mM $MgSO_4*7H_2O$, 50 mM β-merchaptoethanol) and assayed for β-galactosidase activity by using o-nitrophenyl β-D-galactopyranoside (ONPG) as a substrate as previously described [85].

H. Purification of QseA and QseD proteins under native conditions

To purify the His-tagged QseA protein, the *E. coli* strain containing pVS241 was grown at 37°C in LB to an OD_{600} of 0.7, at which the expression of the protein was induced with 0.2% arabinose for three hours at 37°C, and subsequently purified using Nickel-affinity chromatography in native conditions according to manufacturer's instructions (Qiagen) [82].

To purify the His-tagged QseD protein, the *E. coli* strain containing pFS09 was grown at 37°C in LB to an OD_{600} of 0.7, at which the expression of the protein was induced with 1 mM IPTG for three hours at 37°C, and subsequently purified using Nickel-affinity chromatography in native conditions according to manufacturer's instructions (Qiagen) [82].

I. Electrophoretic mobility shift assay

In order to study the direct binding of QseA to the promoter of *ler*, electro-mobility shift assays (EMSA) were performed using the purified QseA-His protein and PCR amplified DNA probes. *Taq* DNA polymerase was used to amplify the *ler* promoter -393

to -42 base pairs, -173 to -42 base pairs, and -393 to -300 base pairs for DNA probe from EHEC using primers orf1 F/ler R1, Ler promoter -173F/Ler promoter -42R, orf1 F/ler R3, respectively. Additionally, the *bla* region, as the negative control, was amplified from pBR322 using primers ApR and ApF. DNA probes were then end-labeled using [γ-^{32}P]-ATP using T4 polynucleotide kinase (Invitrogen). End-labeled probes were run on a 6% polyacrylamide gel, excised, and purified using the Qiagen PCR purification kit.

EMSAs were performed by adding increasing concentrations of purified QseA-His protein (0 to 5 μg) to end-labeled probe (10 ng) equivalent to 2 -15 kcpm per reaction with 5X band shift buffer (5X transcription buffer (60 mM HEPES, pH 7.5, 5 mM EDTA, 3 mM DTT, 300 mM KCl, 25 mM $MgCl_2$), 50 ng/ul poly-dIdC, 500 ug/ml BSA (NEB)) and water for 20 minutes at 4^{o}C. 5X ficol loading buffer (5% ficol, 0.1% bromphenol blue) was added to reactions and immediately loaded to a 5% polyacrylamide gel that is pre-run for 1 hr, 50 V, at 4^{o}C. The gels were electrophoresed at 4^{o}C at 180 V, dried, and exposed to phophorimage cassette 1 hr to overnight at RT [82].

J. Motility assay

Motility assays were performed at 37^{o}C on 0.3% agar plates containing tryptone media (1% tryptone and 0.25% NaCl). The motility halos were measured at 4, 6, and 8 hours [76].

K. TCA precipitation assay

Secreted proteins from EHEC strains 86-24, JS5, and FS55 were prepared by growing the strains in DMEM at 37^{o}C aerobically to an OD_{600} of 1.0. Bacteria were pelleted by

centrifugation (10,000 x g, 10 minutes). Supernatants were filter sterilized with 0.22 μm filters and treated with phenylmethylsulfonyl fluoride (50 μg/ml; Sigma), aprotinin (0.5 μg/ml; Sigma), and EDTA (0.5 μM; Sigma) and precipitated overnight with 10% trichloroacetic acid at 4°C. The precipitated proteins were pelleted by centrifugation (20,000 x g, 1 hour, 4°C) and resuspended in 1 x PBS solution. The proteins were analyzed by sodium dodecyl sulfate-12% polyacrylamide gels by loading total protein collected [86].

L. Flagellin detection assays

Total proteins were extracted from strains 86-24, JS5, MW82, and FS55 grown in DMEM at 37°C aerobically to an OD_{600} of 1.0. Cultures were pelleted by centrifugation (10,000 x g, 10 min). Cultures were resuspended in 1 x PBS solution. The protein concentration was measured using the Bradford assay (Biorad). Equal concentrations of total proteins were electrophoresed in sodium dodecyl sulfate-12% polyacrylamide gels [81].

M. Western analysis

Protein concentrations were measured using the Bradford assay [81]. Equal concentrations of total proteins were electrophoresed in sodium dodecyl sulfate-12% polyacrylamide gels. Western blotting procedures were performed as previously described, and blots were probed with polyclonal antisera directed against the anti-H6 flagellin, anti-EspA, and anti-EspB. [38] (Graciously given by Dr. James Kaper, University of Maryland).

N. Northern analysis

Bacterial cultures were grown in DMEM to an OD_{600} of 1.0. Total RNA was extracted using Trizol reagent as recommended by the manufacturer for 86-24, JS5, and FS55 (Invitrogen). RNA was quantified spectrophometerically and equal concentrations of total RNA were loaded into a 1.2% agarose formaldehyde-morpholinepropanesulfonic acid gel as previously described. After electrophoresis, the RNA was blotted onto Hybond-N nylon membranes (Ambion) by downward transfer and UV cross-linking (Current protocols procedure). Pre-hybridization was performed on all membranes at 42^{o}C with Ultrahyb buffer (Ambion). Samples were probed with single-stranded DNA probes specific for *LEE4 (sepL)*. The probes were amplified with *Taq* DNA polymerase using primers midorf23 F/midorf23 R and orf23RT/espA PE for *orf23/LEE4*. Additionally, 23S RNA was amplified for the negative control using *Taq* DNA polymerase using primers 23S F and 23S R. The primers used are listed in Table 2. Probes were labeled with ready-to-go DNA labeling beads and [α^{32}P]-dCTP (Amersham-Pharmacia Biotech). The signal was detected using phosphoimager and standard autoradiography.

Table 1. *Strains and plasmids used in this study.*

Strain	Genotype/Description	Reference
DH5α	Host *E. coli* strain for cloning experiments	Strategene
TOP10	Host *E. coli* strain for pCR Blunt II TOPO ligations	Invitrogen
86-24	Stx2+ EHEC strain (serotype O157:H7)	Griffin et al (1988)
VS145	86-24 *qseA* mutant	Sperandio et al (2002)
VS151	VS145 with plasmid pVS150	Sperandio et al (2002)
MC4100	*araD*139 D(*araABC-leu*)7679 *galU galK D(lac)*X74 *rpsL thi*	Silhavy and Beckwith (1984)
JS5	86-24 *qseD* mutant	This study
JS5pJS4	JS5 complemented with pJS4	This study
MW82	JS5 complemented with pMW81	This study
FS55	JS5 complemented with pFS09	This study
FS02	MC4100 *qseA* mutant	This study
FSO8	MC4100 *qseD* mutant	This study
FS76	FS02 with plasmid pVS150	This study
FS10	MC4100 with plasmid pVS232Z	This study
FS11	MC4100 with plasmid pVS200	This study
FS12	MC4100 with plasmid pVS204	This study
FS13	MC4100 with plasmid pVS205	This study
FS14	MC4100 with plasmid pVS206	This study
FS15	MC4100 with plasmid pVS224	This study
FS16	MC4100 with plasmid pVS225	This study
FS17	MC4100 with plasmid pVS226	This study
FS86	MC4100 with plasmid pRS551	This study

Strain	Genotype/Description	Reference
FS18	FS02 with plasmid pVS232Z	This study
FS19	FS02 with plasmid pVS200	This study
FS20	FS02 with plasmid pVS204	This study
FS21	FS02 with plasmid pVS205	This study
FS22	FS02 with plasmid pVS206	This study
FS23	FS02 with plasmid pVS224	This study
FS24	FS02 with plasmid pVS225	This study
FS25	FS02 with plasmid pVS226	This study
FS87	FS02 with plasmid pRS551	This study
FS77	FS76 with plasmid pVS232Z	This study
FS78	FS76 with plasmid pVS200	This study
FS79	FS76 with plasmid pVS204	This study
FS80	FS76 with plasmid pVS205	This study
FS81	FS76 with plasmid pVS206	This study
FS82	FS76 with plasmid pVS224	This study
FS83	FS76 with plasmid pVS225	This study
FS84	FS76 with plasmid pVS226	This study
FS88	FS76 with plasmid pRS551	This study
FS106	Single copy fusion of FS10	This study
FS108	Single copy fusion of FS12	This study
FS109	Single copy fusion of FS13	This study
FS110	Single copy fusion of FS14	This study
FS111	Single copy fusion of FS15	This study
FS112	Single copy fusion of FS16	This study
FS122	Single copy fusion of FS86	This study

Strain	Genotype/Description	Reference
FS114	Single copy fusion of FS18	This study
FS116	Single copy fusion of FS20	This study
FS117	Single copy fusion of FS21	This study
FS118	Single copy fusion of FS22	This study
FS119	Single copy fusion of FS23	This study
FS120	Single copy fusion of FS24	This study
FS123	Single copy fusion of FS87	This study
FS125	Single copy fusion of FS77	This study
FS127	Single copy fusion of FS79	This study
FS128	Single copy fusion of FS80	This study
FS129	Single copy fusion of FS81	This study
FS130	Single copy fusion of FS82	This study
FS131	Single copy fusion of FS83	This study
FS134	Single copy fusion of FS88	This study
TEVS232Z	Promoter region of *LEE1*	Sperandio et al 1999
TEVS21	Promoter region of *LEE2*	Sperandio et al 1999
TEVS76	Promoter region of *LEE4*	Sperandio et al 1999
TEVS24	Promoter region of *LEE5/Tir*	Sperandio et al 1999
TEVS26	Promoter region of *LEE3*	Sperandio et al 1999

Plasmid	Genotype/Description	Reference
pRS551	*lacZ* reporter gene fusion vector	Simmons et al (1987)
pCVD442	Suicide vector	Donnenberg et al (1991)
pBADMycHis	C-terminal Myc-His-tag cloning vector	Invitrogen
pQE30	Cloning vector	Qiagen
pBlueScriptII	Cloning vector	Stratagene
pMW50	*Bla* in pBluescript	This study
pVS241	*qseA* in pBADMycHisA	This study
pVS150	*qseA* in pACYC177	Sperandio et al (1999)
pVS232Z	*ler*[*LEE1]::lacZ* in pRS551, base pairs -393 to +323	Sperandio et al (1999)
pVS200	*ler::lacZ* in pRS551, base pairs -393 to -42	This study
pVS204	*ler::lacZ* in pRS551, base pairs -343 to +86	This study
pVS205	*ler::lacZ* in pRS551, base pairs -300 to +86	This study
pVS206	*ler::lacZ* in pRS551, base pairs -218 to +86	This study
pVS224	*ler::lacZ* in pRS551, base pairs -173 to +86	This study
pVS225	*ler::lacZ* in pRS551, base pairs -123 to +86	This study
pVS226	*ler::lacZ* in pRS551, base pairs -123 to +323	This study
pFS133	-173 to -42 bp of *ler* promoter in TOPO	This study
pJS2	*qseD*::Tc construct suicide pCVD442; SM10 λpir	This study
pJS4	*qseD* from K-12 MC1655 into pBADMycHis	This study
pMW81	*qseD* from 86-24 in pACYC184	This study
pFS09	*qseD* from 86-24 in pQE30	This study

Table 2. *Primers used in this study.*

Primers	Oligonucleotide (5' – 3')	Reference
qseAF	CTCGAGGGAACGACTAAAACGCATGTCGG	Russell et al 2005
qseAR	AAGCTTCTTCTCTTTCCCGCGCCCGT	Russell et al 2005
Orf1 F	CGGAATTCATGTGCTGCGACTGCGTTCG	Sperandio et al 2002
Ler 2F	CGGAATTCCTGGGGATTCACTCGCTTGC	This study
Ler 3F	CGGAATTCGTAGAGTATAGTGAAACGGT	This study
Ler 4F	CGGAATTCCGCTTAACTAAATGGAAATGC	This study
Ler 5F	CGGAATTCAGATGATTTTCTTCCATTTAAT	This study
Ler 6F	CGGAATTCGATTTTTTTGTTGAGACACAT	This study
Ler R1	CGGGATCCTCTATCAAATTAGGACACAT	This study
Ler R2	CGGGATCCGTATGGACTTGTTGTATGTG	This study
Ler R3	CGGGATCCGTCGGCCTACGCCCGACC	This study
Ler promoter -173F	CGGGATCCCGATGATTTTCTTCTATATCATTG	This study
Ler promoter -42R	CGGAATTCCGCGACCTTATCAGGAAGGACC	This study
ApR	CGGGATCCGGTGAGCAAAAACAGGAAGG	This study
ApF	GGAATTCGAAAGGGCCTCGTG ATA CGC	This study
QseD 1 F	GGATCCACGCCGCTGCAACTCTCAGA	This study
QseD 1 R	CCCGGGTCAGCTAAGCACAATCTCCAGCTC	This study
QseDiadA F	CAAAGACGGCAAAGCCTGCGTG	This study
QseDiadA R	GCCGCCACGCAGCTCTGCAA	This study
Midorf23 F	AATCAAAACCCCGCATCTGT	This study
Midorf23 R	ACTCTCCAGCAACCAGTCTT	This study
Orf23 RT	AGACTGGTTGCTGGAGAGT	This study

Primers	**Oligonucleotide (5' – 3')**	**Reference**
Esp APE	CGATGTCGAAGAACTCGCAC	This study
23S F	GGATGTTGGCTTAGAAGCAG	This study
23S R	CAGCTGGTATCTTCGACTGA	This study
Tc 1	GGAATTCCTTCTCATGTTTGACAGCTTA	This study
Tc 2	GGGGTACCCCTCAGGTCGAGGTGGCCCGG	This study
yjiE A	ACGGAAGCGGGGCGTCACGTC	This study
yjiE B	CGCGAATTCTCGCACCACAGTCATCCATT	This study
yjiE C	CGGGGTACCGAACTGCGCGAGCTGGAGAT	This study
yjiE D	CCGCTCGAGGTTCCGATAGAGCATACGTC	This study
yjiE B Rev	ATGGATGACTGTGGTGCGA	This study
yjiE C Rev	ATCTCCAGCTCGCGCAGTTC	This study

CHAPTER III. CHARACTERIZATION OF QUORUM SENSING *E. coli* REGULATOR A AND REGULATION OF THE *LEE1* (*LER*) PROMOTER

A. Introduction

Quorum sensing *E. coli* regulator A, QseA, is a key transcriptional regulator within the quorum sensing cascade of enterohemorrhagic *E. coli*. The transcription of *qseA* was significantly down-regulated in the *luxS* mutant, as seen in previous array studies. QseA has significant homology to members of the LysR-family of regulators, as it contains the typical helix-turn-helix DNA binding motif at the amino-terminus [42]. Also characteristic of members of the LysR-family of regulators, QseA is involved in the regulation of its own transcription, repressing its own transcription 84-fold in stationary-growth phase [79]. QseA was shown to activate the transcription of *LEE1,* which encodes Ler, an essential activator of the locus of enterocyte effacement (LEE) [42]. To elucidate the molecular mechanisms by which QseA activates transcription of *LEE1*, genetic and biochemical methods were used as described further in this section of this thesis.

B. Nested Deletion Analysis in Multi-Copy in EHEC

In order to identify the minimal regulatory region of *LEE1/ler* that is necessary for QseA-mediated activation, a series of nested deletion constructs of the *LEE1/ler* promoter in EHEC were generated (Figure 10). These deletions were then fused to a promoterless *lacZ* cassette in pRS551 and used for both multi-copy nested deletion analysis in EHEC

strain 86-24 and K-12 strain MC4100, and single-copy nested deletion analysis in K-12 strain MC4100 using the λRS45-based transducing system. The single-copy nested deletion analysis could not be performed in EHEC strain 86-24 because EHEC is immune to λ transduction.

The multi-copy nested deletion analysis in EHEC strains 86-24 (wild-type), VS145 (*qseA* mutant), and VS151 (*qseA* complemented) is shown in Figure 11. Transcription of *LEE1/ler::lacZ* is decreased in the *qseA* mutant compared to wild-type and complemented strains in promoter fusion constructs -218 to +86 base pairs, -173 to +86 base pairs, -123 to +86 base pairs, and -123 to +323 base pairs, suggesting that there is a dependence on the activation of the *ler* promoter between -218 and +86 base pairs by QseA. Additionally, we observe QseA-dependent regulation of the entire promoter region, as seen using fusion construct -393 to +323 base pair. We observe a 4-fold decrease in *LEE1* transcription in the *qseA* mutant with the -393 to +323 base fragment (Figure 11). There is a 3-fold decrease in the *LEE1* basal level transcription in the wild-type strain in fusion -393 to -42 base pairs as compared to the -393 to +323 base pair construct, although activation of the -393 to -42 base pair fragment is still dependent on QseA. The -393 to -42 base pair fragment lacks the P2 promoter of *LEE1/ler* (Figure 11). These data further suggest that QseA activates transcription of the *ler* promoter through the P1 promoter in EHEC, which validates previous primer extension data [42].

We also observe that the *LEE1* basal level transcription in the wild-type strain decreases 7-fold from the -393 to +323 base pair fragment to the -218 to +86 base pair fragment, suggesting that there is another yet unidentified transcriptional activator acting

between -393 to -218 base pairs (Figure 11). QseA-dependent activation is lost in the region between -343 and -218. Transcription of *LEE1* is highly repressed between regions -343 and -300, suggesting that another unidentified factor may be involved in repressing this region. Of notice, QseA-dependent activation of P1 can be observed until nucleotide -123 (which corresponds to -50 in relation to the -35 region of P1) (Figure 11). This proximity to the promoter may suggest that QseA directly interacts with RNAP near P1, which is a feature of LysR transcriptional regulators.

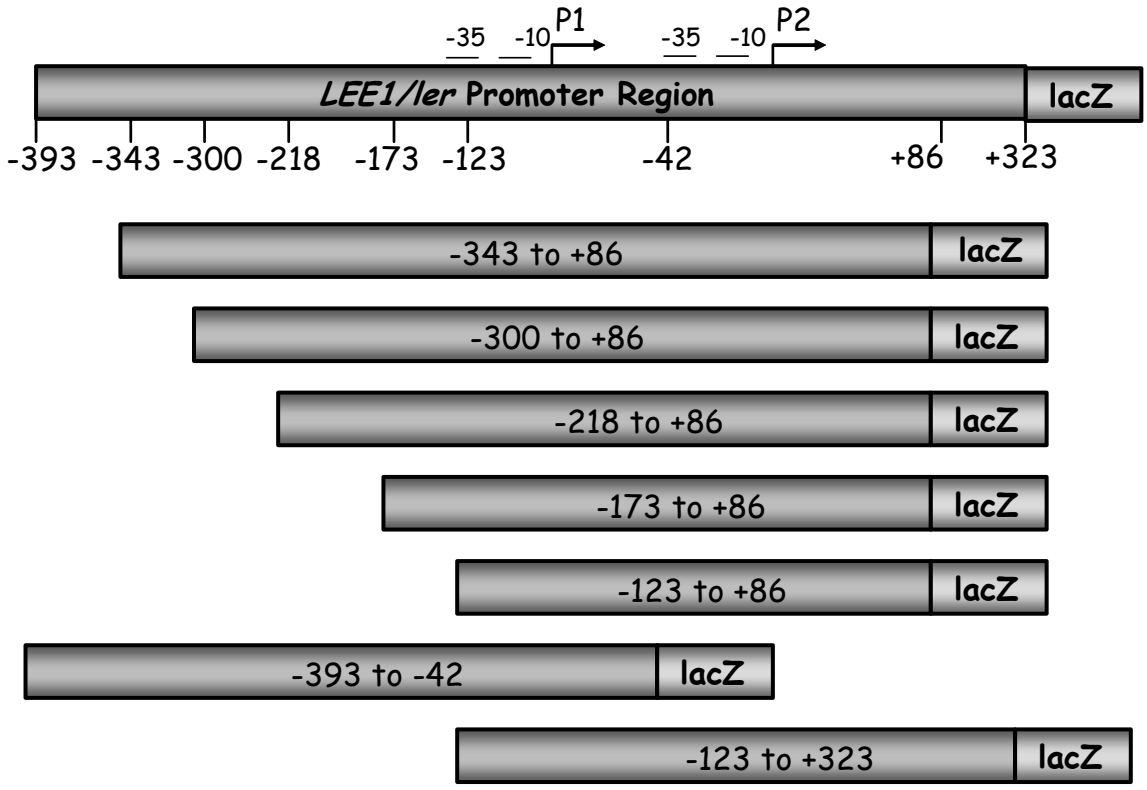
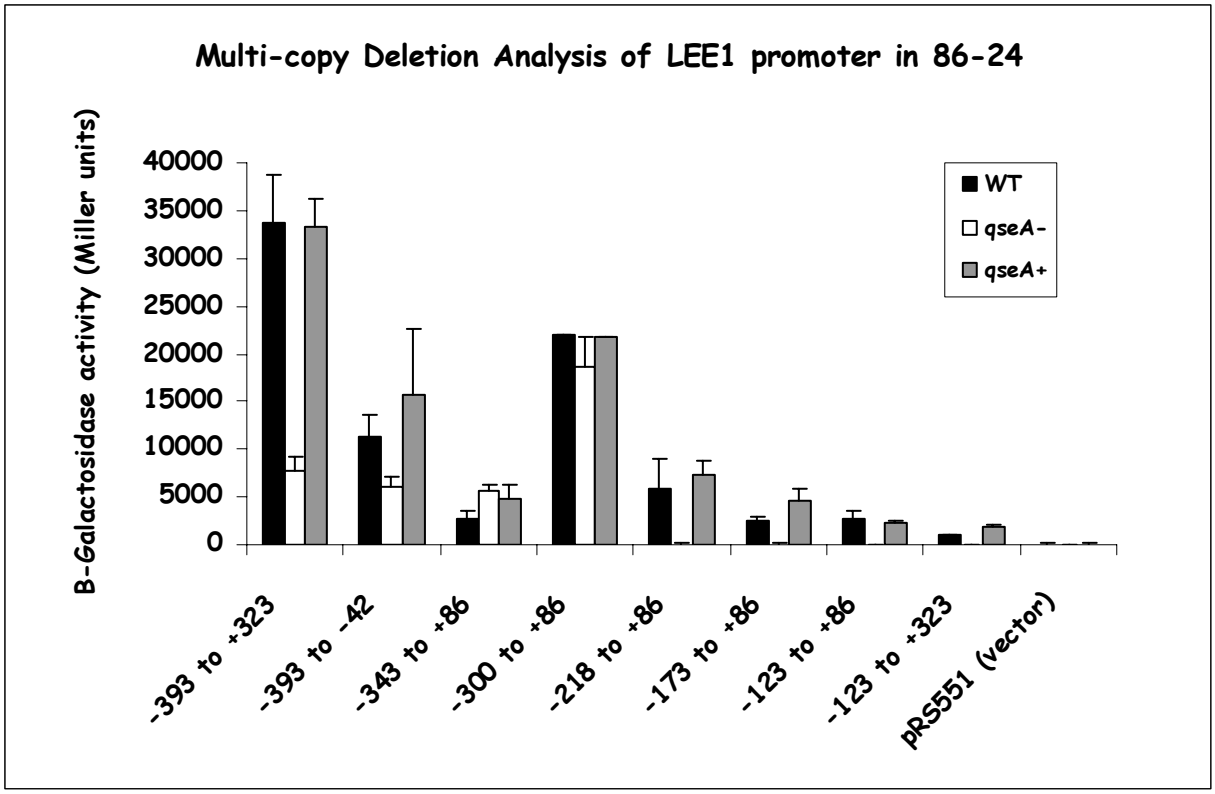


Figure 10. *Schematic of nested deletion analysis. The deletion analysis was stems from data previously generated with primer extension analysis [42]. The numbering of the bases (-393 to +323) is based on the P2 transcriptional start site.*



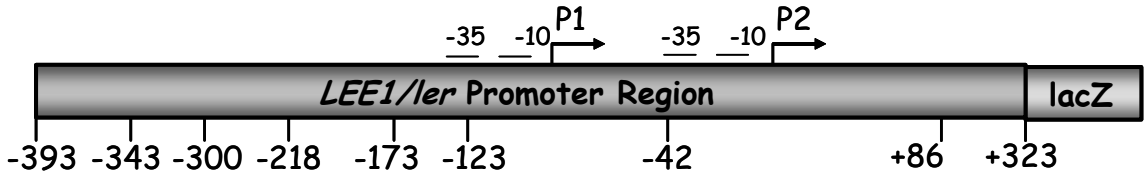


Figure 11. *Multi-copy deletion analysis of LEE1/ler promoter in EHEC. Fragments of the ler promoter were electroporated into 86-24 (wild-type), VS145 (qseA mutant), and VS151 (qseA complemented) and assessed for β-galactosidase activity. Error bars indicate standard deviations.*

C. **Nested Deletion Analysis in Multi-Copy in K-12**

The multi-copy deletion analysis in K-12 strain MC4100 is shown in Figure 12. Using the *qseA* mutant in K-12 (FS02) and the complemented strain (FS76) described in Materials and Methods, we were able to determine whether the regulation of the *LEE1/ler* promoter is different in a strain of *E. coli* K-12 compared to 86-24 [49]. Comparing Figure 11 to Figure 12, one can observe that there is a significant overall decrease (over 10-fold) in the basal transcriptional level of this promoter. This suggests that in K-12 transcription of *LEE1/ler* is repressed compared to EHEC. We did not observe QseA-dependent activation of *LEE1* transcription between base pairs -393 and -218. Of notice, transcription of *LEE1* is highly repressed in this region, suggesting that a transcriptional repressor, which is present in K-12, but absent in EHEC, acts in this repression. Transcription of *LEE1::lacZ* is restored to 5000 Miller units (similarly in EHEC) in the fusion construct -218 to +86. We also observe QseA-dependent *LEE1* activation from nucleotides -218 and -173, similar to the nested deletion analysis performed in EHEC, again, suggesting that this is the region in the *LEE1* promoter where QseA may directly bind to activate P1 transcription (Figure 12). We note a 3-fold loss in activation in the *qseA* mutant -218 to +86 base pairs and -173 to +86 base pairs, which is comparable to the region of regulation in the EHEC deletion analysis (Figures 11 and 12). This suggests that -218 to +86 base pairs of the *ler* promoter may be the direct region of interaction between QseA and the *LEE1/ler* promoter. Of notice, in the multi-copy deletion analysis in K-12, there is no activation of the promoter fragments -393 to +323 base pairs, -393 to -42 base pairs, -343 to +86 base pairs, -300 to +86 base pairs, and -123 to +86 base pairs (Figure 12). We do not observe the same dependence on regulation of -393 to -42 base

pairs in the K-12 deletion analysis seen previously in the EHEC deletion analysis (Figures 11 and 12). This further implies that there may be an EHEC specific factor that is also involved in the regulation of the *LEE1/ler* promoter.

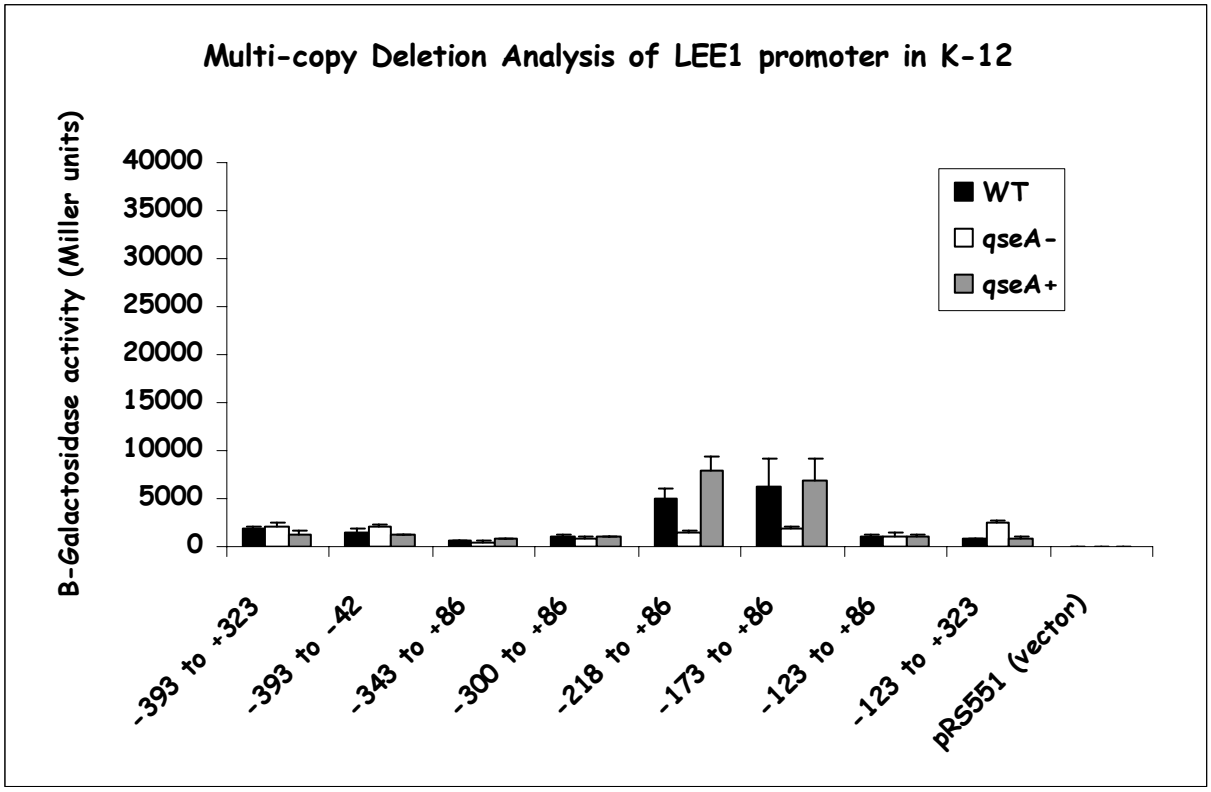


Figure 12. *Multi-copy deletion analysis of LEE1/ler promoter in K-12. Fragments of the ler promoter were transformed into MC4100 (wild-type), FS02 (qseA mutant), and FS76 (qseA complemented) and assessed for β-galactosidase activity. Error bars indicate standard deviations.*

D. Nested Deletion Analysis in Single-Copy in K-12 in LB

To address issues concerning plasmid copy number and coiling effects, we studied the regulation of the *LEE1/ler* promoter using single-copy chromosomal fusions as depicted in Figure 10. Using the previously described K-12 wild-type, *qseA* mutant, complemented strains, we introduced the *ler* promoter fragments in single-copy into the chromosomes using the λRS45-based system described in Materials and Methods. There is a significant 5- to 12-fold decrease in the basal level of transcription of the *ler* promoter in the single-copy compared to multi-copy deletion analysis. We observed a similar regulation pattern in the *LEE1* regulation of the single-copy deletion analysis in K-12 (Figure 13) as in the multi-copy deletion analysis in K-12 (Figure 12). Here again, there is *LEE1* QseA-dependent activation until nucleotide -173 in a *qseA* mutant compared to wild-type. The activation of the -123 to +86 base pair fragment is also QseA-dependent in single-copy (Figure 13). This region is QseA-dependent in multi-copy in EHEC, but not K-12. As previously mentioned, nucleotide -123 in relation to the P1 promoter would be -50, suggesting again that QseA may directly interact with this region in the *LEE1* promoter.

In both multi- and single-copy we did not observe QseA-dependent activation between nucleotides -393 and -343 (Figures 12 and 13), suggesting that QseA activation of this region in EHEC occurs via a transcriptional activator present in EHEC and absent in K-12. One potential candidate would be GrlA, previously reported by Deng et al to activate *LEE1* transcription [47]. In support of this hypothesis, Russell et al has demonstrated that transcription of the *grlRA* operon is activated by QseA in a Ler-independent manner [49].

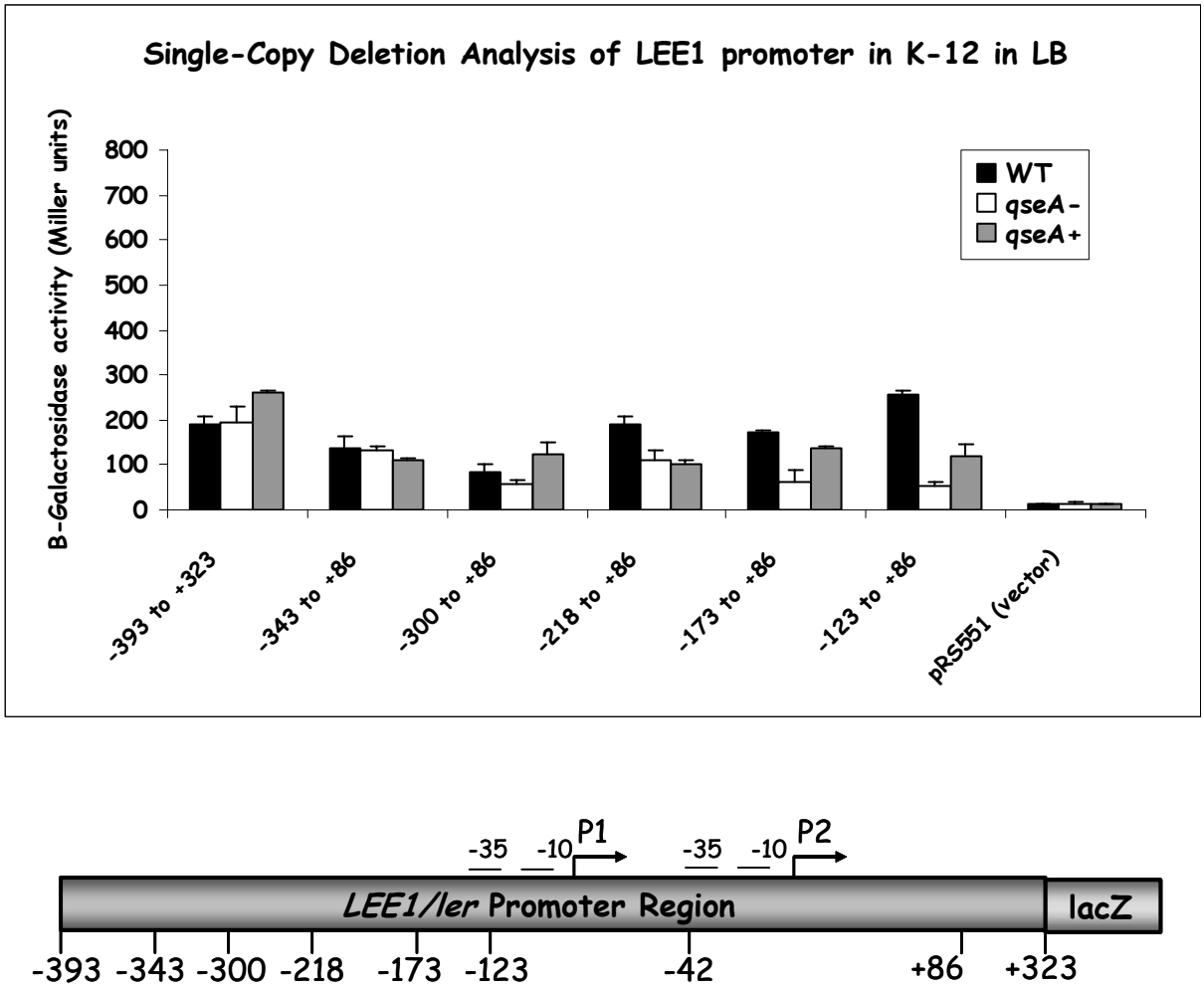


Figure 13. *Single-copy deletion analysis of LEE1/ler promoter in K-12 in LB. Fragments of the ler promoter were introduced into the chromosome of MC4100 (wild-type), FS02 (qseA mutant), and FS76 (qseA complemented) and assessed for β-galactosidase activity in LB. Error bars indicate standard deviations.*

E. Electrophoretic Mobility Shift Assay

In order to assess if QseA directly interacts with the *LEE1/ler* regulatory region, we performed electrophoretic mobility shift assays (EMSA) with QseA purified in native conditions. *qseA* was cloned into pBADMycHis vector to generate a C-terminal myc-his fusion under the control of the *araC* (pBAD) promoter. The C-terminal fusion was chosen because the HTH DNA binding motif of QseA is in the N-terminus. QseA was expressed from the resulting vector, pVS241 using 0.2% arabinose and purified using a Nickel-affinity column under native conditions (Figure 14).

To demonstrate that QseA interacts directly with fragments of the *LEE1/ler* promoter, we used electrophoretic mobility shift assays. We generated probes harboring -393 to -42 base pairs and -173 to -42 base pairs of the *ler* promoter. These regions were shown to be important in the QseA-dependent regulation of the *LEE1/ler* promoter. These probes were PCR amplified and end-labeled using [γ-^{32}P]-ATP and T4 polynucleotide kinase (Invitrogen). The constitutive *bla* promoter fragment was amplified with primers ApR and ApF and used as a negative control. With the addition of increasing concentrations of His-tagged QseA protein a shift of the *LEE1/ler* promoter region -393 to -42 base pairs and -173 to -42 base pairs was observed (Figure 15). The negative control *bla* did not shift with the addition of increasing concentration of QseA protein, suggesting that QseA binding is specific to the *ler* promoter (Figure 15). We also performed an EMSA using a probe harboring the -393 to -300 base pairs of *LEE1* (Figure 15). This region has been shown to be activated by QseA in EHEC, but not in K-12. QseA did not bind to the -393 to -300 region, further suggesting that QseA activation of this region is indirect via a transcription factor present in EHEC and absent in K-12 (Figure 15). These data suggest

that the minimal region necessary QseA binding to the *ler* promoter is located between -173 to -42 base pairs.

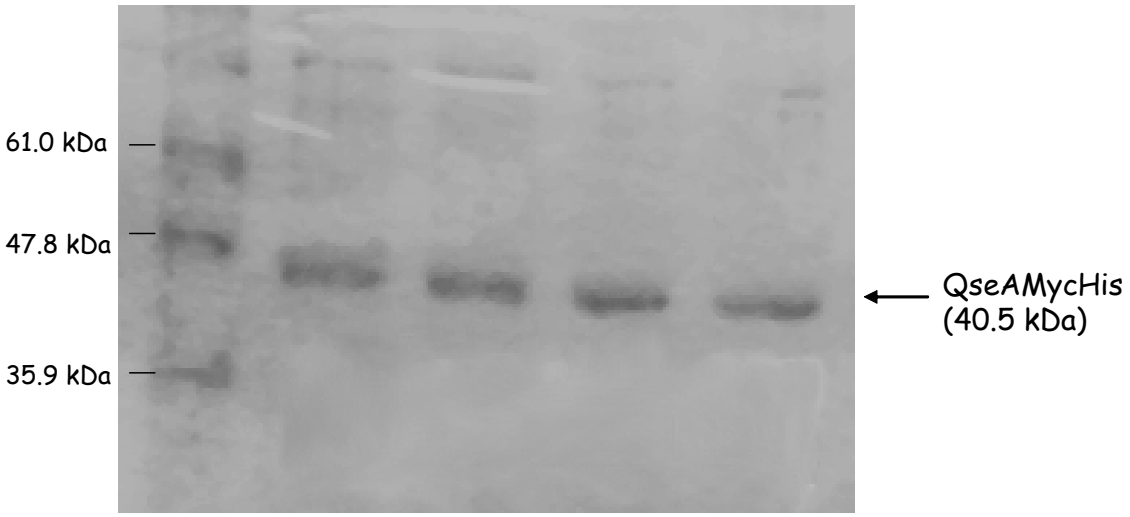


Figure 14. *Coomassie blue staining of nickel-column purification of QseAMycHis on 12% SDS-PAGE. His-tagged QseA protein was estimated to be greater than 95% pure.*

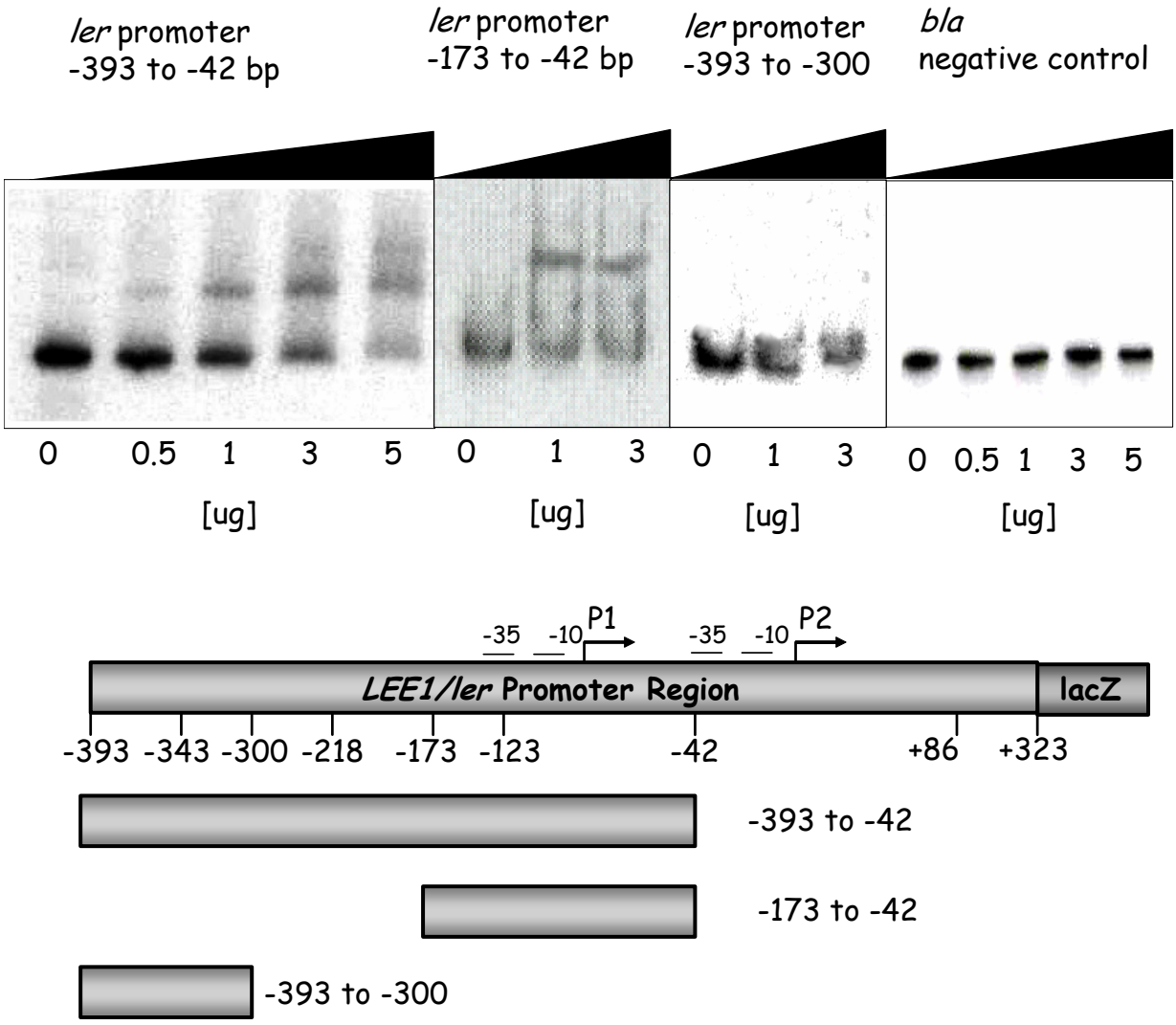


Figure 15. *EMSAs of fragments of ler promoter with QseA protein. Purified QseA was added to radio-labeled fragments of the ler promoter -393 to -42 base pairs, -173 to -42 base pairs, and -393 to -300 base pairs. The constitutive promoter of bla was used as negative control.*

F. Concluding Remarks

Quorum sensing *E. coli* regulator A, QseA, activates the transcription of the *LEE1/ler* promoter. The nested deletion analyses in EHEC and K-12 in multi-copy suggest that QseA acts on the *LEE1/ler* promoter -218 to +86 base pairs (based on the P2 promoter). The nested deletion analysis in single-copy in K-12 suggests that QseA may be directly interacting -173 to +86 base pairs (based on the P2 promoter). We also observe QseA-dependent activation in the constructs -123 to +86 base pairs and -123 to +323 base pairs. The nucleotide -123, corresponding to -50 in relation to the -35 region of P1, undergoes QseA-dependent activation. These data suggest that QseA interacts in close proximity to the P1 transcriptional start site. In the multi-copy deletion analysis in EHEC, we observe another level of regulation. There is QseA-dependent transcriptional activation in the promoter fragments -393 to +323 base pairs and -393 to -42 base pairs in the EHEC deletion analysis, which is not observed in the K-12 multi-copy analysis. These data suggest that another factor, specific to EHEC, may also be responsible for regulation of the *LEE1/ler* promoter, possibly GrlRA (Figure 16). Additionally, in electrophoretic mobility shift assays (EMSA) we observe that QseA directly binds to -173 to -42 base pairs of the *LEE1/ler* promoter. We do not observe binding -393 to -300 base pairs again, suggesting that the activation of this region observed in EHEC may be due to another factor. Recent data shows that QseA activates the expression of *grlRA* indirectly [49]. This study also shows that GrlA activates *ler* [49]. From data generated in our studies, GrlA may be acting as an activator of *LEE1/ler* from -393 to -300 base pairs. Additionally, GrlR may act as a repressor around -300 base pairs (Figure 16). Other factors have been shown to regulate the LEE in EHEC, including Pch, EivF and

EtrA. These EHEC specific transcriptional regulators should be tested to determine whether any of these factors play a role in the transcriptional activation of -393 to +343 base pairs or repression of -300 to +86 base pairs of the *LEE1/ler* promoter. Although from the nested deletion analyses it appears that QseA may activates through the P1 transcriptional start site, another construct -123 to -42 base pairs would assess whether QseA fully activates the transcription of the *LEE1/ler* promoter through the P1 transcriptional start site. Competition assays and DNaseI footprinting studies are necessary to validate the specificity of the binding of QseA to the promoter regions and to identify the specific binding site within -173 to -42 base pairs.

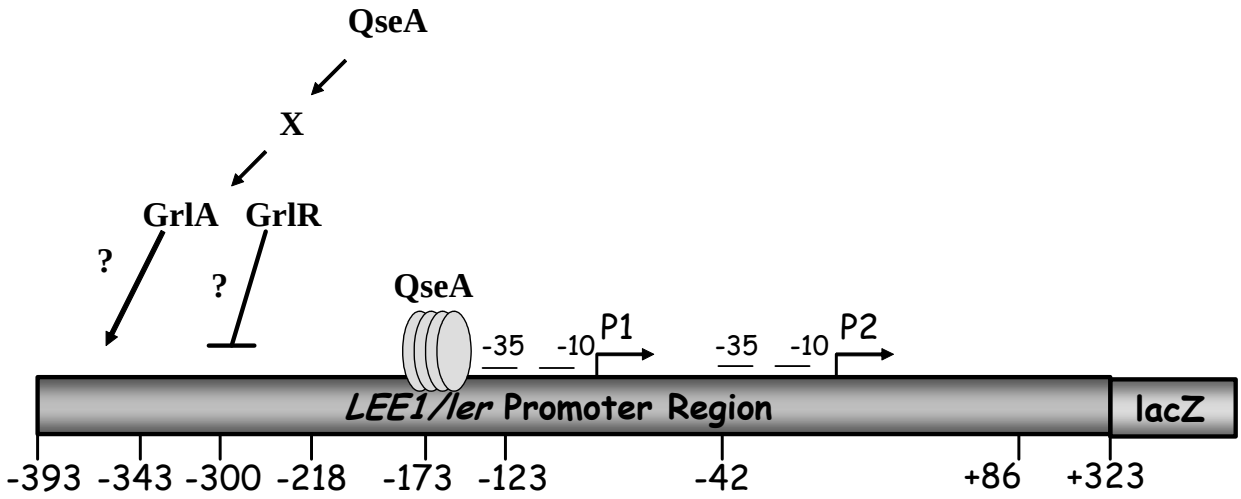


Figure 16. *Model for the regulation of LEE1/ler by QseA and GrlRA. QseA activates grlRA [49]. The activation is indirect, suggesting that another factor is transcriptionally activated by QseA, then acts on grlRA. GrlR represses LEE1/ler, possibly at nucleotide -300, while GrlA activates LEE1/ler, possibly -393 to -300 nucleotides. QseA directly activates the transcription of the LEE1/ler promoter through nucleotides -173 to -42.*

CHAPTER IV. CHARACTERIZATION OF QUORUM SENSING *E. coli* REGULATOR D IN THE OVERALL QUORUM SENSING CASCADE OF EHEC.

A. Introduction

As mentioned previously, EHEC contains many factors that are regulated through quorum sensing and are involved in the intricate regulation of virulence mechanisms, including type III secretion and flagellar regulation. These transcriptional regulators play a role in the precise control of these virulence phenotypes to establish infection within the host. Quorum sensing *E. coli* regulator D, QseD, is a previously uncharacterized regulator in the EHEC quorum sensing cascade. This section of the thesis describes characterization of the role of QseD in EHEC pathogenesis and quorum sensing.

B. E. coli putative regulator *yjiE* is repressed by quorum sensing

Initial reports have shown that quorum sensing regulates the transcription of genes encoding regulators involved in the expression of LEE genes and flagella in EHEC [38, 42, 76, 77]. In addition to *qseA* and *qseBC*, transcription of *yjiE* is also regulated through quorum sensing as observed using gene array technology [42]. Through the array analysis initially reported by Sperandio et al, *yjiE* in the K-12 and EHEC genomes (GenBank accession numbers AE000403 and AE005552, respectively) encodes one of 19 putative regulators under QS control [42]. Transcription of *yjiE* is down-regulated 20–fold in the wild-type compared to the *luxS* mutant (Figure 17). This open reading frame encodes a putative regulator of the LysR family of transcriptional regulators. Due to the

fact that it encodes for a putative transcription factor and that its transcription is repressed via quorum sensing, we renamed *yjiE* as the quorum-sensing *E. coli* regulator D, or *qseD*.

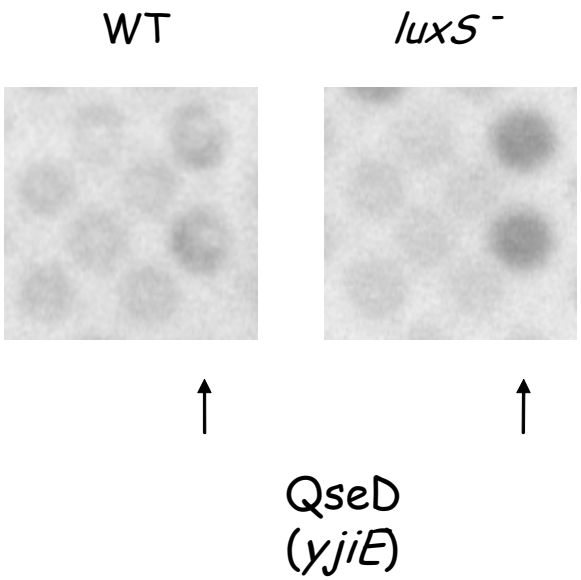


Figure 17. *E. coli array data showing the upregulation of yjiE in the luxS mutant.*

According to sequence alignments, *qseD* from EHEC contains a frameshift mutation that introduces a stop codon and deletes the N-terminal HTH DNA binding domain. Comparing the sequence of MG1655 K-12 strain and 86-24 (GenBank accession numbers AE000403 and AE005552, respectively) shows that the K-12 strain QseD contains the HTH domain (1-65 amino acids), while 86-24 has the introduced stop codon (Figure 18). Interestingly, all other bacterial species that harbor QseD (including K-12, EPEC, *Yersinia*, and *Salmonella* species) have the full-length QseD. The deletion of the HTH domain of this protein seems to be restricted to EHEC from serotype O157:H7, given that the QseD from three O157:H7 strains do not contain the HTH domain (strains EDL933, 86-24 and Sakai).

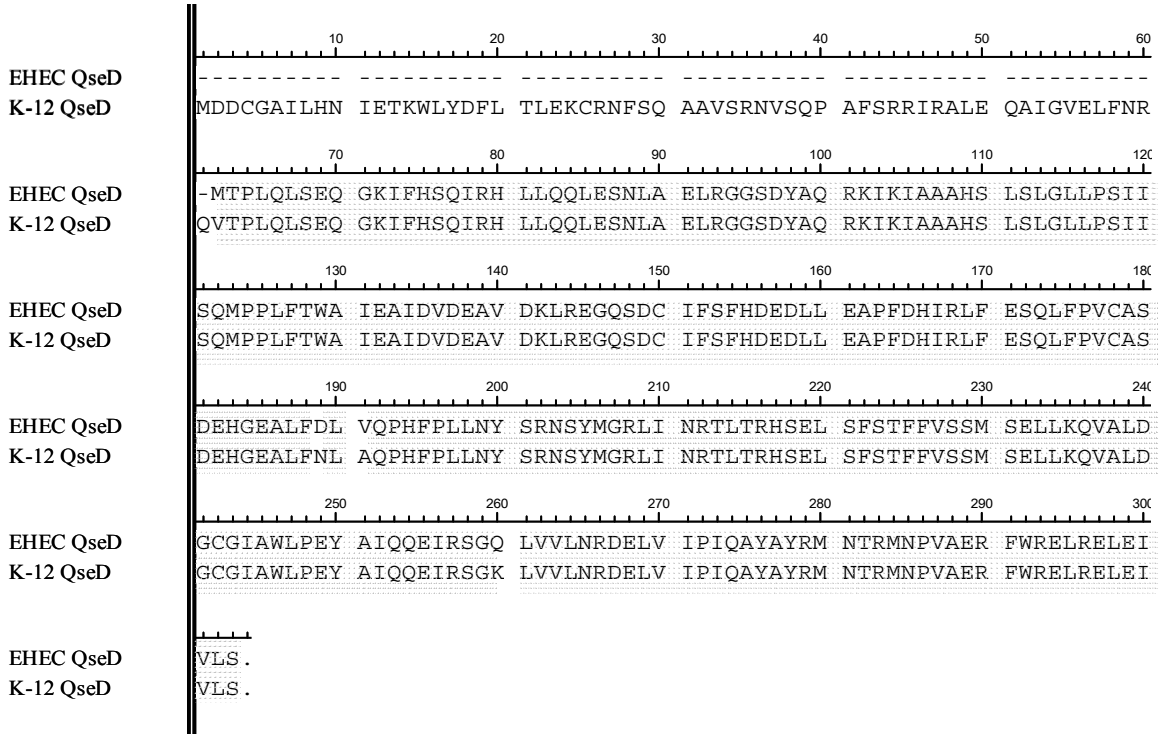


Figure 18. *Protein sequence alignments of EHEC QseD and K-12 QseD.*

C. Motility Assay

Quorum sensing in EHEC has been reported to activate the flagella operon via quorum sensing *E. coli* regulators B and C (QseBC) [76, 77]. To determine whether the putative regulator QseD plays a role in flagella regulation, motility assays were utilized to test the ability of the *qseD* mutant to swim compared to the wild-type EHEC. As observed in the motility assay, the *qseD* mutant shows a significant reduction in the diameter of the motility halo as compared to wild-type and complemented strains (Figure 19). In tryptone semi-solid agar, strains 86-24 (wild-type), JS5 (*qseD* mutant), and FS55 (*qseD* complemented) formed average halos of approximately 30 mm, 20 mm, and 30 mm, respectively, after 8 hours of incubation at 37^{o}C (Figure 19). The reduction has been shown to be statistically significant (Figure 19). The result from the motility assay suggests that *qseD* may have a role in regulation of motility in EHEC.

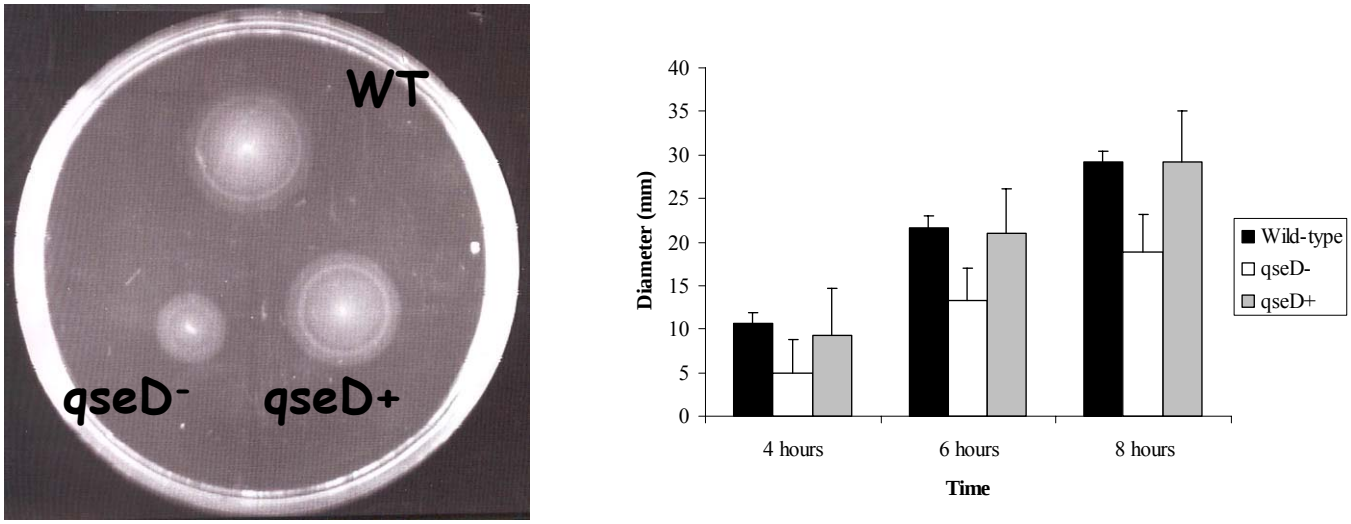


Figure 19. *Motility assay of qseD mutant compared to wild-type 86-24. The wild-type is located at the top of the triangle, qseD mutant at the bottom right side of triangle, and complemented strain at bottom left side of triangle. The graph shows the statistical significance of the reduced motility of the qseD mutant.*

E. Production of Flagellin

Given that the *qseD* mutant had reduced motility, we were interested in determining whether flagellin production was reduced in the *qseD* mutant compared to complemented and wild-type strains. We observed a marked decrease in the production of flagellin in the *qseD* mutant, which is restored upon complementation (Figure 20). This data, along with the motility assays, suggests that QseD may play role in activation of the expression of the flagella regulon in EHEC. Further analysis will be necessary to determine whether QseD acts transcriptionally on the flagellar genes.

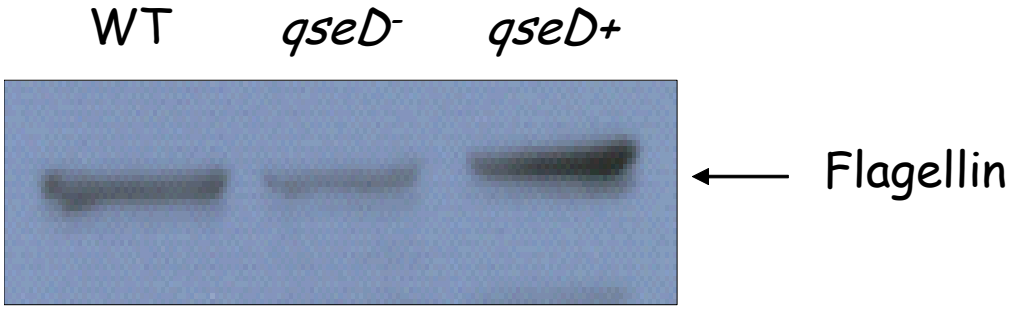


Figure 20. *Anti-H6 flagellin western. Whole cell lysates of wild-type, qseD mutant, and complemented strains were run on 12% SDS-PAGE.*

F. QseD Overexpression and Secretion Studies

Using *Taq* DNA polymerase, *qseD* was amplified using primers QseD 1F and QseD 1R and cloned into the *BamHI/SmaI* site of the expression vector pQE30. Upon induction with 1 mM IPTG, QseD production was determined using anti-histidine monoclonal antisera (Invitrogen) (Figure 21). Then we compared the transcription of several *LEE::lacZ* chromosomal transcriptional fusions (*LEE1, LEE2, LEE3, LEE5/tir,* and *LEE4*) in K-12 in the presence of pFS09 (*qseD* from EHEC in pQE30) and the vector alone. The transcription of the *LEE* operons was also tested comparing vector control pBADMycHis and pJS4, the plasmid containing K-12 QseD (Figure 22).

The QseD from EHEC (without a HTH DNA domain) was compared to a QseD from *E. coli* K-12 (with a HTH domain) in β-galactosidase activity assays determining the transcriptional regulation of the LEE genes. As observed in Figure 22A**,** the transcription of *LEE4* is repressed in the presence of EHEC QseD compared to vector control. This repression is not observed during overexpression of the K-12 QseD (Figure 22B). No other operons within the *LEE* PAI were affected transcriptionally in the overexpression assay. These data suggest that the QseD from EHEC (that lacks the HTH DNA binding motif) represses the transcription of the *LEE4* operon, which encodes for the secreted proteins EspA, B and D. This supports the TCA precipitation assay data showing that a *qseD* mutant has a marked increase in the secretion of EspA and EspB (Figure 23).

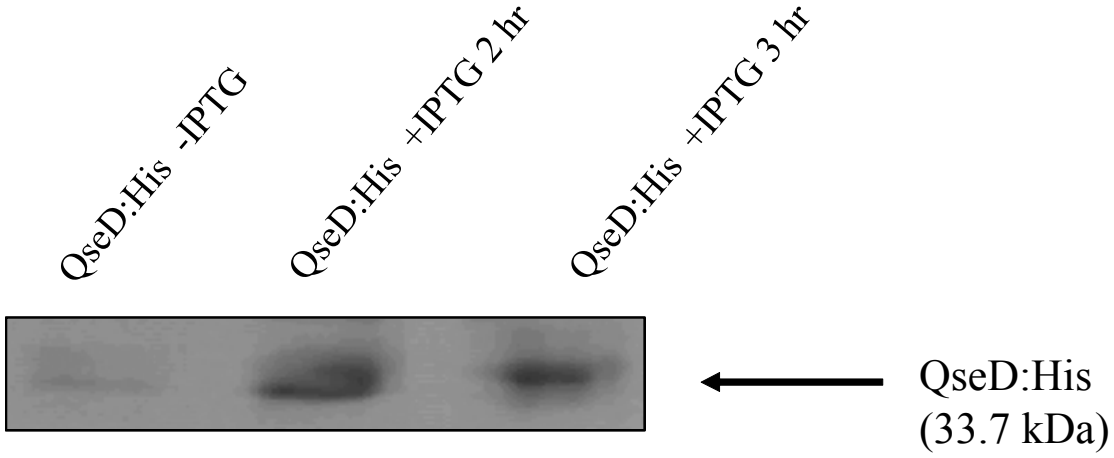


Figure 21. *Expression of EHEC His-QseD from pQE30 (pFS09). Whole cell lysates of pFS09 were un-induced (lane 1), induced with IPTG for 2 hours (lane 2), and induced with IPTG for 3 hours (lane 3) and run on a 12% SDS-PAGE. The predicted size of QseD without histidine tag is 27.7 kDa (33.7 kDa plus Histidine tag).*

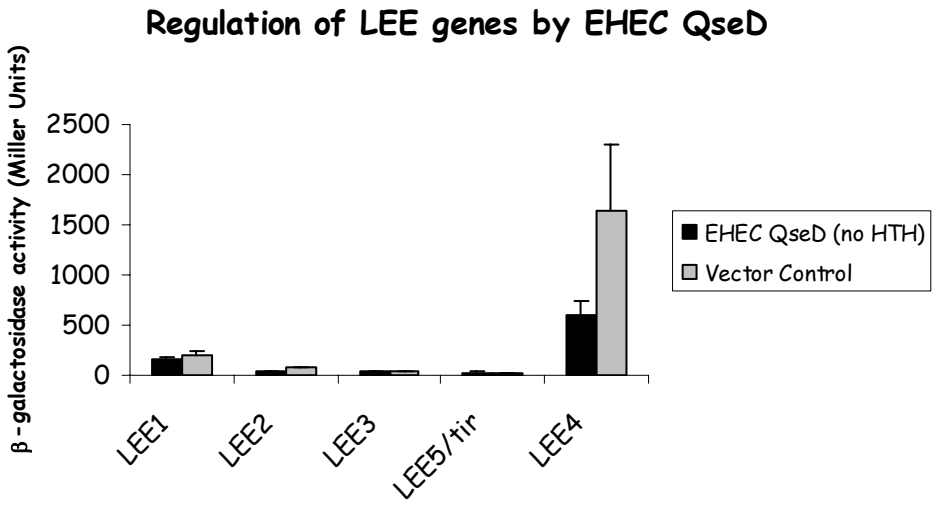


Figure 22A. *Overexpression analysis of the EHEC QseD with the LEE genes. QseD from EHEC lacks the HTH DNA binding motif that is found in other species. Error bars indicate standard deviations.*

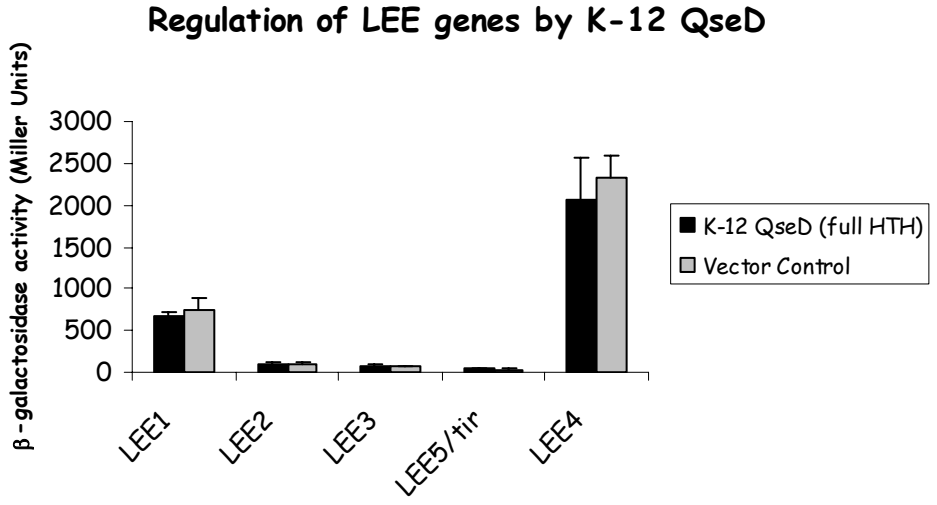


Figure 22B. *Overexpression analysis of K-12 QseD on LEE genes. Error bars indicate standard deviations.*

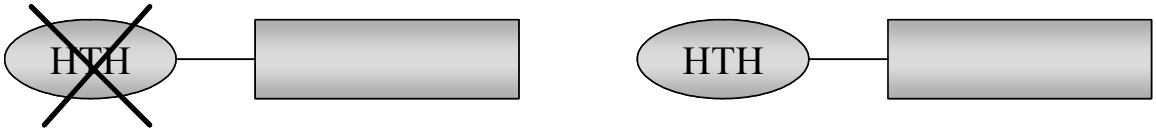


Figure 22C. *Model of QseD from EHEC (without the HTH DNA binding motif) and K-12 (with the HTH DNA binding motif).*

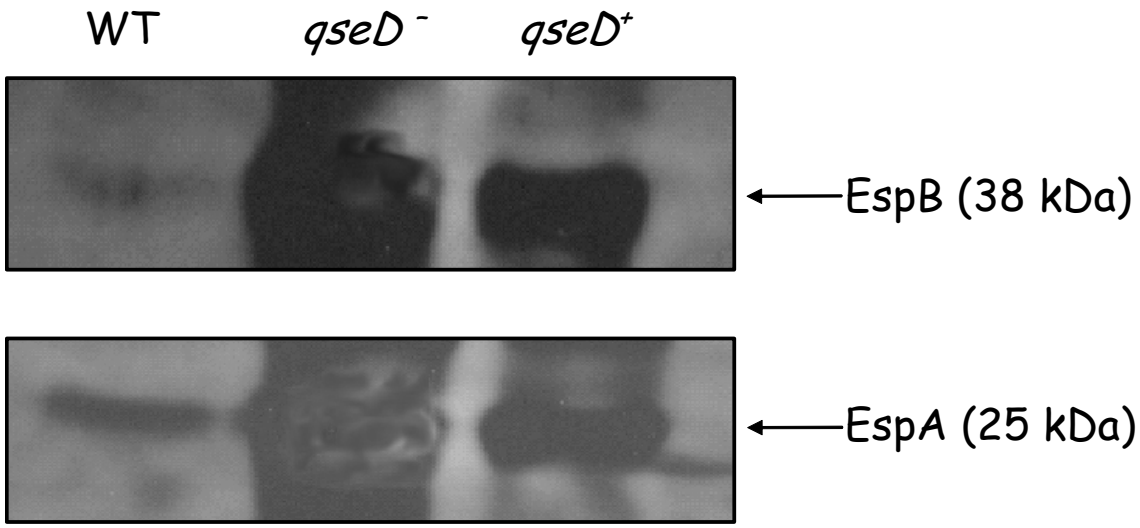


Figure 23. *TCA precipitation of secreted proteins. Supernatants of wild-type, qseD mutant, and complemented strains were run on 12% SDS-PAGE. Westerns were performed with anti-EspA and anti-EspB antibodies.*

F. Northern Analysis of LEE4

Upon the observation that a *qseD* mutation results in over-secretion of the *E. coli* secreted proteins EspA and EspB (Figure 24), we next wanted to determine if the regulation of these secreted proteins is at the level of transcription, using northern blot analysis. In the northern blot analysis, using a probe against *orf23* (*sepL*), which is encoded within *LEE4,* we observe that the wild-type transcript is 1.2 kb, however, the *qseD* mutant transcript sizes is at 5 kb, and the complemented strain has a transcript at 1.2 kb (Figure 24). Roe et al observed a distinct band of 1.2 kb and a faint transcript at 5 kb using a *sepL* probe in RNA extracted from different isolates of EHEC [87]. This data suggests that 86-24 typically produces a 1.2 kb transcript from *orf23*, but the *qseD* mutant produces an unprocessed transcript that is 5 kb (Figure 24). QseD may be involved in the processing of the *orf23* transcript. Further analysis needs to be performed to determine whether QseD is involved in the control and regulation of *LEE4.*

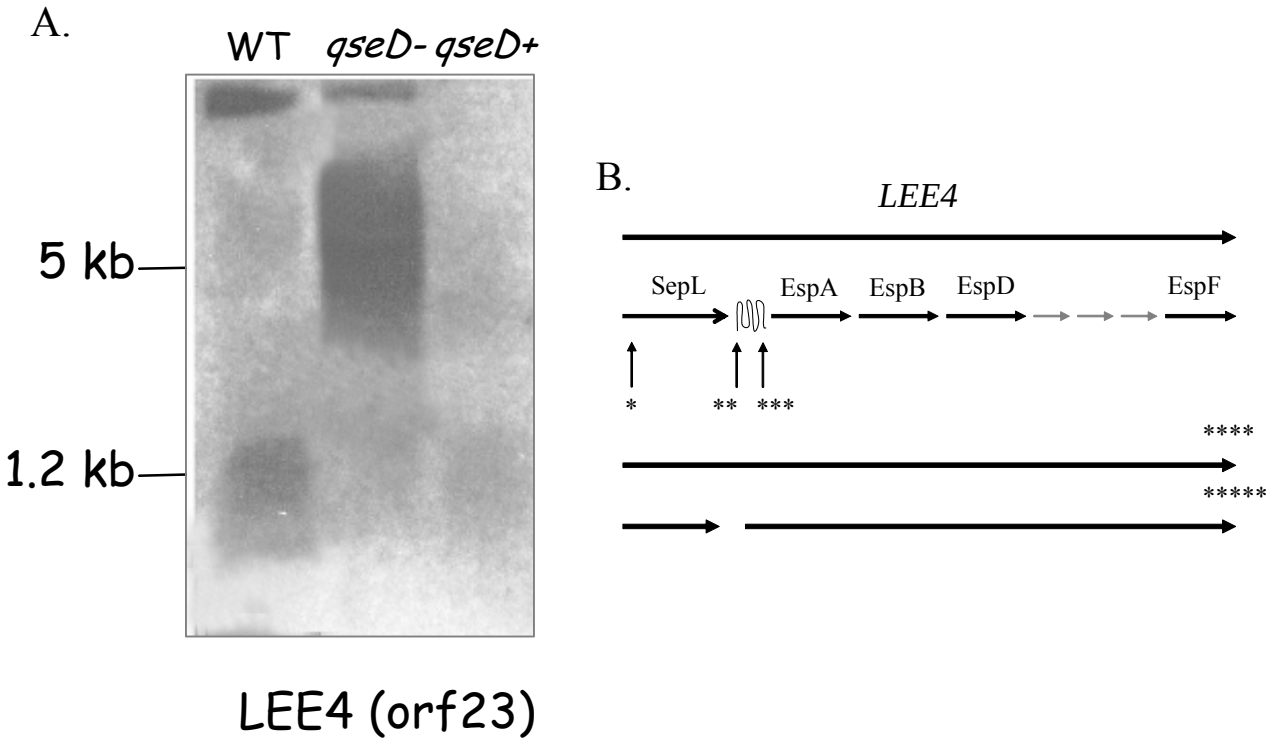


Figure 24. A. *Northern analysis of LEE4. Using a probe against orf23, 86-24 (wild-type), JS5 (qseD mutant) and FS55 (complemented) RNA, transcript size was determined.* ***B.*** *LEE4 operon structure. * LEE4 promoter [14], ** putative transcriptional terminator [14],*** LEE4 promoter [88] **** 5 kb full transcript [87], ***** processed transcript (1.2 kb and 4 kb) [87].*

G. Concluding Remarks

Quorum sensing *E. coli* regulator D, QseD, is involved in the complex regulation of virulence systems in EHEC, including motility and attaching and effacing lesions. We observe that the QseD protein from EHEC lacks the N-terminal HTH DNA binding motif characteristic of QseD proteins from other species. QseD plays a role in modulating the motility in EHEC. We observe a statistically significant decrease in the swimming ability of the *qseD* mutant in motility assays. Additionally, flagellin is produced significantly less in the *qseD* mutant. We also observe a QseD-dependent regulation on the LEE genes. In overexpression and secretion studies, QseD appears to play a repressive role on *LEE4*. Finally, *LEE4* northern analysis comparing the *qseD* mutant to wild-type and complemented strains suggests that QseD may be involved in processing the *LEE4* transcript in EHEC. QseD plays an intricate role in the modulation of both flagella and LEE genes in EHEC, although the exact mechanism is currently unknown.

V. DISCUSSION

Enterohemorrhagic *E. coli* O157:H7 (EHEC) is responsible for severe cases of hemolytic colitis and hemolytic uremic syndrome. Key virulence factors, including Shiga toxin, type III secretion, and flagella and motility aid in the ability of EHEC to cause severe disease in humans. Through quorum sensing, EHEC regulates these virulence factors in an intricate fashion. The results of theses studies describe in greater detail the transcriptional regulation of *LEE1/ler* by quorum sensing *E. coli* regulator A, QseA, and the role of quorum sensing *E. coli* regulator D, QseD, in the overall quorum sensing cascade in EHEC.

QseA was previously described as a transcriptional activator of the locus of enterocyte effacement through the activation of the *LEE1/ler* promoter [42]. Recently, QseA has also been shown to activate the transcription of *grlRA* within the LEE pathogenicity island [49]. It was observed that GrlRA positively activates the expression of *LEE2* and *LEE4* in EHEC independently of *ler* and modulates expression of *LEE1*, adding another level of complexity in the entire system [47, 49]. This proposal aimed to investigate the molecular mechanisms by which QseA activates transcription of *LEE1/ler*. Through nested promoter deletion analyses of *LEE1/ler* in both multi- and single-copy within EHEC and *E. coli* K-12 backgrounds, a region between -123 and +86 base pairs (numbering according to P2 transcriptional start site) was shown to be essential for the QseA-dependent transcriptional activation of the *ler* promoter (Figures 11-13).

QseA has been previously shown (using primer extension) to control transcription of *LEE1/ler* through the distal (P1) promoter. Our nested deletion analyses in EHEC and K-12 in multi- and single-copy confirmed these previous observations. Additionally, through EMSA experiments, we demonstrated that QseA directly interacts with the region between -173 to -42 base pairs. We also observe QseA-dependent transcriptional activation as close as -123 to +86 base pairs of the *LEE1/ler* promoter (Figures 11-13). The -123 nucleotide corresponds to -50 in relation to the -35 region of the P1 transcriptional start site. These data suggest that QseA, a member of the LysR family of transcriptional regulators, binds in close proximity to the P1 promoter, and may interact with the RNAP, in similar fashion to other LysR proteins. QseA was unable to bind in EMSA experiments in the region between -393 to -343 base pairs (Figure 15). This data suggests that another transcriptional regulator, such as Pch, EivF, EtrA, or GrlRA, may be necessary for a second level of QseA-dependent activation of the *LEE1/ler* promoter. QseA may be regulating the transcription of the *LEE1/ler* promoter -393 to -300 base pairs indirectly through the activation of another transcriptional regulator that is specific to EHEC.

QseD, a previously undescribed regulator in EHEC, is repressed by quorum sensing. We were interested in investigating the role QseD played in the overall quorum sensing cascade in EHEC. The data presented suggests that QseD is a novel factor involved in the regulation of both the type III secretion and flagella and motility. In motility assays, we observe that the *qseD* mutant is deficient in its ability to swim compared to wild-type EHEC (Figure 19). Additionally, we observe a slight decrease in the production of flagellin, consistent with the decreased motility observed (Figure 20). In overexpression

studies, QseD from EHEC was shown to repress the transcription of *LEE4*, which encodes for the Esp proteins which are involved in type III secretion (Figure 22A). In contrast, QseD from K-12 had no effect on *LEE4* transcription (Figure 22B). The QseD from EHEC appears to differ from that of K-12 by lacking the HTH DNA binding motif. We predict that QseD (lacking the HTH motif) cannot properly bind to DNA; therefore, QseD may interact with another member of the LysR family of proteins to modulate the expression of these virulence genes. In agreement with these transcriptional studies, the *qseD* mutant has increased secretion of EspA and EspB proteins (encoded within *LEE4*) (Figure 23). These data suggest that QseD is involved in the intricate regulation of the LEE and flagellar genes. Further analysis on the transcriptional or post-transcriptional events related to the regulation of these genes will be necessary to unravel the molecular mechanisms by which QseD influences gene expression.

Further studies on the protein-protein interactions of QseD with other members of the LysR family of proteins must be performed to determine whether this protein forms protein-protein interactions to facilitate DNA binding. Suspected proteins that may bind to QseD include QseA to regulate the expression of LEE genes, and LhrA, which is involved in the expression of flagellar genes [42, 50]. In addition to binding as homotetramers, LysR proteins often bind as heterotetramers. QseD may act by disrupting homotetramers of QseA and LhrA to modulate the expression of the LEE and flagella genes.

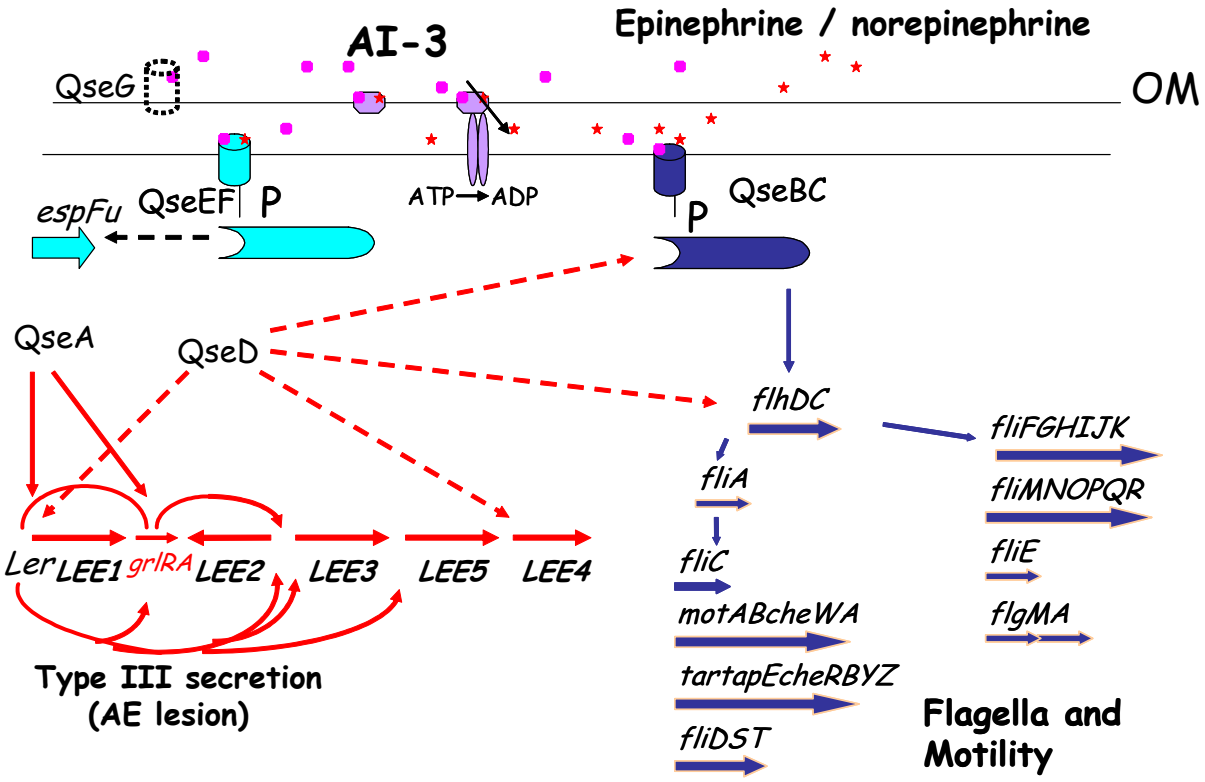


Figure 25. *Current working model of the quorum sensing cascade of Enterohemorrhagic E. coli O157:H7 (EHEC). EHEC responds to AI-3 produced by the host microflora and host epinephrine/norepinephrine. QseBC, a two-component system, activates flagella and motility. QseEF, a putative two-component system, activate attaching and effacing lesions, through the transcriptional activation of $espF_u$. QseA, a member of the LysR family of transcriptional regulators, activates the transcription of LEE1/ler and grlRA. QseD, a putative LysR family transcriptional regulator, represses LEE4 genes and activates motility.*

Studies thus far on the intricate regulation of quorum sensing in EHEC have shown that many factors are involved in the activation and repression of virulence genes. QseA activates the expression of *ler* by binding to a specific region of DNA in close proximity to the promoter P1, close to the binding site of RNAP. This activation by QseA leads to Ler activation of other genes in the LEE pathogenicity island, which are for AE lesions formation. Finally, the data presented concerning QseD, a previously undescribed regulator, suggests that this protein may be involved in the regulation of both the type III secretion and flagella and motility. The quorum sensing cascade in EHEC is complex, but further studies on these transcriptional factors and how they regulate expression of these virulence mechanisms will enable a better understanding of the overall model of quorum sensing signaling in enterohemorrhagic *E. coli* O157:H7 (Figure 25).

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VITA

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