THE FUNCTIONAL CHARACTERIZATION OF THE LYSR-TYPE TRANSCRIPTIONAL REGULATOR QSED AND THE SORC-TYPE TRANSCRIPTIONAL REGULATOR LSRR IN ENTEROHEMORRHAGIC *ESCHERICHIA COLI*

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DEDICATION

To my parents Ted and Alice, for the ceaseless love and support in every endeavor.

To everyone who has inspired me to grasp as much knowledge as possible, and to never give up trying to understand the world around me.

To all my friends who have come and gone, thanks for putting up with me.

In memory of my grandparents: Joe and Mary Habdas, and Clifford and Marge Fox.

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by

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Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 is a human pathogen responsible for numerous outbreaks of hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) throughout the world. EHEC is able to sense and respond to biotic cues from its environment, such as the human host produced catecholamines epinephrine and norepinephrine, through two two-component systems QseBC and QseEF, and abiotic environmental cues, such as phosphate and sulfate levels through QseEF [1-2]. Additionally, quorum sensing (QS) signaling cascades have evolved to sense microbial population density and diversity through the recognition of bacterially produced autoinducers (AI) AI-2, and 3 by LsrR, and QseBC respectively [1, 3]. Through the interpretation and integration of these multiple regulatory signaling networks that often involve intracellular regulatory proteins, such as the lysine regulator (LysR) type

transcriptional (LTTR) family member QseA, EHEC is able to coordinate the expression of its multiple virulence factors [4]. These factors include the production of flagella that confer bacterial motility, the locus of enterocyte effacement (LEE) encoded type three secretion system (TTSS) that facilitates formation of attaching and effacing (AE) lesions on gut epithelium, and is positively regulated by QseA, and Shiga toxin (Stx), which causes cellular damage and HUS.

Here, we show that *yjiE*, renamed Quorum Sensing *E. coli* Regulator D (QseD), which was predicted to encode a transcriptional regulator of the LTTR family, functions in a QS-dependent manner to regulate gene expression in both pathogenic and commensal strains of *E. coli*. LTTRs, the largest known family of prokaryotic DNA binding proteins, contain two functional domains, an N-terminal helix-turn-helix (HTH) and a C-terminal co-factor binding domain which allows for oligomerization [5]. We have demonstrated that QseD indirectly represses transcription of the LEE in EHEC and represses the flagella regulon expression in K-12 *E. coli*. Additionally QseD regulates the expression of *iraD*, which has recently been demonstrated to prevent degradation of RpoS by RssB sequestration, leading to an altered bacterial stress-response [6-7]. However, what is most intriguing is that while *qseD* is prevalent in many enterobacteria it seemingly exists almost exclusively in EHEC O157:H7 isolates as a helix-turn-helix truncated "short" isoform (sQseD). Due to the inability of the sQseD to bind to DNA and the predicted *in silico* ability of LTTR family members to form hetero-dimers in order to bind DNA, a targeted yeast-two-hybrid (Y2H) approach was used to exclude the known LTTR regulators of LEE transcription QseA and LrhA, as QseD interaction partners. Taken together, these results show that QseD regulates alternate targets in EHEC and K-

12 *E. coli*, and that EHEC O157:H7 has evolved to encode a truncated form of this protein.

We also studied the role of the LsrR regulon in EHEC pathogenesis and environmental persistence through biofilm formation. LsrR, a negative regulator of *lsrK* and of the *lsrACDBFG* operon, has been shown to regulate the uptake and removal of AI-2, the cell-to-cell signaling product of LuxS, from the environment through regulation of the LsrACDB AI-2 uptake pump [8-9]. LsrK, an AI-2 kinase, has been shown to alleviate *lsrACDBFG* operon repression by generating the inhibitory ligand of LsrR DNA binding, phospho-AI-2 [10]. In *E. coli*, LsrR has been implicated along with LsrK in AI-2 dependent regulation of biofilm architecture and small-RNA (sRNA) expression [11]. However, while it has been suggested that AI-2 signaling can affect pathogenesis in EHEC, the direct effects of LsrR and LsrK have never been examined [12].

Here we show that in EHEC both LsrR and LsrK regulate virulence expression, and that this regulation is altered in the absence of a functioning LuxS enzyme. In EHEC, while *lsrR* and *lsrK* both positively regulate motility in the presence of *luxS*, in its absence they both repress motility in a temperature dependent manner. Additionally, in the presence of *luxS*, *lsrR* increases biofilm formation. In microarray studies, LsrR was also shown to down-regulate the LEE, and differentially regulate non-LEE effectors (Nle's). Taken together, these results show that both LsrR and LsrK have regulatory roles in the pathogenesis of EHEC and that their effects are altered by the absence of *luxS*.

These findings have given us a more complete and greater understanding of the genetic regulatory networks and their signaling and integration in EHEC.

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PRIOR PUBLICATIONS

Habdas BJ, Smart J, Kaper J, Sperandio V. 2010. The LysR-type transcriptional regulator QseD alters type three secretion in enterohemorrhagic *Escherichia coli* and motility in K-12 *Escherichia coli*. *Cellular Microbiology* (Manuscript Submitted)

Habdas BJ, Sperandio V. 2010. The differential regulation of biofilm production and type three secretion in enterohemorrhagic *E. coli* by LsrR and LsrK. *J.Bact.* (Manuscript in Preparation)

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LIST OF ABBREVIATIONS

86-24	Enterohemorrhagic *E. coli* wild-type strain 86-24
AE	Attaching and effacing
AI	Autoinducer
bp	Base pair
BSA	Bovine serum albumin
c-di-GMP	3, 5- cyclic diguanosine monophosphate
CF	Cystic fibrosis
CFU	Colony forming unit
DEAC	Diffusely adherent *E. coli*
DMEM	Dulbeco's modified Eagle's medium
DNA	Deoxyribonucleic acid
dNTP	dideoxy-nucleotide triphosphate
DPD	4,5-dihydrody-2,3-pentanedione
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EAEC	Enteroaggregative *E. coli*
EDTA	Ethylenediamine-tetraacetic acid
EHEC	Enterohemorrhagic *E. coli*
EPEC	Enteropathogenic *E. coli*
EIEC	Enteroinvasive *E. coli*
ELISA	Enzyme-linked immunosorbent assay
ETEC	Enterotoxigenic *E. coli*

GI	Gastrointestinal
GrlA	Global regulator of LEE-activator
GrlR	Global regulator of LEE-repressor
HC	Hemorrhagic colitis
HUS	Hemolytic uremic syndrome
IPTG	β-D-thiogalactopyranoside
IraD	Inhibitor of RssB activity in response to DNA damage
IRTKS	Insulin receptor tyrosine kinase substrate
kDa	kilo-Dalton
LB	Luria-Bertani broth
LEE	Locus of enterocyte effacement
Ler	LEE encoded regulator
LPS	Lipopolysaccharide
LTTR	LysR type transcriptional regulator
LysR	Lysine Regulator
Map	Mitochondrion associated protein
Nle's	Non-LEE encoded effectors
ORF	Open reading frames
PAI	Pathogenicity Island
O.D.	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction

Qse	Quorum sensing *E. coli* regulator
QseA	Quorum sensing *E. coli* regulator A
QseBC	Quorum sensing *E. coli* regulator B and C
QseD	Quorum sensing *E. coli* regulator D
QseEF	Quorum sensing *E. coli* regulator E and F
RH	5-ribosyl-homocysteine
RNA	Ribonucleic acid
RpoA	RNA polymerase subunit A
RssB	Regulator of Sigma S B
R-THMF	(2R,2S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran
SAH	5-adenosyl-homocysteine
SAM	S-adenosyl-L-methionine
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SorC	sorbitol operon regulator
STTR	SorC type transcriptional regulator
Stx	Shiga toxin
Tir	Translocated intimin receptor
TTP	Thrombotic thrombocytopenic purpura
TTSS	Type three secretion system
Y2H	Yeast-two-Hybrid
WT	Wild type

CHAPTER ONE

LITERATURE REVIEW

TAXONOMY

Escherichia coli originally known as *Bacterium coli* commune, was identified in 1885 by the German pediatrician and bacteriologist, Theodor Escherich [13]. *E. coli* is classified as a member of the Enterobacteriaceae family of gamma-proteobacteria which includes known pathogens such as *Salmonella*, *Shigella*, and *Yersinia*. *E. coli* is a Gram-negative facultative anaerobic bacillus which colonizes the lower gastrointestinal (GI) tract of warm blood animals and humans. Although most strains of *E. coli* are nonpathogenic, they can cause disease in immunocompromised individuals. Enterohemorrhagic *Escherichia coli* or EHEC was first described in 1983 by Ripley *et al*. as a rare non-invasive or toxigenic *E. coli* serotype, O157:H7, which was transmitted by undercooked meat and caused severe hemorrhagic colitis [14]. The "O" in O157:H7 refers to a specific glycan polymer or O antigen of the lipopolysaccharide (LPS) on the bacterial cell surface, while the "H" refers to the flagellar antigen.

HISTORICAL PERSPECTIVE OF ENTEROHEMORRHAGIC *E. COLI*

E. coli is one of the most abundant commensal facultative anaerobes in the human lower GI tract with individuals generally being colonized within a few hours to days of birth. Pathogenic strains of *E. coli* are commensal derivatives that have adapted to stably express additional genetic content that has been acquired through horizontal gene transfer such as phage, plasmid or pathogenicity island (PAI) acquisition. There are six well described categories among human *E. coli* pathogens: enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), and diffusely adherent *E. coli* (DEAC) [15-16]. This research dissertation focuses on EHEC, an emerging pathogen of worldwide concern.

EHEC is a human pathogen that causes major outbreaks of hemorrhagic colitis and hemolytic uremic syndrome (HUS) throughout the world. EHEC causes approximately 73,000 illnesses, 2,000 hospitalizations, and 69 deaths in the U.S. annually [17]. There are two major contributing factors to EHECs extensive mortality and morbidity. First, it possesses a very low infectious dose of approximately 50 colony forming units (CFU) [18]. Secondly, it expresses a chromosomally encoded toxin that can lead to hemolytic anemia, thrombocytopenia, acute renal failure, and death in susceptible individuals, and whose expression is exacerbated by conventional antibiotic treatment [19-23].

EHEC was first recognized as a human pathogen following its first outbreak in 1982, where 47 people came down with a feverless bloody diarrhea after eating undercooked hamburger meat at McDonald's restaurants in Michigan and Oregon. Stool samples confirmed the presence of a concurrently characterized cytotoxigenic *E. coli*

O157:H7 isolates that were associated with hemolytic uremic syndrome (HUS) development [19]. In 1993 EHEC became nationally recognized during the now infamous Jack in the Box outbreak, in which over 73 restaurants were involved, over 700 people were sickened, and four children died. Following these original outbreaks and the labeling of EHEC as the "Hamburger *E. coli*", additional outbreaks have occurred and have been attributed to a diverse array of sources including but not limited to: processed salami [24], wild game meat [25], produce [26-28], unpasteurized milk [29] and juice [30], cheese products [31], petting zoos [32], and fresh water sources [33-36].

CLINICAL PRESENTATION, DIAGNOSIS, AND TREATMENT

EHEC infections generally have a 3-8 day incubation period, following which patients initially develop severe abdominal cramping and a non-bloody watery diarrhea. Within 1-5 days the diarrhea becomes bloody, with 30 % and 50 % of patients developing a mild fever and/or nausea and vomiting respectively [37]. The bloody diarrhea, which can range from streaks of blood in the stool to a complete bloody discharge (tablespoons to cups), generally lasts 2-4 days, following which the patient recovers [38-39]. While EHEC infections generally last only about a week, 2-14 days following the presentation of initial symptoms, HUS develops in 10 % of infected patients (Figure 1.1) [40-41].

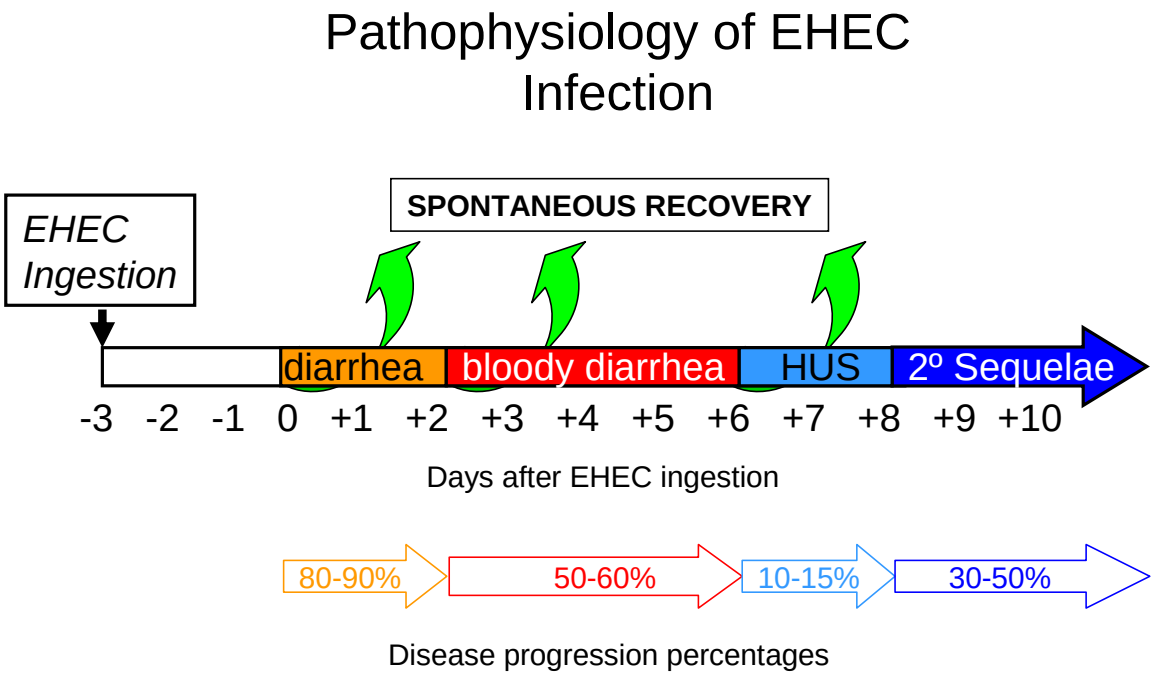


Figure 1.1. Pathophysiology of an EHEC infection. EHEC infections generally have a 3-8 day incubation period, following which a watery diarrhea develops. This diarrhea becomes bloody within 1-5 days before resolving within a week. Approximately 10-15% of infected individuals develop HUS, with 30-50% of those individuals experiencing additional secondary sequelae such as neurological complications. [37]

HUS, which is characterized by microangiopathic hemolytic anemia, thrombocytopenia and thrombotic thrombocytopenic purpura (TTP), renal failure, and neurologic complications, including seizures, coma and hemiparesis, occurs primarily in children and the elderly [42] [43] [44]. While the general mortality rate for an EHEC infection is estimated to be between 5-10 %, with death most often resulting from HUS complications, up to 5 % of survivors can experience end-stage renal failure and permanent neurologic injury [41, 45].

Clinical diagnosis of EHEC infection is most often done by streaking patient samples on sorbitol-MacConkey agar. EHEC cannot ferment sorbitol, and so will remain white on sorbitol-MacConkey agar, while sorbitol fermenting coliforms, such as other

E.coli species will turn red [46]. Secondary verification can be provided by commercially available Shiga toxin and O and H antigen enzyme-linked immunosorbent assays (ELISAs), or more accurately by polymerase chain reaction (PCR) based methods [47-49].

The use of antibiotics to treat EHEC infections is controversial and generally discouraged, as they can induce the expression and release of Shiga toxin (Stx) contributing to HUS development [50-52]. Therefore treatment during the initial stages of infection is generally supportive, with the goals being threefold: (1) to reduce the severity and duration of symptoms, (2) to prevent complications such as HUS, and (3) to reduce the risk of further transmission [53-54]. If patients do progress to HUS, then treatment shifts to the management of renal failure, which includes but is not limited to: fluid and electrolyte replacement and balancing, treatment of anemia, and dialysis [55].

PATHOLOGY

The overall histopathology in patients due to EHEC infections includes mucosal abnormalities ranging from the rectum to the cecum, with erythema, hemorrhages, and edema being the most prominent features [39]. In severe cases, patients may present additional inflammatory gastrointestinal complications including but not limited to: gross fecal peritonitis, colonic perforation and necrosis, toxic megacolon, and rectal prolapse [56]. Due to the predilection for the development of HUS and additionally gastrointestinal complications in children, EHEC has the ability to cause a considerable impact on the utilization of pediatric surgical services [57].

The classical histopathology characteristic of an EHEC infection is its ability to cause attaching and effacing (AE) lesions on intestinal epithelial cells. These lesions are characterized by the effacement of the brush-border microvilli and rearrangement of the sub cellular cytoskeleton forming an actin pedestal that cups the bacterium and forms an intimate adherence between both cells (Figure 1.2) [15].

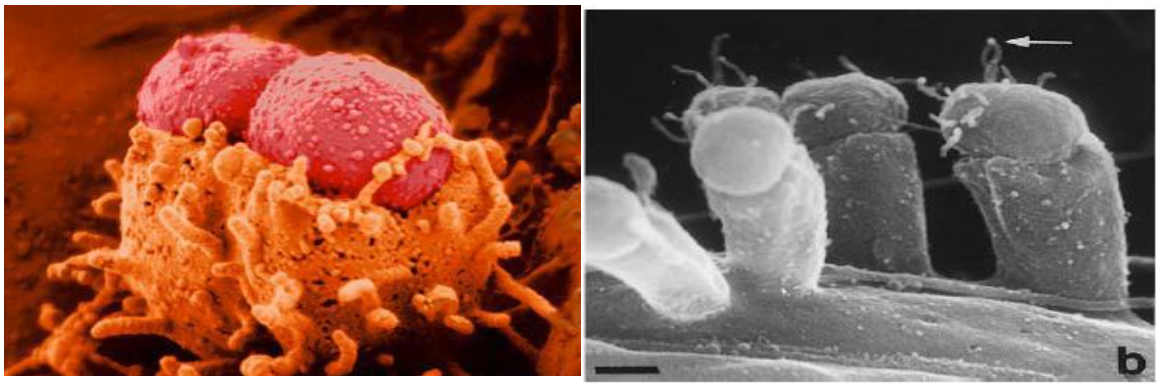


Figure 1.2. A characteristic EHEC AE lesion/pedestal. The electron micrograph depictions of EHEC (left) and EPEC (right) AE lesion/pedistals demonstrate the rearrangement of the actin cytoskeleton around and below the bacterial cells. [15, 58]

VIRULENCE FACTORS OF ENTEROHEMORRHAGIC *E. COLI*

Once EHEC enters the colonic lumen through ingestion of contaminated food or water it coordinately regulates its virulence expression. EHEC produces multiple virulence factors including a phage encoded Stx, and a type III secretion system (TTSS) encoded by the locus of enterocyte effacement (LEE) PAI. Flagellation allows the bacteria to swim into close proximity to the colonic epithelium, where LEE PAI TTSS expression allows for intimate AE lesion formation. Eventually through host or pathogen mediated cell stress, STX is produced and released leading to possible host morbidity and mortality (Figure 1.3) [59].

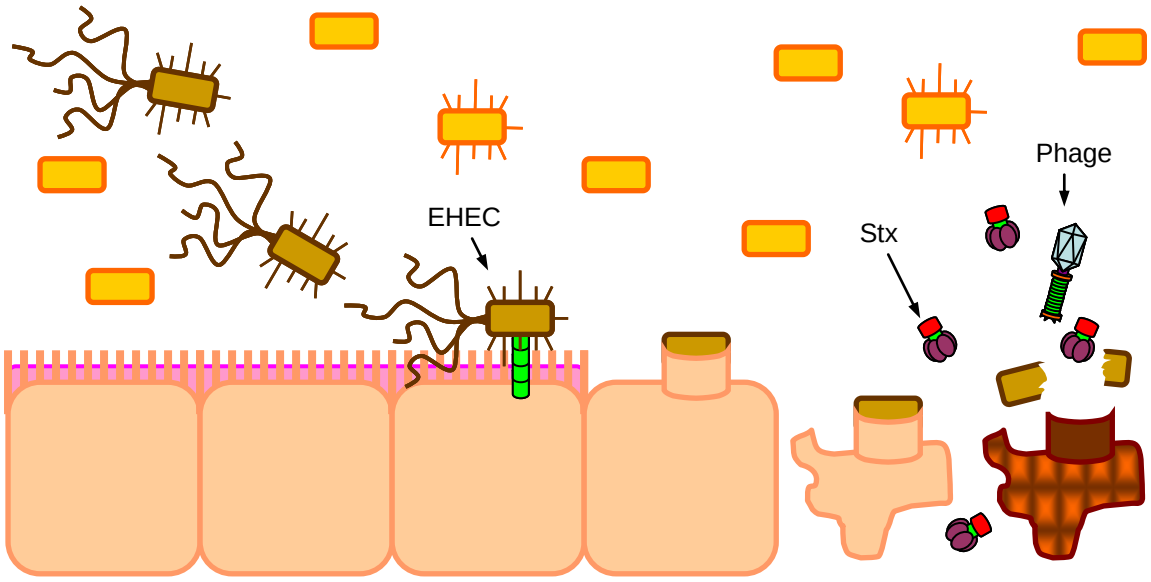


Figure 1.3. Model of EHEC Pathogenesis. The steps involved in EHEC pathogenesis are as follows: (1) flagellation allows for swimming into close proximity to the epithelia barrier, (2) the LEE TTSS allows for the formation of AE lesions, and (3) lysis of the bacteria release STX into the blood stream leading to host morbidity and mortality. [59]

The Locus of Enterocyte Effacement

EHEC belongs to a larger group of enteric pathogens that includes EPEC, a rabbit EPEC, and *Citrobacter rodentium*, all of which are able to cause AE lesions on intestinal epithelial cells [60]. The genes necessary for the formation of these characteristic AE lesions are chromosomally encoded within the 35-Kb LEE PAI [61]. The LEE encodes for structural proteins that form a TTSS, several of the secreted effectors, which are translocated through the TTSS into host cells, and several internally encoded regulators such as Ler, GrlA (global regulator of LEE-activator), and GrlR (global regulator of LEE-repressor) [62-65]. The LEE is composed of 41 genes, most of which are arranged into five major operons termed *LEE1*, *LEE2*, *LEE3*, *LEE4*, and *LEE5*, which are collectively regulated by the LEE encoded regulator (Ler) (Figure 1.4) [66].

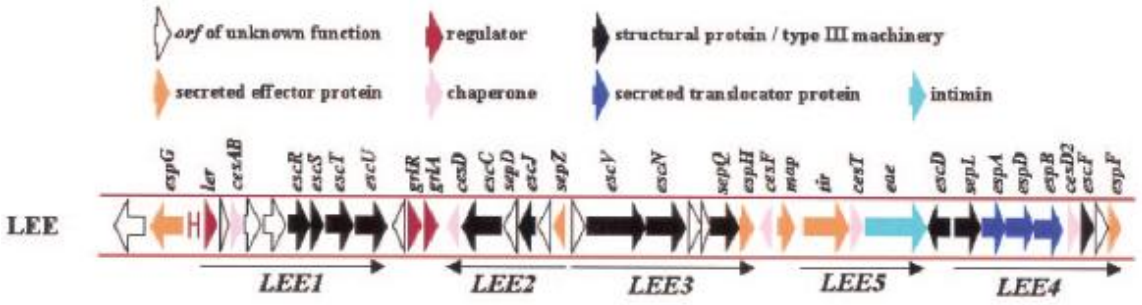


Figure 1.4. The EHEC Locus of Enterocyte Effacement (LEE). The EHEC LEE contains five operons *LEE1*, *LEE2*, *LEE3*, *LEE4*, and *LEE5* which collectively encode for Ler, the structural components of the translocation apparatus, Tir and Intimin, and the EspA filament and EspBD pore complex. [67]

Regulation of the LEE is highly complex and involves multiple regulatory proteins and pathways [4, 63, 66, 68-85]. Transcription of the *ler* gene, encoding the master regulator of the LEE [66], is directly activated by the LysR type transcriptional regulator (LTTR) QseA [4, 86], and negatively by the RNA regulatory chaperone Hfq [84, 87]. Other post-transcriptional regulatory factors such as ClpXP have been shown to increase transcription of *LEE3* by inhibiting its repression by GrlR and by increasing the degradation of the EHEC global regulator stationary-phase sigma factor RpoS [74, 78, 88]. RpoS is itself under a high level of post-transcriptional control by Hfq [89], translational control by Hfq and the LTTR LrhA [90], and post-translational control by RssB [91]. RssB, whose activity is modulated by LrhA, has been shown to sequester RpoS and targets it for ClpXP degradation [92-93]. Finally, IraD, an RssB anti-adaptor protein, is induced in response to DNA damage and prevents RssB mediated ClpXP degradation of RpoS, thereby altering cellular responses to stress [6-7, 94].

The type three secretion system is used by multiple pathogens to translocate bacterial effectors into the host cells that alter cellular functions in order to facilitate pathogenesis and evade immune clearance [95]. While *LEE1* encodes for Ler, *LEE2*, *LEE3*, and *LEE4* encode for structural components of the basal TTSS translocation apparatus, and *LEE5* encodes for Tir and Intimin [65]. The assembly of the TTSS is a multistage sequential process that requires the Sec secretion machinery. The TTSS can generally be broken into six functional parts: the bacterial inner membrane complex composed of EscRSTUV, the bacterial outer membrane pore composed of EscC, the periplasmic bridge EscJ, and the outer membrane needle EscF, the EspA filament, and the host pore complex EspBD (Figure 1.5) [96-99].

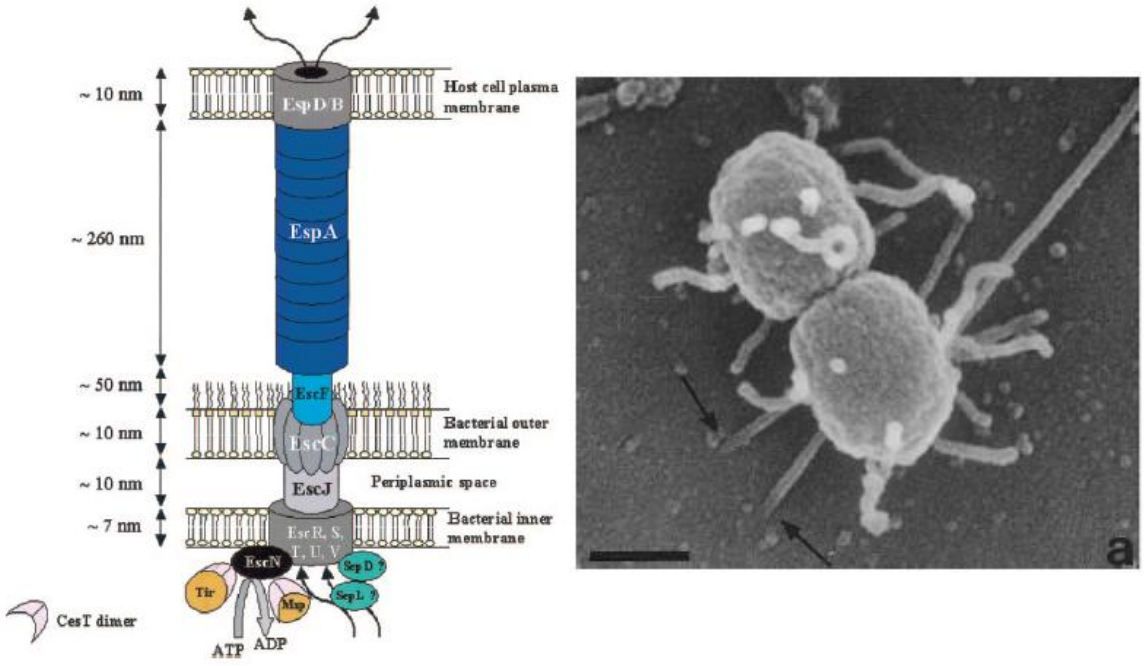


Figure 1.5. The EHEC Type three secretion system (TTSS). The assembled LEE encoded TTSS needle allow for ATP dependent translocation of encoded protein effectors into eukaryotic host cells (left: the structural componets, right: an electron micrograph depiction of the needle in contact with eukaryotic cells). [58, 67]

Additionally there are several proteins that while encoded by the LEE are not found in the TTSS structure itself but instead serve more functional roles. These proteins can generally be broken into two categories: accessory proteins such as SepD, SepL and EscN, and chaperones such as CesF, CesT, CesD, CesD2, and CesAB. SepD and SepL are accessory proteins of unknown function, which interact with each other to regulate protein secretion through the TTSS [63, 100-101]. EscN is an ATPase involved in protein transport through the TTSS [102]. CesF and CesT are chaperones involved in EspF, and Tir and Map translocation, respectively [103-106]. CesAB, CesD, and CesD2 are chaperones involved in EspA, EspB, and EspD translocation and assembly [107-109].

Intimin and translocated intimin receptor (Tir), which are the two proteins that are integral components of AE lesion formation, are encoded together within *LEE5*. Intimin, a 94 kDa protein encoded by the gene *eae*, is a an EHEC outer membrane protein that has been demonstrated to be necessay for colonization, and pathogenesis [110-113]. Tir, once thought to be a mammalian host protein, is translocated through the TTSS into the host cell [114]. Once there, it embeds itself in the host membrane, where its extracellular domain serves as a docking point for its cognate bacterial receptor, intimin, thus allowing for intimate attachment and formation of the characteristic AE lesions on eukaryotic cells [62]. Interestingly, while both EHEC and EPEC produce, secrete, and utilize Tir in AE lesion formation, both isoforms of the protein are not functionally equivalent [115-116]. In EPEC, Tir is the only protein required for pedestal formation, which when phosphorylated on Tyr474 recruits Nck to promote actin polymerization [116-118]. Alternatively, in EHEC Tir is not phosphorylated, and instead through interactions with the mammalian proteins IRTKS and IRSp53, recruits the type three secreted effector

EspFu [119-121]. EspFu, which is required for EHEC pedestal formation, recruits N-WASP, which in turn activates Arp2/3 and stimulates actin nucleation [122]. Additionally, the mammalian protein cortactin, which is recruited to sites of Tir and EspFu interactions, has been demonstrated to have a phosphorylation stated dependent role in regulating actin polymerization [123-124].

There are additional LEE encoded translocated effectors that have been demonstrated to be required for EPEC and EHEC pathogenicity [125]. They include: Map, EspF, EspG, EspH, and EspZ. Map (mitochondrion associated protein) has three known functions: (1) it inhibits mitochondrion function [126-127], (2) it disrupts intestinal epithelial tight junctions [128], and (3) it affects actin assembly and arrangement [129]. EspF also has three known functions: (1) it localizes to mitochondria and is possibly involved in apoptosis induction [130], (2) it disrupts intestinal tight junctions [131], and (3) it is thought to be involved in intestinal ion exchange and stimulation of diarrhea [132]. While EspG and EspH are thought to modulate the cellular actin cytoskeleton [133-134], the function of EspZ is not known [135].

Other EHEC Effectors

There are several additional non-LEE (Nle) TTSS secreted effectors involved in EHEC pathogenesis. While there are at least 39 Nles encoded within the EHEC genome, only a few of these including: EspFu, EspJ, EspL2, and NleA have been characterized to any extent [136]. EspFu, which serves as a bacterial adaptor protein linking Tir to N-WASP, is involved in actin pedestal formation [122]. EspJ and EspL2 have been demonstrated to disrupt receptor mediated phagocytosis [137], and alter the actin

cytoskeletal arrangement repectively [138]. NleA was demonstrated to disrupt protein vesicle transport and ER secretion [139].

pO157

EHEC O157:H7, the serotype that is by far the most prevalent and virulent [28, 140], carries a characteristic 92 Kb F-like plasmid (pO157) that contains 100 putative open reading frames (ORF's), 19 of which have been characterized as putative virulence determinants [141]. This plasmid has been demonstrated to modulate multiple characteristic of EHEC pathogenicity including but not limited to: modulation of epithelial cell adherence [142], increased bovine colonization and persistence [143-144], increased development of hemorrhagic colitis [145], and altered biofilm formation and surface adherence [146]. Additionally, pO157 encodes a type II secretion system which has been shown to increase intestinal colonization in animal models [147]. The putative virulence factors encoded on this plasmid include but are not limited to: *ehxA*, *etpCDEFGHIJKLMNO*, *espP*, *katP*, *toxB*, *ecf*, and *stcE* [148]. Of these, StcE is the most characterized and has been demonstrated to function as a metalloprotease that cleaves mucin-type glycoproteins [149] to alter neutrophil mediated inflammation and cellular response [149]. The pO157 plasmid also contains the EHEC enterohemolysins [150].

Shiga toxin

EHEC contains a phage-encoded Stx, which is the causative agent of hemorrhagic colitis, cellular necrosis, and HUS [151]. Stx production is closely associated with severity of disease [152]. The Stx family is composed of two major subgroups, Stx1 and

Stx2, which share 57% and 60% similarity in their A and B subunits, respectively [153-154]. The EHEC strain 86-24 (used throughout this dissertation) does not contain Stx1, which is antigenically identical to the Stx from *Shigella dysenteriae* type 1 [155], but does contain the antigenically unique Stx2 [156]. Although Stx1 has a 10-fold higher eukaryotic cellular affinity [157], Stx2, which has been demonstrated in tissue culture to be 1000 times more cytotoxic [158], is 7 times more likely than Stx1 to cause HUS in EHEC infected patients [159].

Stx is a classic AB_5 cytotoxin (Figure 1.6). It contains five ~7.7 kDa non-covalently bound B subunit polypeptides that bind to the eukaryotic glycolipid receptors globotriaosylceramide (Gb3Cer) and globotetraosylceramide (Gb4Cer) and mediate clathrin-dependent endocytosis [160-161]. Gb3Cer and Gb4Cer are enriched on kidney cellular membranes and vascular endothelial cells [162-163].

Once delivered to cells and endocytosed, Stx is shuttled through the golgi by retrograde transport to the ER where the ~32 kDa A subunit is cleaved by the membrane anchored protease furin into the catalytically active ~27.5kDa A1 and ~4.5 kDa A2 fragments [164-167]. The catalytically active A subunit then exerts its rRNA N-glycosidase acitivty to remove a specific adenine residue from the cellular 28S rRNA resulting in protein synthesis inhibition and cell death [168-170]. The potency of Stx is so high, that the release of one cleaved catalytically active A1 subunit into the cytosol of a cell will lead to death [171].

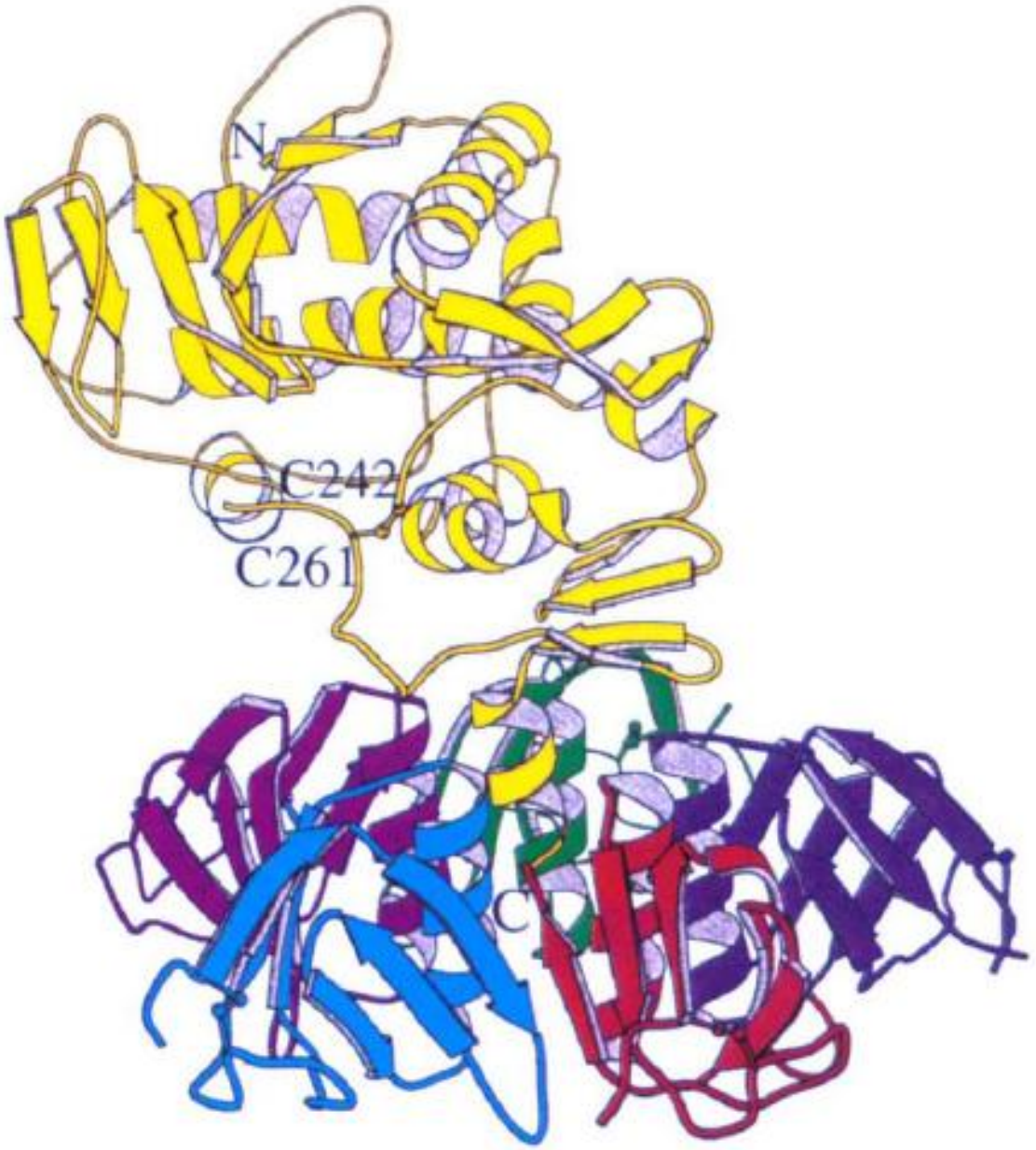


Figure 1.6. The AB_5 cytoxin, Stx from *Shigella dysenteriae*. Cystal structure of $StxAB_5$ (Colored in yellow is the catalytically active A subunit, with the B pentamers attached). [172]

The *stx* gene in located within a λ prophage in the EHEC genome, which is normally maintained in a quiescent state by the prophage encoded repressor, cI [173-175]. During an SOS response DNA damage generates ssDNA, which increases the activity of RecA. Activated RecA induces the autocleavage of LexA, a cellular DNA repair repressor, leading to the de-repression of cellular DNA repair machinery expression [176-177]. RecA also is able to cleave the prophage repressor cI, which leads to induction of the phage lytic cycle [178] and expression of *stx* [179]. Antibiotics are generally discouraged for the treatment of EHEC infections as they are known to induce a bacterial SOS response and therefore may increase the likelihood of Stx expression and HUS development [180].

THE FLAGELLAR REGULON

The bacterial flagellum is a proteinaceous filament, structurally related to the TTSS, which allows for ATP-dependent motility [181-182]. Flagella regulation and expression is broken into three hierarchical classes which includes over 50 genes and 15 operons [183-184]. In *E. coli*, the first class (the early genes) is composed of *flhD* and *flhC*, which when expressed, form a transcriptional master regulator complex that binds to σ^{70}-dependent promoters of the middle and late flagella genes and activates their expression [183, 185-186]. The second class (the middle genes) can be subdived into three groups: (1) the hook basal body, (2) the σ^{28} alternative sigma factor *fliA* [187], which is required for late gene expression, and (3) the anti-sigma factor *flgM* [188]. The third class (late genes), includes the flagellar motor, flagellins, and chemotaxis proteins

[189]. Late flagella gene expression is dependent on σ^{28} (FliA) transcriptional activation and therefore require a functional hook basal body for export of the anti-sigma factor FlgM [190-191].

Bacterial flagellar expression is a costly endeavor, and as such its regulation is complex and tightly controlled through transcriptional and translational regulation of *flhDC* [185, 192]. There are several systems and factors involved in the regulation of *flhDC* including: quorum sensing [68, 193], temperature [194], osmolarity (OmpR) [195], cell cycle [196], LrhA [197], GrlR [198], cAMP-CRP [199], RcsCDB [200], H-NS [201], and IHF [202].

GENETIC CONTENT

EHEC is believed to have diverged from the common laboratory *E. coli* K-12 strain approximately 4.5 million years ago [203]. The EHEC genome is approximately 5.5 megabases and contains a 4.1 megabase backbone that is conserved in K-12. The additional 1.34 megabases of genetic material, which is known to contain PAIs and virulence factors, is scattered throughout the genome in what are referred to as O-islands [204-205]. There is 0.54 megabase of genetic material unique to K-12 in what are referred to as K-islands. While K-12 and EHEC share most of a common genetic core, EHEC O157:H7 is believed to have descended more recently from a common EPEC O55:H7 ancestor [206].

CELL-TO-CELL SIGNALING IN BACTERIA

Cell-to-cell signaling is a mechanism in which bacteria produce and secrete diffusible autoinducer molecules and peptides in order to: communicate with each other, sense their surrounding population density and diversity, signal their cellular metabolic state, and regulate gene expression [207-208]. The four recognized categories include: the Gram negative bacterial AI-1 and AI-3, the Gram positive bacterial autoinducing peptides, and AI-2 which is used by both Gram negative and Gram positive bacteria. Additional bacterial receptors have evolved to sense not only bacterial but eukaryotic signaling molecules, such as the mammalian catecholamine hormones epinephrine and norepinephrine [1].

THE AI-2 SYSTEMS

The AI-2 QS system was first indentified in the bacterium *Vibrio harveyi* and *Vibrio fisheri* where it was shown to regulate bioluminescence [209-210]. The AI-2 precursor 4,5-dihydroxy-2,3-pentanedione (DPD) spontaneously cyclizes in solution to form several furanones [211]. Two of which (2R,4S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (R-THMF) and S-THMF-borate are the active signaling molecule for a number of bacterial species including *E.coli, Salmonella,* and *Vibrio* species, respectively. Due to its widespread use in the bacterial world, AI-2 is proposed to promote interspecies signaling in a broad range of bacterial species [9]. LuxS is the bacterial enzyme involved in S-adenosylmethionine (SAM) metabolism that converts S-

ribosylhomocysteine into homocysteine and the AI-2 precursor 4,5-dihydroxy-2,3-pentanedione (DPD) [212]. SAM is required for cellular growth and is a necessary substrate in the methylation of cellular metabolites, proteins, RNA, and DNA [213].

AI-2 is recognized generally by three cognate sensor proteins: LuxP in *Vibrio* species [209], RbsB in *Actinobacillus actinomycetemcomitans* [214], and LsrB and possibly LsrB orthologs in *Salmonella typhimurium*, *Escherichia coli*, and other enteric species (Figure 1.7) [8, 211, 215]. LuxP, in the presence of AI-2, interact with the histidine kinase LuxQ to induce its phosphase activity. Dephosphorylated LuxQ is no longer able to phorphorylate LuxU, which is then prevented from phosphorylating LuxO [216]. In the absence of phospho-LuxO, σ^{54}-dependent LuxR destabilizing small regulatory RNAs (srRNA) are not produced, and LuxR, the transcriptional regulator of luminescence [209], induces light production [217-219].

LsrR, a member of the SorC type transcriptional regulator (STTR) family, has been shown in *Salmonella typhimurium* and *E. coli* to repress the up-take, phosphorylation, and degradation of AI-2, by the direct negative transcriptional regulation of the genes encoding the LsrACDB ABC tranporter, LsrK, and LsrFG respectively [8-10]. While direct DNA binding has never been demonstrated, LsrR has been implicated in AI-2 dependent regulation of biofilm architecture, motility, and small-RNA (sRNA) expression [11]. Due to the recently increase in the understanding of the breadth of its regulatory role in *E. coli* and other species [11, 220], LsrR interference has become an area of intense focus in the fight against bacterial infections [221].

LuxS mutants have been used in multiple bacterial studies to explore the effect of AI-2 loss on cellular signaling and virulence [222-225]. Due to the integral involvement

of LuxS in central metabolism and cellular methyl cycling, the lack of an AI-2 sensor in several *luxS* positive bacteria [226], and the apparent spontaneous formation of AI-2 in the absence of *luxS* [227], these studies should be reexamined experimentally [211]. A more current and accurate approach considers AI-2 signaling and *luxS* to be a somewhat independent entities and therefore require that comparisons of the Δ*luxS* strains to wild type backgrounds include the presence of exogenous *in vitro* synthesized AI-2 as a control [228-229].

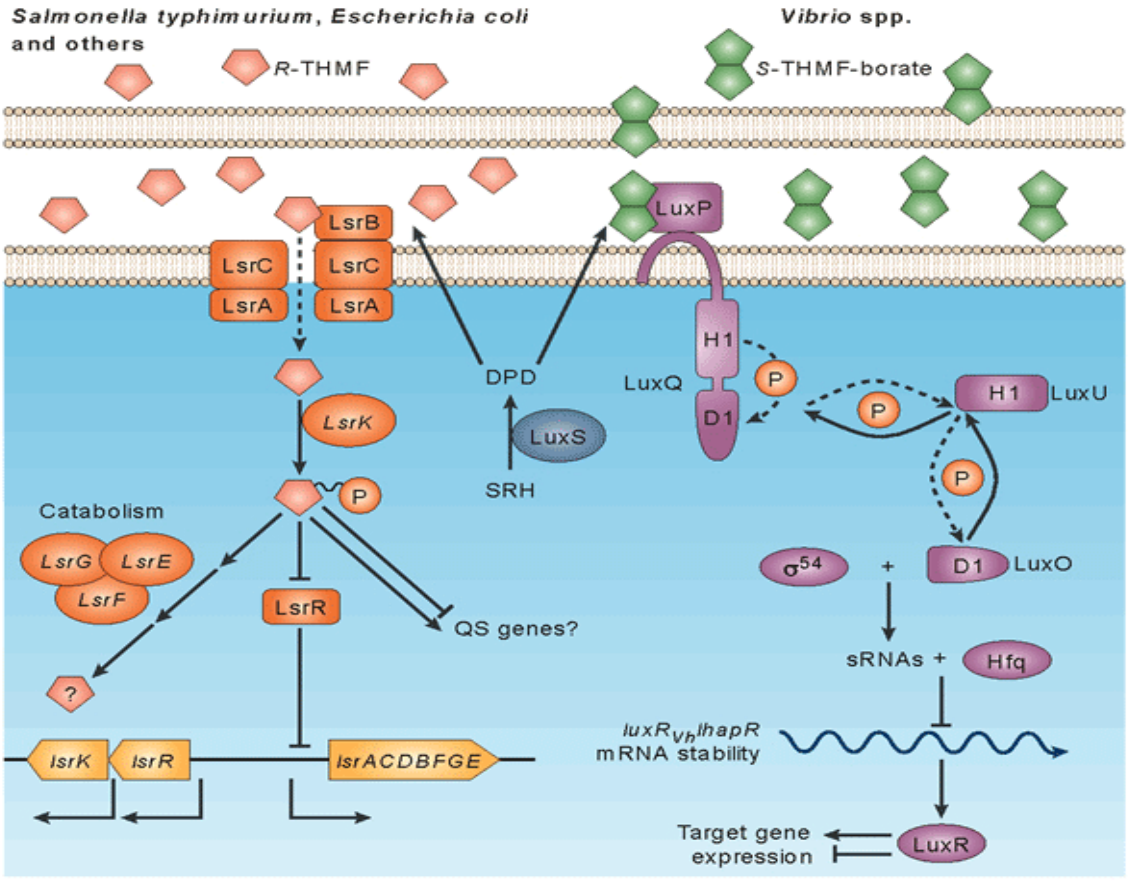


Figure 1.7. The AI-2 regulatory network. Cartoon representation of the AI-2 signaling networks in *E. coli*, *Salmonella*, and *Vibrio* spp. [211]

THE AI-3 SYSTEM

The AI-3 regulatory system, originally identified in EHEC, was detected initially due to the presence of a non-AI-2 QS molecule's ability to regulate flagella and LEE PAI expression [230]. Compared to the AI-2 regulatory system, characterization of this system is still in its infancy, as even the structure of the AI-3 signaling molecule, which has been predicted to possibly constitute a collection of chemical quasi species [231], remains unknown. AI-3 has been shown, due to the ability of culture supernatants to activate a *LEE1* promoter fusion, to be produced by a variety of pathogenic and non-pathogen Gram-negative bacterial species [232]. The only currently characterized AI-3 cell-signaling receptor is the sensor kinase QseC [1]. Besides AI-3, QseC has also been demonstrated to sense the host signals epinephrine and norepinephrine [1]. QseC and its cognate response regulator QseB constitute a two-component regulatory system. In response to AI-3, the inner membrane sensor kinase QseC autophosphorylates on a conserved histidine residue. This phosphate group is then transferred to an aspartate residue on cytoplasmic response regulator QseB, which is then able to interact with the promoter region of its target gene leading to their altered expression [233]. In addition to phosphorylating QseB, QseC also phosphorylates two non-cognate response regulators: KdpE and QseF [82]. The flagella regulon is activated by QseB, while the LEE genes and expression of the EspFU effector are activated through KdpE and QseF, respectively [82]. QseC activates transcription of the *qseEF* genes encoding the QseEF two component system [2]. QseE is a histidine kinase that senses epinephrine and norepinephrine, but not AI-3. QseE also senses sulfate, and phosphate ions [85]. The

intracellular LTTR QseA is also part of this signaling cascade [4] and directly binds to the LEE1 promoter to activate expression of the LEE (Figure 1.8) [86].

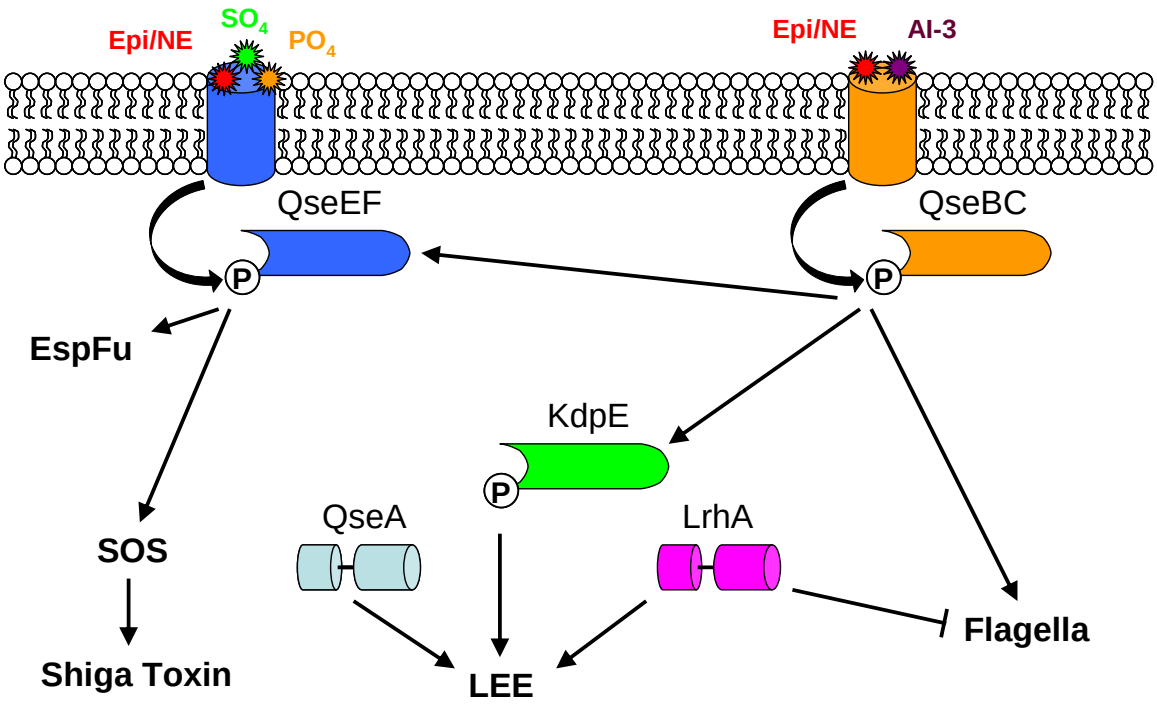


Figure 1.8. The AI-3 signalling pathway in EHEC. AI-3 or Epinephrine/ Norepinephrine (Epi/ NE) binding to QseC leads to increased phosphorylation of QseB and the downstream activation of Flagella, LEE, and Shiga Toxin expression.

THE LYSR TYPE TRANSCRIPTIONAL REGULATORY FAMILY

LTTR's are the largest family of prokaryotic DNA binding regulatory proteins [5]. LTTR's control many diverse regulatory pathways including but not limited to: biofilm formation [234], motility [197], virulence [235], TTSS [4], and amino acid metabolism and synthesis [236], in a variety of bacterial species such as: *E. coli*, *Salmonella enterica serovar Typhimurium* [237], and *Yersinia enterocolitica* [238]. LTTRs are about 300

amino acids in length and contain two functional domains, an amino-terminal helix-turn-helix (HTH) DNA-binding transcriptional regulatory domain, and a carboxy-terminal co-inducer binding, oligomerization domain [239]. LTTRs can act as either transcriptional activators or repressors generally dependent upon the location of the HTH domain. In addition, some dual functioning LTTRs have been described [240]. In the classical model for LTTR dependent transcription, the LysR protein binds the promoter of the *lysR* gene and represses its transcription while simultaneously binding the upstream promoter of the divergently transcribed target gene, where in the presence of co-inducer, tetramerization, commonly referred to a "dimer of dimers", occurs, and transcription in initiated (Figure 1.9). Generally LTTR co-inducers are small organic molecules that are a product or substrate of one of the target regulated genes, thus allowing for the formation of positive and/or negative feedback loops.

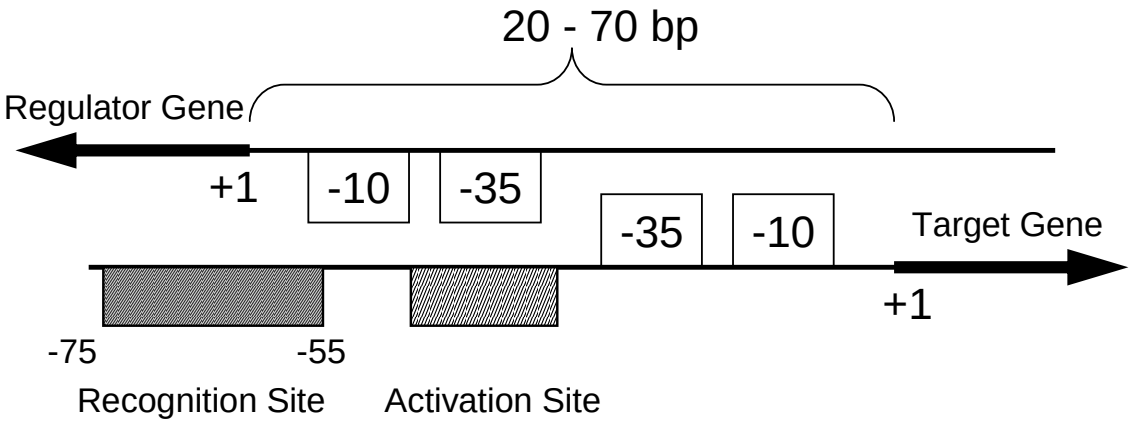


Figure 1.9. Cartoon representation of a typical divergently regulated LTTR promoter. Both the classical recognition and activation binding sites of the LTTR "dimer of dimers", as well as the divergent -10 and -35 promoter elements are depicted. [5]

Recently, the regulatory prowess of LTTRs has been demonstrated to extend beyond the local genetic level, and both the scope and manner in which these global transcriptional regulators were thought to function has been drastically altered [241]. LTTR are known to require homo-dimer and thereby tetramerization formation in order to regulate transcription, although hetero-dimerization with additional LTTRs has also been predicted [5, 242-243]. Additionally, novel classes of LTTRs, such as those which require octamerization instead of dimerization to alter target gene transcription, are constantly being discovered [244].

BIOFILMS

Ever since the discovery that microbial agents were the mediators of many classical diseases it was assumed that by studying bacteria grown in pure culture suspensions it was possible to reproduce physiological disease state conditions. It was eventually realized that idealized laboratory *in vitro* conditions do not reproduce the natural *in vivo* bacterial microenvironments [245-246] and that adherent bacterial biofilms were an important mediator of disease [247-248].

Biofilms are defined as single or multi bacterial species aggregates imbedded in a self produced extracellular polysaccharide matrix [249]. Biofilms form on both inorganic, and organic surfaces and are mediators of infectious disease pathologies [250], and persistence [251-253]. The formation of biofilms occurs in a multifaceted hierarchical fashion that can be broken down into five stages: (1) initial attachment, (2)

irreversible attachment, (3) microcolony formation, (4) macrocolony formation and maturation, and (5) dispersion. (Figure 1.10) [254].

Multiple genetic regulatory pathways and factors have been implicated in biofilm regulation and formation including but not limited to: QS [255-256], 3, 5- cyclic diguanosine monophosphate (c-di-GMP) [257-258], guanosine pentaphosphate (p)ppGpp [259-261], type 1 fimbriae expression [262], type 4 pili [263], and cAMP and catabolite repression [264-265].

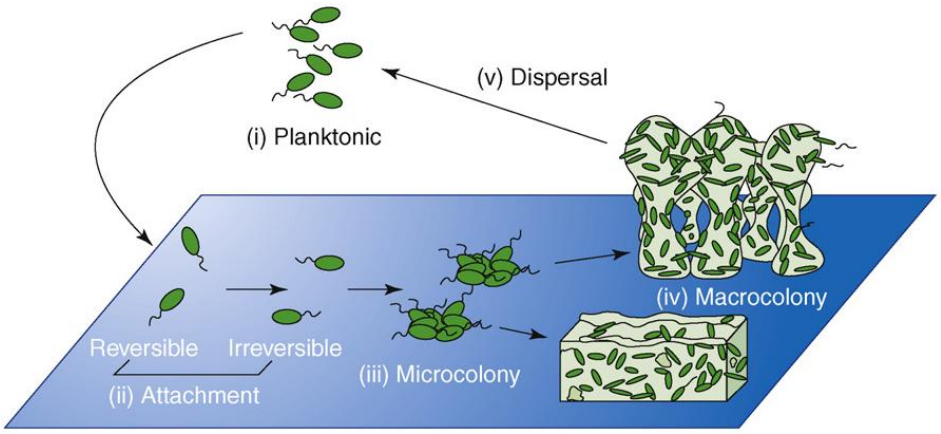


Figure 1.10. A Cartoon representation of biofilm formation. Biofilms form in five stages: (1) initial attachment, (2) irreversible attachment, (3) microcolony formation, (4) macrocolony formation and maturation, and (5) dispersion. [254]

Biofilms have been demonstrated to: increase antibiotic resistance [266-267], increase the carriage and transmission of nosocomial infections [268], alter bacterial transcriptomes [269], and mediate multiple microbial infections including: dental plaques [270], urinary tract infections (UTI) [271-272], and cystic fibrosis (CF) [273]. This makes them excellent targets for the development of antimicrobials [274] with current strategies including: antimicrobial peptides [275], quorum sensing peptide analogs [276], novel antibiotics [277], and numerous inhibitory plant extracts [278-279].

CHAPTER TWO

OVERALL OBJECTIVE AND SYNOPSIS

EHEC is a human pathogen that colonizes the large intestine, causing the development of hemorrhagic colitis and potentially HUS. EHEC infection leads to over 70,000 illnesses in the U.S.A. and is the leading cause of acute renal failure in children. Bacterial quorum sensing (QS) signaling cascades have evolved to sense microbial population density and diversity through the recognition of bacterially produced autoinducers AI-2, and AI-3. EHEC not only senses both these signals but, through the two two-component sensor kinases, QseBC and QseEF, is also able to dectect and respond to environmental cues. Through the interpretation and integration of these multiple regulatory signaling networks EHEC is able to regulate the expression of its multiple virulence factors including: the LEE TTSS, Shiga toxin (Stx), and the expression of flagella. In order to better understand and characterize the regulatory cascades leading to virulence gene expression in EHEC, we undertook a series of experiments that targeted two separate regulatory proteins, QseD and LsrR. Although these genes were shown to have vastly unique target gene repertoires, they are both integral components of the cell-to-cell signaling regulatory network in EHEC.

The gene *yjiE*, which was predicted to encode a LysR-type transcriptional regulator (LTTR), was originally identified in a microarray study as being a regulatory target of QseBC, and in a spotted array as being transcriptionally regulated by LuxS. These results suggest that this gene is regulated in a cell density-dependent manner, and thus we renamed it quorum sensing *E. coli* regulator D (*qseD*). QseD is present in all enterobacteria but, due to a point mutation generating a stop codon, exists almost exclusively in O157:H7 isolates as a helix-turn-helix (HTH) truncated isoform. Upon closer examination we noticed that downstream of the stop codon in EHEC there was a compensatory mutation that generated an alternative start site following a ribosome binding site. We therefore reasoned that *yjiE* may still generate a protein product in EHEC, although truncated and missing a HTH as compared to K-12 *E. coli*, which could lead to altered gene regulation. To examine if *yjiE* still possessed a regulatory function in EHEC, and what its original function was in *E. coli*, we constructed a non-polar mutation in O157:H7 strain 86-24 and obtained an additional K-12 *E. coli* mutant, and examined the effect on the bacteria transcriptome in its absence. The EHEC Δ*qseD* was shown to exhibit increased expression of all LEE operons and deregulation of AE lesion formation. While the loss of *qseD* in EHEC does not affect motility, the K-12 Δ*qseD* is hyper-motile. QseD was also shown to represses transcription of *iraD*, leading to altered bacterial stress responses. While the K-12 full length long QseD (lQseD) directly binds to the *ler* promoter, encoding the LEE master regulator, to repress LEE transcription, the EHEC truncated short QseD (sQseD) does not. LTTRs bind to DNA as tetramers, and these data suggest that sQseD regulates *ler* by forming hetero-tetramers with another LTTR. The LTTRs known to regulate LEE transcription, QseA and LrhA, do not interact

with sQseD, suggesting that sQseD acts as a dominant-negative partner with an yet unidentified LTTR.

LsrR, a member of the SorC type transcriptional regulator (STTR) family, has been shown to negatively regulate the import and degradation of AI-2. In *E. coli*, AI-2 is imported by the LsrACDB ABC transporter and is phosphorylated by LsrK to generate the LsrR inhibitory ligand phospho-AI-2. Increased expression of the LsrACDB transporter beyond basal levels leads to the depletion of external AI-2 signal through importation and eventual degradation by LsrFG, thus returning the entire system to basal expression levels. We hypothesized that LsrR and LsrK have additional regulatory roles beyond *lsrACDBFG* transcription which contribute to the overall epidemiology of EHEC infections. We constructed all single and combinatorial EHEC *luxS*, *lsrK*, and *lsrR* mutants, which allow for production and phosphorylation of AI-2, as well as LsrR-AI-2 transcriptional regulation, respectively. The *luxS*, *lsrR*, and *lsrK* single mutants displayed decreased swimming motility, while the *luxS* and *lsrR* single mutants and *lsrRK* double mutant demonstrated a reduction in biofilm production. Additionally, the addition of *de-novo* synthesized AI-2 was able to restore biofilm production in the *luxS* single mutant. The transcriptome of an EHEC *lsrR* mutant also suggests that *lsrR*/AI-2 signaling is involved in TTSS expression. These data suggest that the AI-2/LsrR/LsrK regulatory system is much more complex than previously appreciated, and that together they are involved in several additional regulatory pathways which contribute to motility, biofilm, and TTSS expression in EHEC.

By analyzing two separate and distinct components of the cell-to-cell signaling regulatory network in EHEC we have both expanded our overall knowledge of the

integral players involved, and broadened our understanding of the breadth of their regulatory cascades. Such studies are necessary as they will allow us to better understand both the mechanism by which EHEC is able to infect and cause disease within its human host, as well as survive within environmental biofilms that facilitate cross contamination of food products. This knowledge will aid the development of more effective treatments for EHEC.

CHAPTER THREE

MATERIALS AND METHODS

STRAINS AND PLASMIDS

All bacterial and yeast strains, plasmids, and oligonucleotides utilized in this study are listed in Tables 3.1 and 3.2. All *E. coli* strains were grown aerobically in Luria–Bertani (LB) broth, M9 with 0.4% glucose, or DMEM at 37°C unless otherwise stated. All yeast strains were grown aerobically in yeast minimal media at 30°C as previously described [280] unless otherwise stated. Where appropriate, media were supplemented with ampicillin (100μg/ml), chloramphenicol (30 μg/ml), and kanamycin (50 μg/ml). The enterohemorrhagic *E. coli* O157:H7 wild-type strain, 86-24, is streptomycin-resistant and is referred to as "wild-type" in all studies. *E. coli* DH5α (New England Biolabs) was used as a host for all plasmid constructions.

Table 3.1. Bacterial Strains and Plasmids Used in this Study.

Strain/plasmid	Genotype/description	Reference
Strains		
86-24	Wild-type EHEC strain (serotype O157:H7)	Griffen *et al.* (1988)
DH5α	*E. coli* cloning strain *supE44 lacU169 (80 lacZ M15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Stratagene
VS94	86-24 *luxS* mutant	Sperandio *et al.* (1999)
BH68	86-24 *qseD* mutant	This study
BH85	BH68 complemented with pBH22	This study
BH86	BH68 complemented with pBH23	This study
BW25113 (K-12)	*rrnB3 ΔlacZ4787 hsdR514 Δ(araBAD)567 Δ(rhaBAD)568 rph-1*	Datsenko and Wanner (2000)
D7	BW25113 *qseD* mutant, Keio collection *YjiE7*	Baba *et al*. (2006)
BH51	D7 complemented with plasmid pBH22	This study
BH52	D7 complemented with plasmid pBH23	This study
D166	Michigan State *E. coli* strain TW05550	NIAID STEC Center
TB226A	Michigan State *E. coli* strain TW04257	NIAID STEC Center
B2F1	Michigan State *E. coli* strain TW07506	NIAID STEC Center
VP08	Michigan State *E. coli* strain TW04584	NIAID STEC Center
DEC 16A	Michigan State *E. coli* strain TW02918	NIAID STEC Center
78/92	Michigan State *E. coli* strain TW05608	NIAID STEC Center
BH294-10	Michigan State *E. coli* strain TW02898	NIAID STEC Center
BH262C-8	Michigan State *E. coli* strain TW02897	NIAID STEC Center
D55	Michigan State *E. coli* strain TW04549	NIAID STEC Center
VP12	Michigan State *E. coli* strain TW04588	NIAID STEC Center
97-3250	Michigan State *E. coli* strain TW07814	NIAID STEC Center
493/89	Michigan State *E. coli* strain TW06555	NIAID STEC Center
DEC 5B	Michigan State *E. coli* strain TW02719	NIAID STEC Center
DEC 5A	Michigan State *E. coli* strain TW00587	NIAID STEC Center
DEC 5C	Michigan State *E. coli* strain TW01959	NIAID STEC Center
DEC 5D	Michigan State *E. coli* strain TW00947	NIAID STEC Center
DEC 5E	Michigan State *E. coli* strain TW00962	NIAID STEC Center
5905	Michigan State *E. coli* strain TW05353	NIAID STEC Center
G5101	Michigan State *E. coli* strain TW05356	NIAID STEC Center
413	Michigan State *E. coli* strain TW05359	NIAID STEC Center
EDL931	Michigan State *E. coli* strain TW02303	NIAID STEC Center
EDL932	Michigan State *E. coli* strain TW02299	NIAID STEC Center
EDL933	Michigan State *E. coli* strain TW02302	NIAID STEC Center
L40	Yeast reporter strain *MATa, trp1, leu2, his3, LYS2::lexA-HIS3, URA3::lexA-LacZ*	Hollenberg et al. (1995)
B2HPC	L40 transformed with pLX-YopJ + pVP-MEKK "Yeast-2-Hybrid Positive Control"	Orth *et al.* (1999)
B2HNC	L40 transformed with pLX-Laminin + pVP16 "Yeast-2-Hybrid Negative Control"	Orth *et al.* (1999)
BH129	L40 transformed with pVPSA + pLXSA	This study
BH130	L40 transformed with pVPSA + pLXLA	This study
BH131	L40 transformed with pVPSA + pLXSD	This study
BH132	L40 transformed with pVPSA + pLXLD	This study
BH133	L40 transformed with pVPLA + pLXSA	This study
BH134	L40 transformed with pVPLA + pLXLA	This study
BH135	L40 transformed with pVPLA + pLXSD	This study

BH136	L40 transformed with pVPLA + pLXLD	This study
BH137	L40 transformed with pVPSD + pLXSA	This study
BH138	L40 transformed with pVPSD + pLXLA	This study
BH139	L40 transformed with pVPSD + pLXSD	This study
BH140	L40 transformed with pVPSD + pLXLD	This study
BH141	L40 transformed with pVPLD + pLXSA	This study
BH142	L40 transformed with pVPLD + pLXLA	This study
BH143	L40 transformed with pVPLD + pLXSD	This study
BH144	L40 transformed with pVPLD + pLXLD	This study
BH157	L40 transformed with pVPSL + pLXSL	This study
BH158	L40 transformed with pVPSL + pLXLL	This study
BH159	L40 transformed with pVPSL + pLXSD	This study
BH160	L40 transformed with pVPSL + pLXLD	This study
BH161	L40 transformed with pVPLL + pLXSL	This study
BH162	L40 transformed with pVPLL + pLXLL	This study
BH163	L40 transformed with pVPLL + pLXSD	This study
BH164	L40 transformed with pVPLL + pLXLD	This study
BH165	L40 transformed with pVPSD + pLXSL	This study
BH166	L40 transformed with pVPSD + pLXLL	This study
BH167	L40 transformed with pVPLD + pLXSL	This study
BH168	L40 transformed with pVPLD + pLXLL	This study
BH29	BH68 complemented with pBH24	This study
BH31	BH68 complemented with pBH26	This study
VS94	86-24 *luxS* mutant	Sperandio *et al.* (1999)
BH36	86-24 *lsrR* mutant	This study
BH58	86-24 *lsrK* mutant	This study
BH59	86-24 *lsrR*, *lsrK* double mutant	This study
BH60	86-24 *luxS*, *lsrR* double mutant	This study
BH61	86-24 *luxS*, *lsrK* double mutant	This study
BH62	86-24 *luxS*, *lsrR*, *lsrK* triple mutant	This study
BH70	86-24 transformed with pMW195	This study
BH71	BH36 transformed with pMW195	This study
BH72	BH58 transformed with pMW195	This study
BH73	BH59 transformed with pMW195	This study
BH74	VS94 transformed with pMW195	This study
BH75	BH60 transformed with pMW195	This study
BH76	BH61 transformed with pMW195	This study
BH77	BH62 transformed with pMW195	This study
BH81	BH36 transformed with pBH78	This study
BH82	BH58 transformed with pBH79	This study
BH83	BH59 transformed with pBH80	This study
BH95	BH60 transformed with pBH78	This study
BH96	BH61 transformed with pBH79	This study
BH97	BH62 transformed with pBH80	This study
Plasmids		
pACYC184	Cloning vector	NEB
pBAD-MycHIS (A)	C-ter MycHIS expression vector	Invitrogen

pBH22	K-12 qseD region inserted into Tet of pACYC184	This study
pBH23	8624 qseD region inserted into Tet of pACYC184	This study
pKD46	λRed helper plasmid	Datsenko and Wanner (2000)
pKD3	λRed template plasmid	Datsenko and Wanner (2000)
pCP20	λRed resolvase plasmid	Datsenko and Wanner (2000)
pVP16	Yeast library plasmid	Hollenberg et al. (1995)
pLex-ADE	Yeast bait plasmid, pBTM116 with the insertion of *ADE2* at the PvuII site	Hollenberg et al. (1995)
pLX-YopJ	pLEX-ADE expressing YopJ	Orth *et al*. (1999)
pVP-MEKK	pVP-16 expressing MEKK	Orth *et al*. (1999)
pLX-Laminin	pLEX-ADE expressing Laminin	Orth *et al*. (1999)
pVPSA	pVP16 expressing “short” QseA	This study
pVPLA	pVP16 expressing “long” QseA	This study
pVPSD	pVP16 expressing “short” QseD	This study
pVPLD	pVP16 expressing “long” QseD	This study
pVPSL	pVP16 expressing “short” LrhA	This study
pVPLL	pVP16 expressing “long” LrhA	This study
pLXSA	pLEX-ADE expressing “short” QseA	This study
pLXLA	pLEX-ADE expressing “long” QseA	This study
pLXSD	pLEX-ADE expressing “short” QseD	This study
pLXLD	pLEX-ADE expressing “long” QseD	This study
pLXSL	pLEX-ADE expressing “short” LrhA	This study
pLXLL	pLEX-ADE expressing “long” LrhA	This study
pET28	N-terminal HIS tagged T7 expression construct	Novagen
pBH24	K-12 QseD in pBAD-MycHIS (A)	This study
pBH26	86-24 QseD in pBAD-MycHIS (A)	This study
pMK208	QseA-HIS (N-ter) in pET28	This study
pBH78	LsrR in pACYC184 "BamH1/Sal1"	This study
pBH79	LsrK in pACYC184 "EcoRV/Sal1"	This study
pBH80	LsrRK in pACYC184 "EcoRV/Sal1"	This study
pMW195	*Pseudomonas aeruginosa* PA01 SahH cloned into pACYC177	Walters *et al.* (2006)

Table 3.2. Oligonucleotides Used in this Study.

Primer Name	Sequence
QseDλRedF	5-TTGTCAGCCGCCCTAATGGACGTAGAATGCCCCCAAGGGCGGCTGACAGAGTAAAACGTAGTGTAGGCTGGAGCTGCTTC-3
QseDλRedR	5-CTAACGTTTGATCTGGTCTGGGATAATGGCGGTTGGCGCAGTGCGACGCTTGAGAATGTCCATATGAATATCCTCCTTAG-3
LsrRλRedF	5-TGCGCAAGAACTGAACAATTGCATTAAAGATTTAAATATGTTCAAAGTGAAGAATGAATTGTGTAGGCTGGAGCTGCTTC-3
LsrRλRedR	5-TGCGTTCTGTCTGTTCCTCTATACGTTCTCCATCATTCCCGGTAATAAGGTCATGCAAATCATATGAATATCCTCCTTAG-3
LsrKλRedF	5-CCGGGAATGATGGAGAACGTATAGAGGAACAGACAGAACGCATAAGCCGAGGATAATCTAGTGTAGGCTGGAGCTGCTTC-3
LsrKλRedR	5-AAGAATAACTATACTCAAAAACTAACAGCCACGGTCATCATGATGTGGCTGTCAATGAAACATATGAATATCCTCCTTAG-3

LsrRKλRedF	5-TGCGCAAGAACTGAACAATTGCATTAAAGATTTAAATATGTTCAAAGTGAAGAATGAATTGTGTAGGCTGGAGCTGCTTC-3
LsrRKλRedR	5-AAGAATAACTATACTCAAAAACTAACAGCCACGGTCATCATGATGTGGCTGTCAATGAAACATATGAATATCCTCCTTAG-3
QseDCompF	5-GCGGTCGACTCAGCTAAGCACAATCTC-3
QseDCompR	5-CGGATCCCAAAGACGGCAAAGCCTG-3
QseDRTF	5-CGGAGTATGCCATCCAACAA-3
QseDRTR	5-TCGTCCCGATTCAGCACAA-3
KptARTF	5-GCGATAAAAGCGTTTTAGTTATTCCA-3
KptARTR	5-GAAGTCGAATGCCCCTGAAC-3
YjiHRTF	5-TTTCGCCGCATATCAAACC-3
YjiHRTR	5-GCGACGCCGGAAGAGAA-3
YjiGRTF	5-GCTGCCAAACGTGGTGATG-3
YjiGRTR	5-CGAGCAGGCCGGTAATTTT-3
IadaRTF	5-GCTAATATGGCGGCAGAATCC-3
IadaRTR	5-TGTCGCCCATGTGGAACAC-3
IraDRTF	5-AATGCTGTACCACGACGATGAA-3
IraDRTR	5-GCGCCAACCGCCATTA-3
YjiCRTF	5-GCCCTTTCGATCCTGTTGAG-3
YjiCRTR	5-GCGTAACCTGGAACATTGCA-3
FliCRTF	5-TCCATCGACAAATTCCGTTCT-3
FliCRTR	5-TGGTGACTGCGGAATCCA-3
MotARTF	5-GAAGAGATTGAGACGCACGAAA-3
MotARTR	5-CGACCAGCGCCAGACTGT-3
LQseDMycHISF	5-CCCCATGGATGACTGTGGTGCG-3
LQseDMycHISR	5-GCGAAGCTTGCTAAGCACAATCTCCAG-3
SQseDMycHISF	5-CCCCATGGTGACGCCGCTGCAACTC-3
SQseDMycHISR	5-GCGAAGCTTGCTAAGCACAATCTCCAG-3
QseDOperF	5-CGGCGGCATCTGGCTGATAATG-3
QseDOperR	5-GTTCAGGCGGGTATTCCGCTGG-3
QseDSequF	5-CGAAATTCTGCCAGGCAATGACGCAGAC-3
QseDSequR	5-GCTGGTATTGCCGTCAGCTGCG-3
VPLEXlQseDF	5-GGGGGATCCTTATGGATGACTGTGGTGCG-3
VPLEXsQseDF	5-GGGGGATCCTTGTGACGCCGCTGCAACTC-3
VPslQseDR	5-GGGGCGGCCGCTCAGCTAAGCACAATCTC-3
LEXslQseDR	5-GGGCTGCAGTCAGCTAAGCACAATCTCCAG-3
VPLEXlQseAF	5-GGGGGATCCTTATGGAACGACTAAAACGCATGTCGG-3
VPLEXsQseAF	5-GGGGGATCCTTGGCTGCCGTCGTATGCTTCATGAAG-3
VPslQseAR	5-GGTGCGGCCGCTTACTTCTCTTTCCCGCG-3
LEXslQseAR	5-GGGCTGCAGTTACTTCTCTTTCCCGCGCCC-3
VPLEXlLrhAF	5-GGGGGATCCTTATGATAAGTGCAAATCGTCCG-3
VPLEXsLrhAF	5-GGTGGATCCTTGCCAGGAAAATCCTGCG-3
VPslLrhAR	5-GGTGCGGCCGCTTACTCGATATCCCTTTCAATC-3

LEXslLrhAR	5-GGGCTGCAGTTACTCGATATCCCTTTCAATCAAC-3
LsrRCompF	5-CGGATCCATGAGCATGAACTGGCGTTAATCTGG-3
LsrRCompR	5-GCGGTCGACTTAACTACGTAAAATCGCCGC-3
LsrKCompF	5-GCGGATATCTGACATCCTCCGCTGACATTGCCCG-3
LsrKCompR	5-GCGGTCGACCTATAACCCAGGCGCTTTCC-3
LsrRKCompF	5-GCGGATATCTGAATGAGCATGAACTGGCGTTAATC-3
LsrRKCompR	5-GCGGTCGACCTATAACCCAGGCGCTTTCC-3
LsrARTF	5-GGCATCGATTTTACGTTGCAT-3
LsrARTR	5-CGATTTACCGGCACCATTG-3

RECOMBINANT DNA TECHNIQUES

Standard methods were used to perform plasmid purification, PCR, restriction digest, ligation, transformation and gel electrophoresis [281].

ISOGENIC MUTANT CONSTRUCTION AND COMPLEMENTATION

Construction of the isogenic mutants was performed as previously described [282]. Briefly, either 86-24 or VS94 (Δ*luxS*) cells containing pKD46 were electroporated with a PCR product generated by primers QseDλRedF and QseDλRedR for *qseD*, LsrRλRedF and LsrRλRedR for *lsrR*, LsrKλRedF and LsrKλRedR for *lsrK*, or LsrRKλRedF and LsrRKλRedR for *lsrRK* (Table 2) using pKD3 as a template. After electroporation, cells were incubated at 22°C overnight in SOC media. Cells were then plated on media containing chloramphenicol (30 μg/ml) and incubated at 22°C overnight once again. Resulting colonies were patched for chloramphenicol resistance and ampicillin sensitivity and PCR verified for the absence of either *qseD*, *lsrR*, *lsrK*, or both *lsrRK*. The chloramphenicol cassette was then resolved using pCP20 mediated recombination, with additional patching for chloramphenicol and ampicillin sensitivity.

The generated *qseD* mutant, BH68, was then complemented with either pBH22 (lQseD) or pBH23 (sQseD) for complementation studies, or pBH24 (lQseD) or pBH26 (sQseD) for protein expression and purification. Plasmids pBH22 and pBH23 were constructed from the PCR product of primer set QseDCompF/R, using K-12 or EHEC as a template, which was digested with *Sal*I and *Bam*HI, and inserted into pACYC184. Plasmids pBH24 and pBH26 were constructed from the PCR product of the primer sets

LQseDMycHISF/R and SQseDMycHISF/R, using K-12 or EHEC as a template, which was digested with *Hind*III and *Nco*I, and inserted into pACYC184.

The generated mutants, BH36 (Δ*lsrR*), BH58 (Δ*lsrK*), BH59 (Δ*lsr*RK), BH60 (Δ*luxS* and Δ*lsrR*), BH61 (Δ*luxS* and Δ*lsrK*), and BH62 (Δ*luxS* and Δ*lsrRK*), were then complemented with either pBH78 (*lsrR*), pBH79 (*lsrK*) or pBH80 (*lsrRK*) for complementation studies. Plasmid pBH78 was constructed from the PCR product of primer set LsrRCompF/R, using EHEC as a template, which was digested with *Sal*I and *Bam*HI, and inserted into pACYC184. Plasmid pBH79 was constructed from the PCR product of primer set LsrKCompF/R, using EHEC as a template, which was digested with *Sal*I and *Eco*RV, and inserted into pACYC184. Plasmid pBH80 was constructed from the PCR product of primer set LsrRKCompF/R, using EHEC as a template, which was digested with *Sal*I and *Eco*RV, and inserted into pACYC184.

RNA EXTRACTION AND QUANTITATIVE REAL-TIME RT-PCR

Cultures were grown aerobically in LB medium at 37°C overnight, diluted 1:100 in LB (for *flhD*, *fliC*, and *motA*) or DMEM (for *ler*, *escV*, *escC*, *espA*, *stx2a*, *nleA* and *lsrA*) and grown aerobically at 37°C. RNA from three biological replicate cultures of each strain was extracted at lag phase (OD_{600} of 0.2), mid-log phase (OD_{600} of 0.5), late-log phase (OD_{600} of 1.0), and stationary/death phase (OD_{600} of 1.5) using the RiboPure Bacteria RNA isolation kit (Ambion) according to the manufacturer's guidelines. Primers were designed using Primer Express v1.5 (Applied Biosystems) (Table 2), the primers for the LEE genes, and *rpoA* [283], *stx2*a, and *nleA* [82] have been previously described. Quantitative real-time reverse transcription-PCR (QRT-PCR) was performed

in a one-step reaction using an ABI 7500 sequence detection system (Applied Biosystems). Amplification efficiencies for all primer sets were validated by standard curves with varying concentrations of RNA template. To ensure template specificity, products were heated to 95°C for 15 seconds, cooled to 60°C, and heated to 95°C while fluorescence was monitored. Relative quantification analysis was used to compare gene expression in BH68, BH85, BH86, BH36, BH58, BH59, BH60, BH61, and BH62 with 86–24 Escherichia coli. Parameters for cDNA generation and amplification were as follows: 1 cycle at 48°C for 30 min, 1 cycle at 95°C for 10 min, and 40 cycles at 95°C for 15 seconds and 60°C for 1 min. The *rpoA* (RNA polymerase subunit A) gene was used as the endogenous control. Each 20 µL reaction consisted of: 10 µL of 2x SYBR master mix, 0.1 µL of Multiscribe reverse transcriptase (Applied Biosystems), 0.1 µL of RNase inhibitor (Applied Biosystems), 2 µL of target RNA (50 ng/µl) and 8 µL of ddH_2O. Expression is shown in graphs as n-fold change in expression level compared with wild-type levels. Error bars represent the standard deviations of the ΔΔCT value. The Student t test was performed to assess statistical significance. A P value of less than 0.05 was considered significant.

MOTILITY ASSAYS

Assays were performed as previously described [193]. Briefly, static overnight (OVN) cultures were stabbed on tryptone soft agar (1% tryptone, 0.25% NaCl, and 0.3% agar) motility plates and incubated at either 37º C or 30º C, with motility halos being measured at either 8 or 24 hours respectively. Graphical comparisons were based upon triplicate plate quantifications.

MICROARRAYS

Microarrays and analysis were performed as previously described [284]. The GeneChip *E. coli* Genome 2.0 array (Affymetrix) was used to compare the gene expression in strain BH68 to that in 86-24, strain D7 to that in strain BW25113, and strain BH36 to that in 86-24. The array includes 10,208 probe sets for all 20,366 genes present in the following four strains of *E. coli*: K-12 MG1655 (lab strain), CFT073 (uropathogenic strain), EDL933 (O157:H7 enterohemorrhagic strain), and Sakai (O157:H7 enterohemorrhagic strain) (http://www.affymetrix.com/products/arrays/specific/ecoli2.affx). The RNA-processing, labeling, hybridization, and slide-scanning procedures were preformed as described in the Affymetrix Gene Expression Technical Manual (http://www.affymetrix.com/support/technical/manual/expression_manual.affx). The resulting data were compared to determine features whose expression was increased or decreased in response to inactivation of the *qseD* gene. Custom analysis scripts were written in Perl to complete multiple array analyses. We note that the isolate used in these studies has not been sequenced and thus is not fully contained on the array and that differences in genome content are evident. Expression data can be accessed using accession number (pending) at the NCBI GEO database.

WHOLE CELL LYSATES AND WESTERN BLOTTING

Whole-cell lysates from WT EHEC 86-24, BH68, BH85, BH86, BW25113 (K-12), D7, BH51, and BH52 were prepared by sonication from strains grown in LB (FliC) or DMEM (EspA, and EspB) to an OD_{600} of 1.0. SDS-PAGE and immunoblotting were

completed as previously described [281]. Protein concentration was determined using a Nano-Drop (Thermo Scientific). Samples were probed by Western blot analysis using polyclonal antisera against EspA, EspB, and FliC (a gift from James B. Kaper, University of Maryland) and monoclonal antisera against RpoA (Neoclone). Ponceau Red staining was used to visualize BSA loading controls.

AE LESION FLUORESCENT ACTIN STAINING (FAS)

FAS was performed as previously described [285]. Briefly, overnight cultures that were grown aerobically in LB at 37°C were diluted 1:100 and used to infect confluent monolayers of HeLa cells grown on glass coverslips at 37°C and 5% CO_2. Cell infections were allowed to progress for 6 hours at 37°C and 5% CO_2. At 6 hours the coverslips were washed, fixed, permeabilized with 0.2% Triton X-100, and treated with fluorescein isothiocyanate-labeled (FITC) phalloidin to visualize actin accumulation, and propidium iodide (PI) was added to stain bacteria. Samples were visualized by immunofluorescence with a Zeiss Axiovert microscope. The entire field of at least six coverslips from each strain was examined, and images of AE lesions were taken and processed using ImageJ.

PROTEIN PURIFICATION

One liter each of LB media was inoculated from BH29, BH31, and MK208 LB overnight growths at 1:100 and grown at 37°C to OD_{600} of 0.6. The cultures were then induced with either 400 µM IPTG (Sigma) or 0.2% arabinose and grown for 6 h at 25°C. Cells were harvested, suspended in lysis buffer (50 mM phosphate buffer pH 8, 1 M

NaCl, and 20 mM imidazole) and lysed by homogenization. The cell lysates were centrifuged and loaded onto to a Ni(2+)-NTA-agarose gravity column (Qiagen). The column was washed with lysis buffer and protein was eluted with elution buffer (50 mM phosphate buffer pH 8, 1 M NaCl, 250 mM imidazole). Fractions containing purified protein were confirmed by SDS-PAGE and concentrated for further use.

ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

EMSAs were used as previously described [82] to explore the possible binding of either isoform of QseD to the *ler* promoter. Briefly, a *ler* DNA probe was generated with primers Ler-173 and Ler-42 [86] using 86-24 as a template. DNA probes were then end-labeled with [γ-32P]-ATP (NEB) using T4 polynucleotide kinase using standard procedures [281]. End-labeled fragments were run on a 5% polyacrylamide gel, excised and purified using the PCR purification kit (Qiagen). EMSAs were performed by adding increasing amounts of purified QseA (MK208) or QseD protein (BH29, BH31) to end-labeled probe (20 ng) in binding buffer (500 µg/ml BSA (NEB), 300 ng/µl poly-dIdC, 30 mM Tris-HCl pH 7.4, 1 mM EDTA, 50 mM KCl, 5 mM NaCl) for 30 min at 22°C. In competitions, both proteins (QseA and QseD) were mixed and incubated on ice for 20 min before addition to probe and binding buffer. Immediately before loading, a 5% ficol solution was added to the mixtures. The reactions were electrophoresed for approximately 14 h at 65 V on a 5% polyacrylamide gel, dried and exposed to KODAK X-OMAT film.

YEAST TWO HYBRID ANALYSIS

A yeast two-hybrid assay was used as previously described [286] to test for possible protein-protein interaction between QseD and the LTTR family members QseA and LrhA. Briefly, the yeast reporter strain L40 was transformed [287] with all combinations of the short "s" and long "l" variants of QseD, QseA, and LrhA in both the bait (pLEX-ADE) and the library (pVP16) vector. Plasmids pVPLD and pLXLD were constructed from the PCR products of the primer VPLEXlQseDF with VPslQseDR or LEXslQseDR, using K-12 as a template, that was digested by *Bam*HI with *Not*I or *Pst*I and inserted into pVP16 or pLEX-ADE. Plasmids pVPSD and pLXSD were constructed from the PCR products of the primer VPLEXsQseDF with VPslQseDR or LEXslQseDR, using EHEC as a template, that was digested by *Bam*HI with *Not*I or *Pst*I and inserted into pVP16 or pLEX-ADE. Plasmids pVPLA and pLXLA were constructed from the PCR products of the primer VPLEXlQseAF with VPslQseAR or LEXslQseAR, using K-12 as a template, which was digested by *Bam*HI with *Not*I or *Pst*I and inserted into pVP16 or pLEX-ADE. Plasmids pVPSA and pLXSA were constructed from the PCR products of the primer VPLEXsQseAF with VPslQseAR or LEXslQseAR, using EHEC as a template, which was digested by *Bam*HI with *Not*I or *Pst*I and inserted into pVP16 or pLEX-ADE. Plasmids pVPLL and pLXLL were constructed from the PCR products of the primer VPLEXlLrhAF with VPslLrhAR or LEXslLrhAR, using K-12 as a template, which was digested by *Bam*HI with *Not*I or *Pst*I and inserted into pVP16 or pLEX-ADE. Plasmids pVPSL and pLXSL were constructed from the PCR products of the primer VPLEXsLrhAF with VPslLrhAR or LEXslLrhAR, using EHEC as a template, which was digested by *Bam*HI with *Not*I or *Pst*I and inserted into pVP16 or pLEX-ADE. Dual

transformants were selected on yeast minimal media lacking leucine, and tryptophan and then assayed for protein-protein interactions using the integrated *LacZ* and *HIS3* reporters. Association of the two protein fusions was determined by growth on yeast minimal media lacking histadine. In this manner all combinations of QseD, QseA, and LrhA (Table 6) were screened for protein-protein interactions.

GROWTH CURVES

Cultures were grown aerobically in LB medium at 37°C overnight, diluted in triplicate at 1:100 in low glucose DMEM or 0.4% glucose M9 and grown aerobically at 37°C. OD_{600} measurements, for all three biological replicate cultures, were recorded every 30 minutes. Barring a 24-hour reading, once cultures reached stationary phase, measurements ceased.

AI-2 UPTAKE

AI-2 uptake assays and analysis were performed as previously described [8]. Briefly, EHEC, BH36 (Δ*R*), BH58 (Δ*K*), BH59 (ΔRK), BH60 (Δ*SR*), BH61 (Δ*SK*), and BH62 (Δ*SRK*) cultures were grown aerobically in LB medium at 37° C overnight. Cultures were pelleted and washed with fresh LB, and then resuspended, to their original ODs, in LB containing 40 μM commercial AI-2 (Omm scientific) and incubated at 37° C for 1 hour. Cell free supernatants prepared from each strain were then diluted 1:100 into AB media, which was inoculated 1:10 from *Vibrio harveyi* 30° C OVNs growths. These cells were grown at 30° C for 4 hours and then bioluminescence was measured and compared to AB media alone, and AB media supplemented with 40 μM commercial AI-

2. Autoinducer bioassay (AB) medium: (pH 7.5 with KOH) 0.3 M NaC1, 0.05 M MgSO4, and 0.2% vitamin-free casamino acids (Difco). After sterilization, 1 ml of sterile 1 M potassium phosphate (pH 7.0), 1 ml of 0.1 M L-arginine (free-base) and 2ml of 50% glycerol were added per 100 ml of AB medium.

CRYSTAL VIOLET BIOFILM ASSAY

Crystal violet biofilm assays were performed as previously described [288]. Briefly, EHEC, BH36 (Δ*lsrR*), BH58 (Δ*lsrK*), BH59 (Δ*lsr*RK), BH60 (Δ*luxS* Δ*lsrR*), BH61 (Δ*luxS* Δ*lsrK*), and BH62 (Δ*luxS* Δ*lsrRK*) cultures or their pMW195 complemented clones were grown anaerobically in LB medium at either 37° or 30° C overnight. Cultures were diluted 1:100 into 12-well cell culture plates and grown anaerobically at 37° or 30° C for 24 hours. Wells were washed twice with water, and stained with 0.1 % crystal violet for 15 minutes, before being washed with water twice more and photographed. The stained biofilms were solubilized with 100 % DMSO and the optical density was then measure at OD_{550}.

HELA CELL ADHERENCE

HeLa cell adherence assays were performed as previously described [289]. Briefly, EHEC, BH36 (Δ*lsrR*), BH58 (Δ*lsrK*), BH59 (Δ*lsr*RK), BH60 (Δ*luxS* Δ*lsrR*), BH61 (Δ*luxS* Δ*lsrK*), and BH62 (Δ*luxS* Δ*lsrRK*) cultures were grown anaerobically in LB medium at 37° overnight. HeLa cells were seeded in 12 well cell culture plates and allowed to reach 80 % confluency before being washed and infected with 1 x 10^9 bacteria for an hour with or without the addition of 1% mannose. Wells were washed twice with

PBS and then HeLa cells were lysed in the wells using 0.1% Triton X-100 in PBS. Serial dilutions of the cell suspensions were plated on LB plates and enumerated.

CHAPTER FOUR

QseD IS A MULTI-STATE REGULATOR IN E. COLI

INTRODUCTION

Enterohemorrhagic *Escherichia coli* (EHEC) is the causative agent of outbreaks of hemorrhagic colitis and hemolytic uremic syndrome (HUS) throughout the world. Of the multiple pathogenic serotypes of clinical importance, O157:H7, a serotype that is believed to have evolved recently from an O55:H7 atypical enteropathogenic *E. coli* (EPEC) strain, is by far the most prevalent and virulent [140, 290-291]. EHEC strains are part of a larger group of enteric pathogens that includes EPEC, a rabbit EPEC, and *Citrobacter rodentium*, all of which are able to cause attaching and effacing (AE) lesions on intestinal epithelial cells [60].

The genes necessary for the formation of these characteristic AE lesions are chromosomally encoded within the locus of enterocyte effacement (LEE) pathogenicity island (PAI) [61]. The LEE is composed of 41 genes, including the LEE encoded regulator (*ler*) that activates transcription of all LEE genes [66]. The majority of the LEE genes are arranged into five major operons that encode both structural proteins that form

a type three secretion system (TTSS), and several of the secreted effectors, such as the translocated intimin receptor (Tir), EspH, and Map, which are translocated through the TTSS into host cells [62-65]. Once translocated Tir embeds itself in the host membrane, where its extracellular domain serves as a docking point for its cognate bacterial receptor, intimin (also encoded within the LEE), thus allowing for intimate attachment and formation of the characteristic AE lesions on eukaryotic cells [62]. Tir, EspH, and Map have also been shown to regulate the length and number of pedestals, as well as eukaryotic filopodia formation by altering actin assembly [129, 134, 292].

EHEC is able to sense and respond to biotic cues from its environment, such as the human host produced catecholamines epinephrine and norepinephrine, through two two-component systems, QseBC and QseEF [1, 85]. EHEC also senses abiotic environmental cues, such as phosphate and sulfate levels through QseEF [85]. Additionally, quorum sensing (QS) signaling cascades have evolved to sense microbial population density and diversity through the recognition of bacterially produced autoinducers (AI) AI-2, and AI-3 [230, 293]. AI-2, the enzymatic product of LuxS, is proposed to promote interspecies signaling in a broad range of bacterial species [9], whereas the breadth of AI-3 signaling has not been as extensively characterized [232]. Through the interpretation and integration of these multiple regulatory signaling networks that often involve intracellular regulatory proteins, EHEC is able to regulate the expression of its multiple virulence factors. These factors include the LEE TTSS, Shiga toxin (Stx), the causative agent of HUS, and the expression of flagella, through its master regulator *flhDC*, which allow for bacterial motility [230].

Regulation of the LEE is highly complex and requires the involvement of multiple regulatory proteins and pathways [4, 63, 66, 68-85]. Transcription of the *ler* gene, encoding the master regulator of the LEE (Mellies et al. 1998), is directly activated by the LysR type transcriptional regulator (LTTR) QseA [4, 86], and negatively by the RNA regulatory chaperone Hfq [84, 87]. Other post-transcriptional regulatory factors such as ClpXP has been shown to increase transcription of *LEE3* by inhibiting its repression by GrlR and by increasing the degradation of the EHEC global regulator stationary-phase sigma factor RpoS [74, 78, 88]. RpoS is itself under a high level of post-transcriptional control by Hfq [89], translational control by Hfq and the LTTR LrhA [90], and post-translational control by RssB [91]. RssB, whose activity is modulated by LrhA, has been shown to sequester RpoS and targets it for ClpXP degradation [92-93]. Finally, IraD, an RssB anti-adaptor protein, is induced in response to DNA damage and prevents RssB-mediated ClpXP degradation of RpoS, thereby altering cellular responses to stress [6-7, 94]

LTTRs are the largest family of prokaryotic DNA binding regulatory proteins [5]. LTTRs control many diverse regulatory pathways including: biofilm formation [234], motility [197], and TTSS [4], in a variety of bacterial species including: *E. coli*, *Salmonella enterica serovar Typhimurium* [237], and *Yersinia enterocolitica* [238]. LTTRs are about 300 amino acids in length and contain two functional domains, an amino-terminal helix-turn-helix (HTH) DNA binding transcriptional regulatory domain, and a carboxy-terminal co-inducer binding, oligomerization domain [239]. LTTRs can act as either transcriptional activators or repressors generally dependent upon the location of the HTH domain. In addition, some dual-functioning LTTRs have been described

[240]. In the classical model for LTTRs dependent transcription, the LysR protein binds the promoter of the *lysR* gene and represses its transcription while simultaneously binding the upstream promoter of the divergently transcribed target gene, where in the presence of co-inducer, tetramerization occurs, and transcription in initiated. Recently, the regulatory prowess of LTTRs have been demonstrated to extend beyond the local genetic level, and both the scope and manner in which these global transcriptional regulators were thought to function has been drastically altered [241]. LTTR are known to require homo-dimer and thereby tetramerization formation in order to regulate transcription, although hetero-dimerization with additional LTTR's has also been predicted [5, 242-243]. Additionally, novel classes of LTTR's, which require octamerization instead of dimerization to alter target gene transcription, are constantly being discovered [244].

In this chapter, we report the identification of a LTTR YjiE, herein renamed QseD (quorum sensing *E. coli* regulator D), which although prevalent in enterobacteria seemingly exists almost exclusively in EHEC O157:H7 isolates in a helix-turn helix (HTH) truncated isoform. This truncated "short" isoform (sQseD) confers EHEC with altered cellular regulatory consequences as compared with the full length K-12 *E. coli* "long" isoform (lQseD). In EHEC QseD down-regulates the LEE and *iraD* transcription, and alters AE lesion formation, while in K-12 QseD represses motility.

RESULTS

Identification of the quorum sensing regulated LTTR QseD.

The gene y*jiE* encodes a 34.7-kDa putative LysR-type transcriptional regulator (LTTR) that is found at the end of a four-gene operon (Figure 4.1A). This gene was originally identified in a microarray study as being a regulatory target of QseBC signaling, and in a spotted array as being transcriptionally regulated by LuxS in a quorum sensing-dependent manner (Figure 4.2) [82, 294]. *yjiE* is found throughout the *Enterobacteriaceae* family, but it contains a point mutation generating a stop codon, and was predicted to be a pseudogene almost exclusively in EHEC O157:H7 and its suggested parent EPEC O55:H7 (Figure 4.1B, Table 4.1). Upon closer examination we noticed that downstream of the stop codon in EHEC there was a compensatory mutation that generated an alternative start site (1*) following a ribosome binding site (RBS*) (Figure 4.1B). Therefore to confirm that *yjiE* was still transcribed as part of a functional operon in EHEC we performed RT-PCR using a primer set, P_1/P_2, that flanked the untranslated region (one primer within the upstream *iadA* gene, and the second within *yjiE*) and that should generate a ~700 bp product. PCR products of the predicted size were observed when using both extracted genomic DNA (positive PCR control) and cDNA generated from RNA as a template thus confirming that *yjiE* is still co-transcribed within its operon in EHEC (Figure 4.1C). We therefore reasoned that *yjiE* may still generate a protein product in EHEC, although truncated and missing a HTH as compared to K-12 *E. coli*, which could lead to altered gene regulation (Figure 4.1D).

To confirm that transcription of the *yjiE* gene was regulated in a cell density-dependent manner, we performed quantitative real-time RT-PCR (qRT-PCR) using cDNA synthesized from RNA extracted from both WT K-12 *E. coli* and WT EHEC during lag phase growth ($O.D._{600}$ of 0.2), mid-log growth ($O.D._{600}$ of 0.5), late-log growth ($O.D._{600}$ of 1.0), and stationary growth phases ($O.D._{600}$ of 1.5). Transcription of *yjiE* in K-12 *E. coli* increases throughout growth peaking during stationary phase, while in EHEC transcript levels drop temporarily during mid-log phase and then similarly peak during stationary phase (Figure 4.1E). These results suggest that this gene is regulated in a cell density-dependent manner, and thus we renamed *yjiE* quorum sensing *E. coli* regulator D (*qseD*).

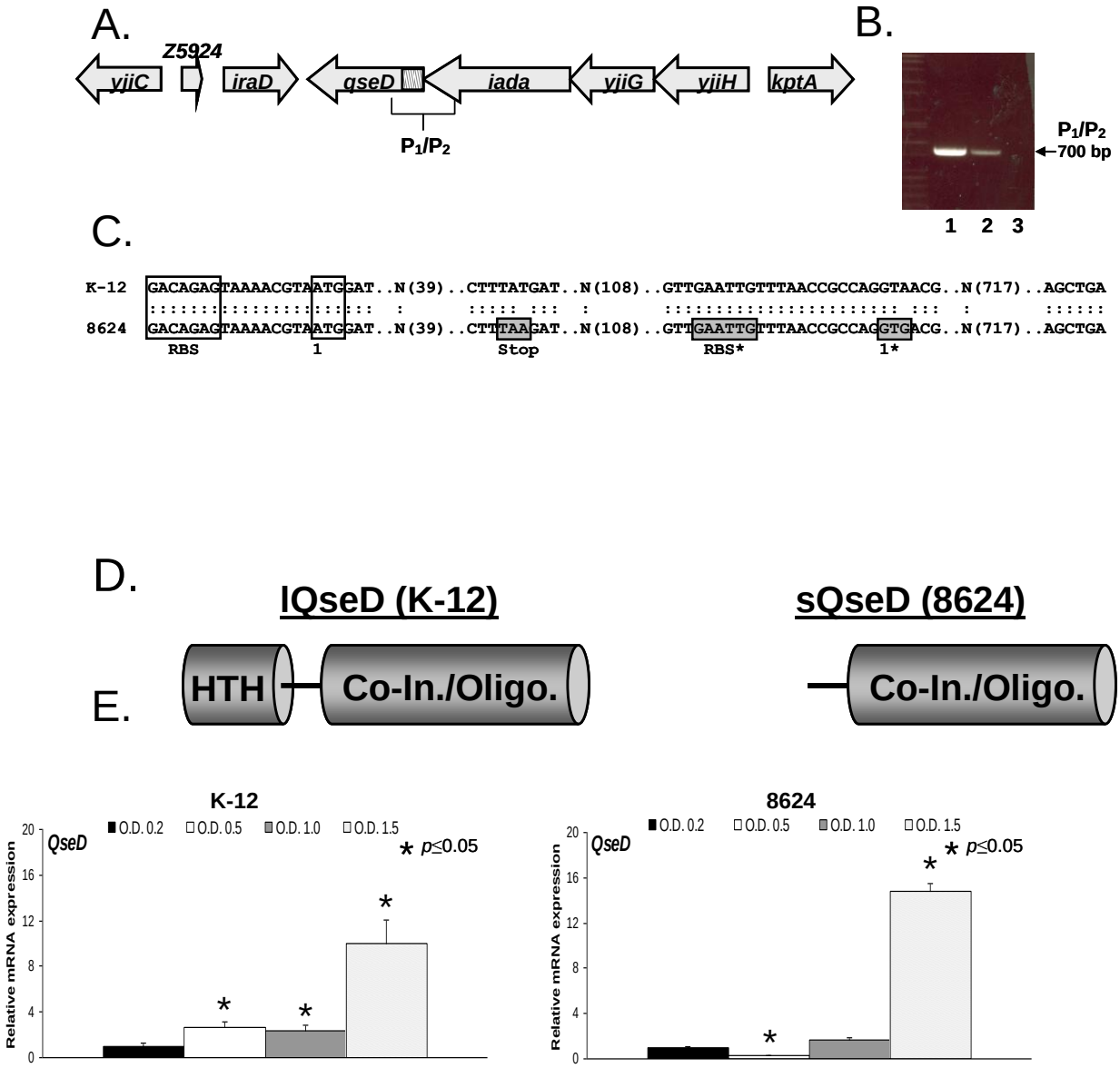


Figure 4.1. **In EHEC 86-24 the intact *qseD* operon encodes a truncated QseD protein. (A)** Cartoon representation of the *qseD* operon (untranslated region shaded) and the surrounding genes. **(B)** RT-PCR using the P_1/P_2 primer set and either cDNA (1), gDNA (2), or RNA (3) harvested and/or synthesized from EHEC 86-24 as a PCR template. **(C)** Comparison of the *qseD* sequence of EHEC 86-24 and K-12 *E. coli* where boxed regions include the ribosome binding site (RBS) and translational start site, and boxed and shaded regions include the stop codon, alternative RBS and translational start codon in EHEC 86-24. **(D)** Cartoon representation of the full length QseD (lQseD) and truncated QseD (sQseD) protein products from K-12 *E. coli* and EHEC 86-24 respectively. In EHEC loss of the translated helix-turn-helix (HTH) does not appear to affect translation of the co-factor recognition/oligomerization domain (Co-In./Oligo.).

(E) QRT-PCR of *qseD* expression levels in DMEM at lag phase (OD_{600} of 0.2), mid-log phase (OD_{600} of 0.5), late-log phase (OD_{600} of 1.0), and stationary phase (OD_{600} of 1.5) in K-12 *E. coli* and EHEC 86-24.

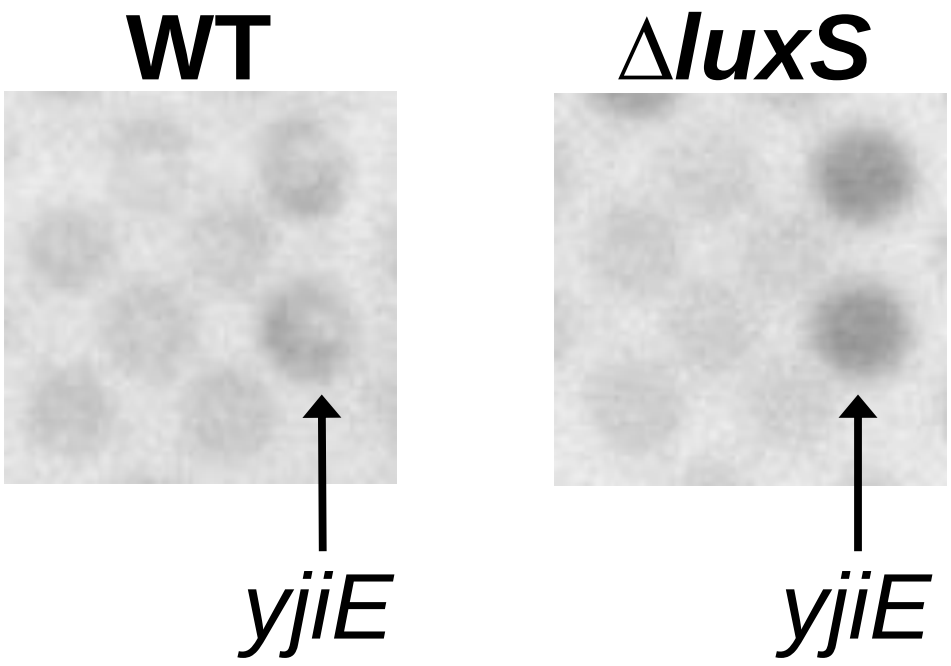


Figure 4.2. *QseD* expression is regulated by cell-to-cell communication. Genomic spotted array depicting the altered expression levels of *qseD* (*yjiE*) in the Δ*luxS* versus WT EHEC 86-24.

Table 4.1. Strain/serotype distribution of QseD isoforms.

Organism	Strain	Serotype	QseD Isoform
EHEC	86-24	O157:H7	S "short"
EHEC	Sakai	O157:H7	S
EHEC	EDL933	O157:H7	S
EHEC	"Spinach"	O157:H7	S
EHEC	EC4115	O157:H7	S
EHEC	TW14588	O157:H7	S
EHEC	TW05356 G5101	O157:H7	S
EHEC	TW02303 EDL931	O157:H7	S
EHEC	TW02299 EDL932	O157:H7	S
EHEC	TW02302 EDL933	O157:H7	S
EHEC	TW05359 413	O157:HNM	S
EHEC	TW06555 493/89	O157:H-	S
EHEC	TW02719 DEC 5B	O55:H7	S
EHEC	TW00587 DEC 5A	O55:H7	S
EHEC	TW01959 DEC 5C	O55:H7	S
EHEC	TW00947 DEC 5D	O55:H7	S
EHEC	TW05353 5905	O55:H7	S
UPEC	UTI89	O18:K1:H7	S*
APEC	O1	O1:K1:H7	S*
K-12	MG1655	OR:H48:K-	L "long"
K-12	BW25113	OR:H48:K-	L
"Commensal"	HS	O9:H4	L
EHEC	TW02897 BH262C-8	O142:H10	L
EHEC	TW05550 D166	O121:H-	L
EHEC	TW02918 DEC 16A	O113:H21	L
EHEC	11128	O111:H-	L
EHEC2	TW04257 TB226A	O111:H-	L
EHEC2	TW07506 B2F1	O111:H-	L
EHEC2	TW05608 78/92	O111:H-	L
EHEC	12009	O103:H2	L
EHEC	TW02898 BH294-10	O76:H7	L
EHEC	TW00962 DEC 5E	O55:H7	L
EHEC	TW07814 97-3250	O26:H11	L
EHEC	11368	O26:H11	L**
EHEC	TW04588 VP12	O26:H-	L
EHEC	TW04584 VP08	O26:H-	L
EPEC	E2348/69	O127:H6	L
EPEC	TW04549 D55	O127:H-	L
EPEC	11128	O111:H-	L
aEPEC (atypical)	E110019	O111:H9	L
ETEC	B7A	O148:H28	L
ExPEC	S88	O45:K1:H7	L
ExPEC	UMN026	O17:K52:H18	L
ExPEC	IAI39	O7:K1	L

EAEC	55989		L
UPEC	536	O6:K15:H31	L
UPEC	CTF073	O6:K2:H1	L**
Shigella flexneri 5	8401		L
Shigella boydii	CDC 3083-94		L
Shigella flexneri 2a	301		L
Shigella boydii	Sb227		L

* An alternative A→T stop codon generating point mutation upstream of the one herein described for all other short QseD's that should also generate the same truncated protein product

** Homologous QseD protein products with an extended C-terminus

QseD alters gene transcription.

LTTR's are known to positively and/or negatively regulate transcription at both the local and global genetic level. To determine the regulatory role of QseD in EHEC gene expression and/or pathogenesis, we constructed a non-polar mutation in *qseD* and examined its effect on the expression of genes co-transcribed with or adjacent to *qseD* in the genome by qRT-PCR. QseD does not autoregulate transcription of its own operon, however it does repress the transcription of the adjacent genes *yjiC* and *iraD* as their transcripts were up-regulated two-fold and 13-fold in the *qseD* mutant respectively (Figure 4.3). While YjiC has no predicted homology to any known protein, IraD has recently been demonstrated to prevent degradation of the stress-alternative sigma factor RpoS, by RssB sequestration, leading to an altered bacterial stress response and increased mutation rates [7, 295].

An *E. coli* K-12 *qseD* mutant was also constructed in order to further broaden the scope of our analysis and to compare and contrast the effect of the HTH truncation in EHEC on genetic regulation. To search for targets of QseD regulation, a transcriptome approach using the Affymetrix *E. coli* 2.0 microarrays was used to compare expression profiles of both the WT EHEC and WT K-12 *E. coli* to their corresponding isogenic *qseD* mutants. These arrays contain ~10,000 probe sets, covering the genomes of two sequenced EHEC strains (EDL933 and Sakai), K-12 strain MG1655, uropathogenic *E. coli* (UPEC) strain CFT073, and 700 probes to intergenic regions that can encode non-annotated ORF's, or small reguatory RNAs. In EHEC during growth in DMEM, a condition know to induce virulence gene expression, 477 probe sets were up-regulated (93 EHEC specific), and 505 were down-regulated (128 EHEC specific) in the *qseD*

mutant (Table 4.2). The largest number of genes with altered expression levels were also found in the *E. coli* K-12 strain MG1655 genome (43%), which represent a common *E. coli* backbone conserved among *E. coli* pathovars (Table 4.3) [296]. In comparison, in K-12 *E. coli* grown in DMEM, there were considerably fewer genes with altered expression levels, 109 up-regulated, and 135 down-regulated in the *qseD* mutant. This trend was partially reversed when the same mutant was grown in LB, where there were 150 genes up-regulated, and 321 genes down-regulated. Taken together these data suggest that the truncated QseD EHEC isoform has a greater regulatory repertoire than the full length QseD, and that a larger proportion of these genes are on the conserved *E. coli* backbone.

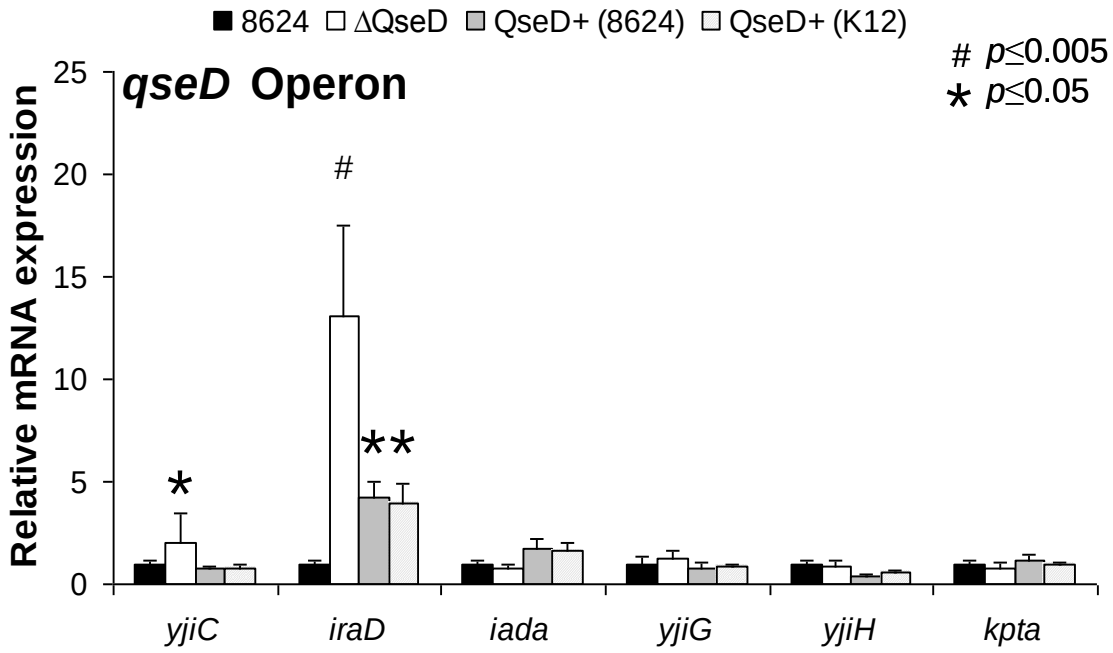


Figure 4.3. QseD does not regulate the *qseD* operon but regulates the surrounding genes in *E. coli*. QRT-PCR of the *qseD* operon (*iadA*, *yjiG*, and *yjiH*) and the surrounding genes (*kptA*, *yjiC*, and *yjiD*) in EHEC 86-24, the Δ*qseD*, the Δ*qseD* complemented *in trans* with *qseD* (86-24), and the Δ*qseD* complemented *in trans* with *qseD* (K12) grown in DMEM (OD_{600} of 1.0).

Table 4.2. Comparison of WT EHEC 86-24 and K-12 BW25113 to their respective Δ*qseD* under various growth conditions.

	Increased	Marg. Incr.	Decreased	Marg. Decr.	No Change
EHEC-	477	368	505	272	8586
K-12-	109	337	135	517	9110
K-12-LB	150	515	321	908	8314

Increased and decreased are at least two fold changes in expression levels. Marginally increased or decreased are changes that are either less than two fold or designated as such by the Affymetrix anaylsis software GCOSv1.4. Both EHEC and K-12 were grown to an OD_{600} of 1.0 in either low glucose DMEM or LB media.

Table 4.3. Pathovar distribution of altered gene expression in the Δ*qseD's*.

	MG1655	EDL933	Sakai	CTF073	Intergenic
EHEC "86-24"-					
Decreased	151	128	29	114	54
Marg. Decreased	93	80	19	48	30
Increased	222	93	21	85	30
Marg. Increased	213	85	11	32	26
No Change	3391	1401	293	2207	1157
K-12 "BW25113"-					
Decreased	88	9	6	16	11
Marg. Decreased	383	27	8	47	41
Increased	79	4	0	15	10
Marg. Increased	240	10	4	42	40
No Change	3280	1737	355	2366	1195
K-12 "BW25113"-					
Decreased	124	32	7	67	89
Marg. Decreased	589	53	13	96	156
Increased	121	9	0	12	7
Marg. Increased	431	9	9	26	17
No Change	2805	1684	344	2285	1028

Increased and decreased are at least two fold changes in expression levels. Marginally increased or decreased are changes that are either less than two fold or designated as such by the Affymetrix anaylsis software GCOSv1.4. Both EHEC and K-12 were grown to an OD_{600} of 1.0 in either low glucose DMEM or LB media.

QseD also differentially regulates genetic pathways in EHEC as compared to K-12 *E. coli*. One of the most striking examples of this differential regulation is the flagella regulon, which while unaltered in the EHEC *qseD* mutant is up-regulated in the K-12 *qseD* mutant (Figure 4.4A). Array findings were validated by observation of increased motility halos for the K-12 *qseD* mutant, but not the EHEC *qseD* mutant in tryptone soft agar motility plates (Figure 4.4B). Congruent with the motility studies, we observed increased transcription of the *flhD*, *motA*, and *fliC* flagellar genes by qRT-PCR in the K-12 *qseD* mutant (Figure 4.4C), as well as increased levels of FliC in whole cell lysates of the K-12 *qseD* mutant, but not the EHEC *qseD* mutant, by Western blot analysis (Figure 4.4D). The observation that both isoforms of QseD (short EHEC QseD and long K-12 QseD) were able to complement the K-12 *qseD* mutant *in trans* (Figure 4.4) suggest that both isoforms are still able to regulate transcription of the flagellar regulon in K-12. The differential regulation between the flagella regulons of EHEC and K-12 by QseD may not be a result of the different isoforms of this protein, but could be a result of the presence of an insertion sequence (IS) in the *flhDC* regulatory region of K-12 *E. coli*, which is absent in EHEC. The presence of this IS has been shown to alter *flhDC* transcription and consequently motility in *E. coli* K-12 [297].

In addition to differentially regulating the flagella regulon between EHEC and K-12, QseD also repressed expression of pathogenesis-specific pathways in EHEC, absent in K-12, such as the expression of the LEE genes (Figure 4.5). The EHEC *qseD* mutant exhibited up-regulation of expression of the *ler* (encoded within the *LEE1* operon), *escV* (*LEE3*), *espA* (*LEE4*), and *eae* (*LEE5*) genes but no effect on *stx2A* (encoding Shiga

toxin) gene expression (Figure 4.5A and B). Here again, both isoforms of QseD were able to complement this mutantion *in trans*.

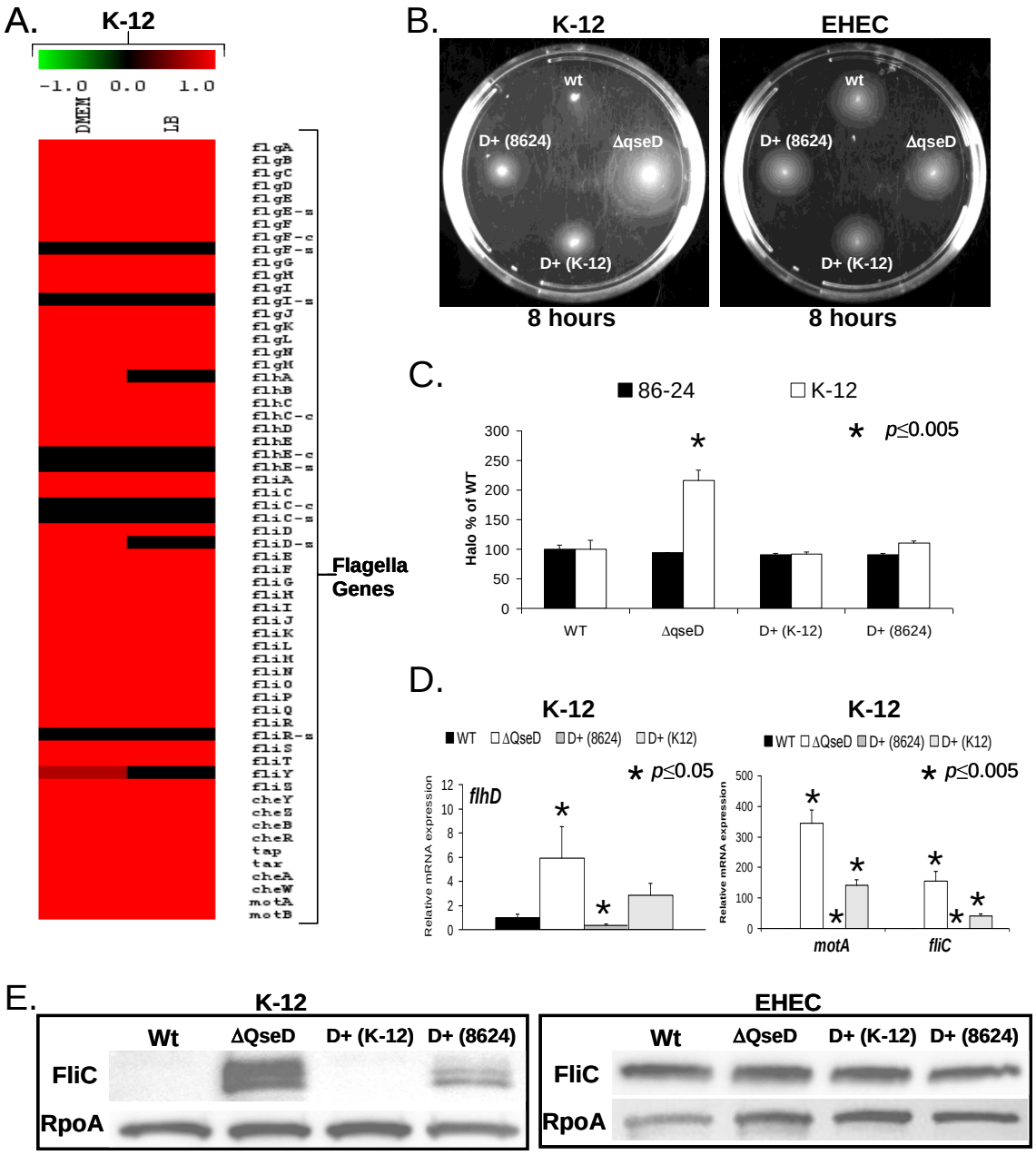


Figure 4.4. QseD affects motility in K-12 *E. coli* but not in EHEC 86-24. (A) Heat maps generated from microarray analysis depicting the differential regulation of the flagellar regulon in the K-12 Δ*qseD* versus WT K-12 *E. coli*. **(B)** Motility plates of the WT K-12 *E. coli* and WT EHEC 86-24 and their corresponding Δ*qseD*, Δ*qseD* complemented *in trans* with *qseD* (K-12), and Δ*qseD* complemented *in trans* with *qseD* (86-24). **(C)** Graphical representation of triplicate motility halos experiments. **(D)** QRT-PCR of *flhD*, *motA*, and *fliC* in WT K-12 *E. coli*, Δ*qseD*, Δ*qseD* complemented *in trans* with *qseD* (K-12), and Δ*qseD* complemented *in trans* with *qseD* (86-24) grown in LB (OD_{600} of 1.0). **(E)** Western blot of FliC from WT K-12 *E. coli* and WT EHEC 86-24 and their corresponding Δ*qseD*, Δ*qseD* complemented *in trans* with *qseD* (K-12), and Δ*qseD* complemented *in trans* with *qseD* (86-24).

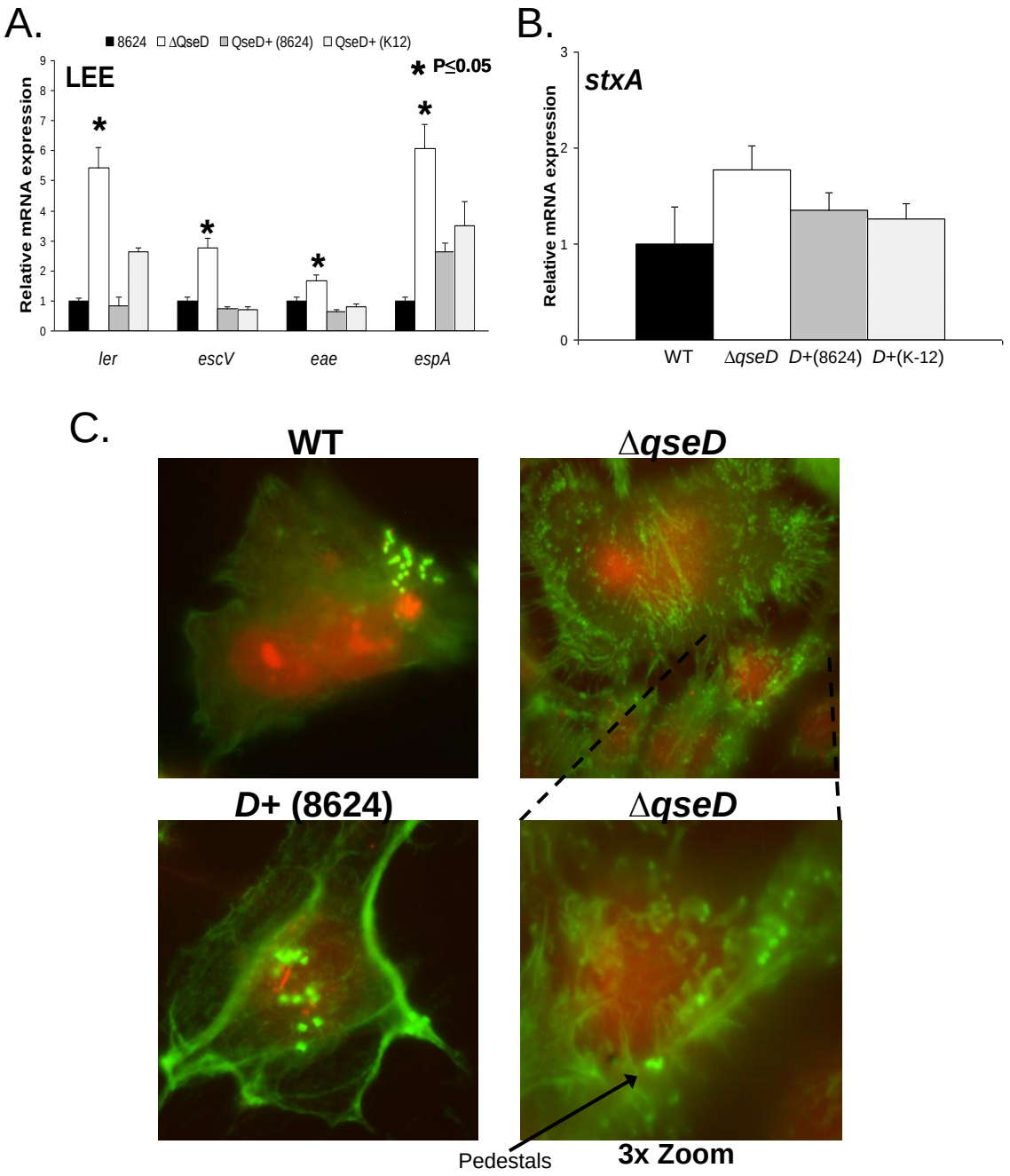


Figure 4.5. QseD regulates the LEE pathogenicity island but not Stx in EHEC 86-24. (A) QRT-PCR of *ler*, *escV*, *escC*, and *espA* in WT EHEC 86-24, the Δ*qseD*, the Δ*qseD* complemented *in trans* with *qseD* (86-24), and the Δ*qseD* complemented *in trans* with *qseD* (K-12) grown in DMEM (OD_{600} of 1.0). **(B)** QRT-PCR of *stx2a* in WT EHEC 86-24, the Δ*qseD*, the Δ*qseD* complemented *in trans* with *qseD* (86-24), and the Δ*qseD* complemented *in trans* with *qseD* (K-12) grown in DMEM (OD_{600} of 1.0). **(C)** FAS assays depicting formation of AE lesions on HeLa cell monolayers by WT EHEC, the EHEC Δ*qseD*, the EHEC Δ*qseD* complemented *in trans* with *qseD* (86-24). Bottom panel shows the EHEC Δ*qseD* at 3x zoom.

As QseD represses transcription of *ler*, and therefore LEE expression *in vitro*, we then assessed AE-lesion formation in the EHEC *qseD* mutant by fluorescence microscopy, as previously described [298]. We observed that while the mutant was still able to form actin pedestals, deregulation of normal AE-lesion formation was occurring. HeLa cells infected with the EHEC *qseD* mutant exhibited increased cellular filopodia formation, as compared to cells infected with the WT and complemented strains (Figure 4.5C). In HeLa cells infected by EHEC, it has been reported that the LEE-encoded TTSS effectors Tir, EspH, and Map can lead to alterations in filopodia formation and pedestal elongation [129, 134]. Given that expression of the genes encoding these effectors are controlled by Ler [299], and transcription of *ler* is repressed by QseD, the alterations in cellular architecture of HeLa cells infected by the *qseD* mutant could be due to overexpression of these secreted effectors in this mutant. These results suggest that QseD is a repressor of EHEC virulence expression.

Differential mechanisms of *ler* transcriptional regulation by s/lQseD.

To investigate how both the long and short (HTH truncated) forms of QseD are able to regulate LEE transcription in EHEC we performed EMSAs to look for potential direct DNA binding. We hypothesized that QseD may bind to the same regulatory region of the *ler* promoter (-173 to -42) as the LTTR QseA [86]. We observed that both purified QseA and lQseD, directly bound to the *ler* promoter, but that purified sQseD did not (Figure 4.6A-C).

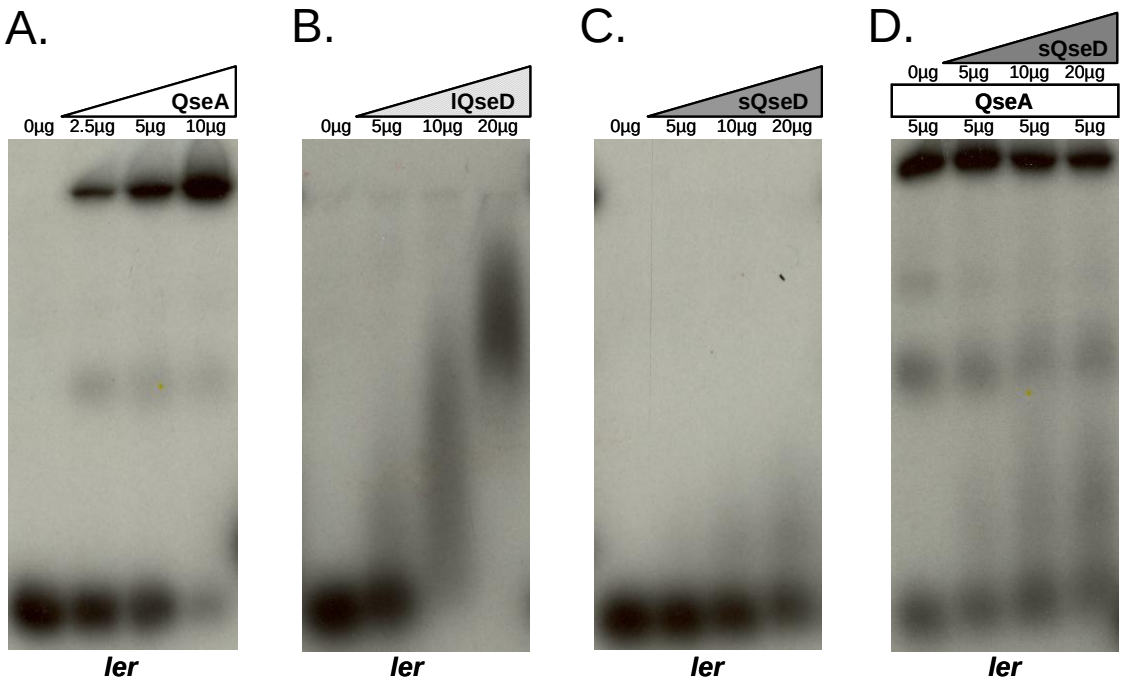


Figure 4.6. The K-12 *E. coli* lQseD binds to the *ler* promoter. EMSA's of the *ler* promoter with purified **(A)** QseA, **(B)** K-12 *E. coli* lQseD, **(C)** EHEC 86-24 sQseD and **(D)** an attempt to prevent QseA binding of *ler* by heterodimer formation with sQseD.

These results were expected, given that sQseD lacks the HTH domain necessary for DNA-biding. While the down-regulation of LEE transcription by lQseD can be accounted for by direct binding to the *ler* promoter, a mechanism for the down-regulation of LEE transcription by sQseD was not apparent. LTTRs are known to require dimer formation in order to bind DNA and regulate transcription. To date most studies assessed the need of homo-dimerization of LTTRs for function, but hetero-dimerization with additional LTTRs has been predicted *in silico* [5, 242-243]. We therefore hypothesized that the HTH truncated sQseD might be more promiscuous in its protein-protein interactions, and that potential hetero-dimers with known LEE regulating LTTRs such as QseA [86] and LrhA [300] could account for LEE regulation in the absence of direct DNA binding by sQseD. However, we did not observe any significant inhibition of binding to *ler* by QseA when it was preincubated with purified sQseD (Figure 4.6D). To test this hypothesis, we assessed whether sQseD could form hetero-dimers with LhrA and/or QseA using a yeast-two-hybrid (Y2H) system. The yeast-two hybrid data showed that LhrA, QseA and QseD form homo-dimers, which is an expected result, but that sQseD or lQseD could not form heterodimers with neither LhrA nor QseA (Table 4.4, Figure 4.7A-C). These results agree with our inability to identify interaction partners between QseA and LhrA in sQseD affinity tagged pull-down experiments (data not shown). We therefore concluded that sQseD does not form protein-protein interactions with these known LEE LTTR regulators, and must therefore regulate LEE transcription through interactions with another yet unidentified LTTR.

Table 4.4. Summary of Yeast-two-Hybrid results.

Strain	Constructs	Growth (HIS-)	Blue Colonies[1]
BH125	VP + LXSA	---	---
BH126	VP + LXLA	---	---
BH127	VP + LXSD	---	+
BH128	VP + LXLD	---	---
BH129	VPSA + LXSA	Yes	+++
BH130	VPSA + LXLA	Yes	+++
BH131	VPSA + LXSD	---	++
BH132	VPSA + LXLD	---	---
BH133	VPLA + LXSA	Yes	+++
BH134	VPLA + LXLA	Yes	+++
BH135	VPLA + LXSD	---	++
BH136	VPLA + LXLD	---	---
BH137	VPSD + LXSA	---	---
BH138	VPSD + LXLA	---	---
BH139	VPSD + LXSD	Yes	+++
BH140	VPSD + LXLD	Yes	+++
BH141	VPLD + LXSA	---	---
BH142	VPLD + LXLA	---	---
BH143	VPLD + LXSD	---	+
BH144	VPLD + LXLD	---	---
BH155	VP + LXSL	Yes	++
BH156	VP + LXLL	---	++
BH157	VPSL + LXSL	Yes	+++
BH158	VPSL + LXLL	Yes	+++
BH159	VPSL + LXSD	---	---
BH160	VPSL + LXLD	---	++
BH161	VPLL + LXSL	Yes	+++
BH162	VPLL + LXLL	Yes	+++
BH163	VPLL + LXSD	---	+
BH164	VPLL + LXLD	---	---
BH165	VPSD + LXSL	Yes	++
BH166	VPSD + LXLL	---	++
BH167	VPLD + LXSL	Yes	++
BH168	VPLD + LXLL	---	++

[1]Refers to the intensity and incubation time required for chromogenic substrate (X-Gal) conversion with (+++) being intense and under 30min, (++) being somewhat intense and within 2-3 hours, and (+) being faint and requiring overnight incubation.

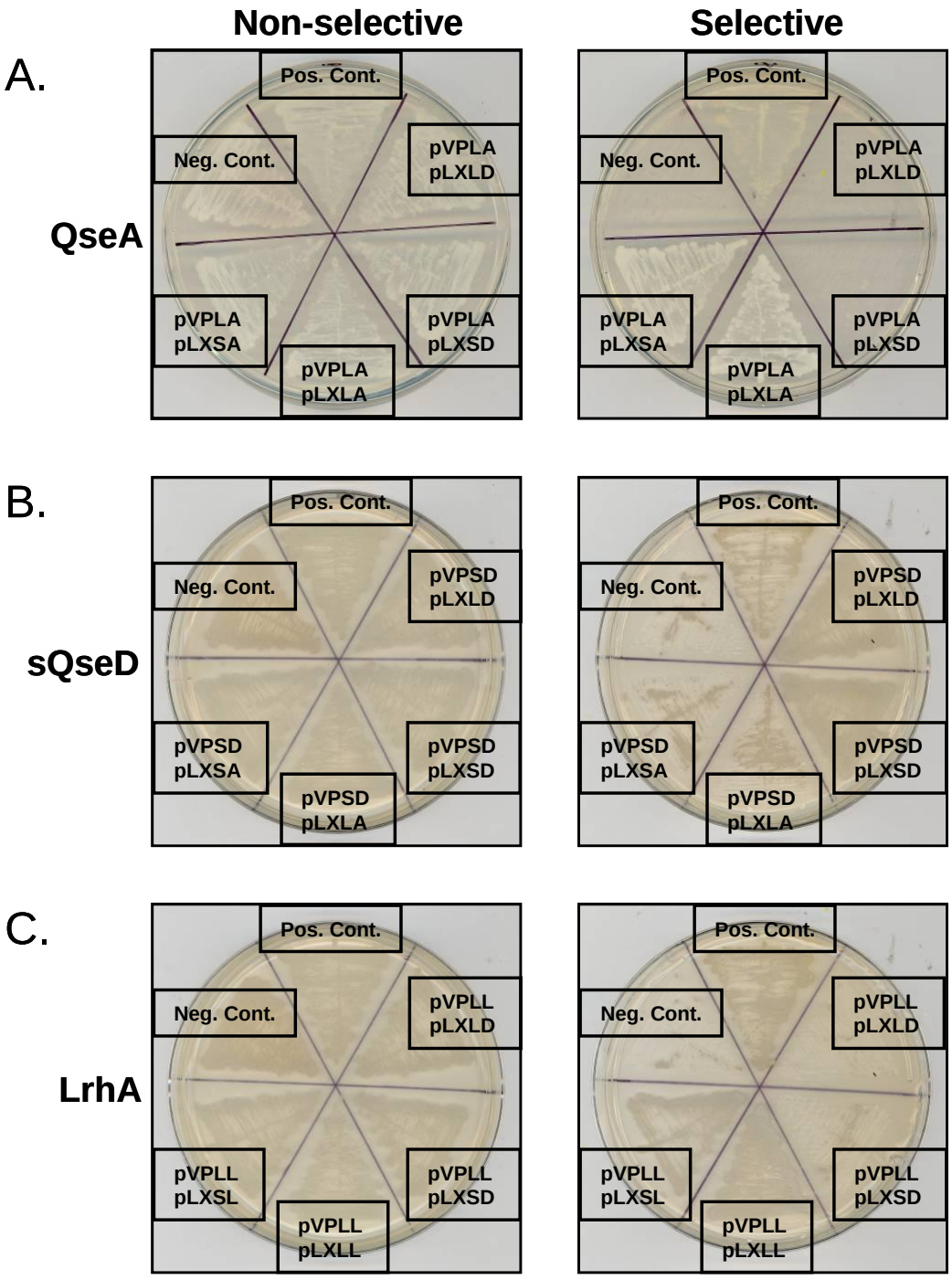


Figure 4.7. EHEC 86-24 sQseD, and K-12 *E. coli* lQseD do not interact with QseA or LrhA. Representative yeast two hybrid non-selective (+Histadine) plates, and selective (-Histadine) plates, depicting the potential protein-protein interactions of the LysR-like proteins **(A)** QseA, **(B)** sQseD, and **(C)** LrhA.

DISCUSSION

Genetic regulation in human pathogens is a complex process, and regulation in EHEC is no exception. EHEC contains multiple levels of hierarchical signal transduction pathways that converge with intracellular regulatory proteins in order to alter genetic expression. In EHEC, virulence factor expression is regulated through the sensing of epinephrine, norepinephrine and AI-3 by the two two-component sensor kinases QseC and QseE [1, 85]. These signals are then integrated using intracellular regulatory proteins such as the LTTR QseA, in a complex process where numerous aspects remain unresolved.

Here we describe QseD, a cell-to-cell communication regulated LTTR in EHEC. Expression of *qseD* is most highly induced in stationary phase. In EHEC, QseD represses expression of *iraD*, the LEE (Figure 4.3), and alters AE lesion formation (Figure 4.5). However, in K-12 *E. coli*, but not in EHEC, QseD down-regulates expression of the flagella regulon, decreasing motility (Figure 4.4). Flagella expression is regulated in a hierarchical fashion, coupled with this machines assembly in *E. coli* [184]. The decision to make a flagellum starts at the level of regulation of the *flhDC* genes, encoding the flagella master regulators. Most of the transcriptional regulation of the flagella regulon occurs at the *flhDC* regulatory region [184]. It should be noted that there are considerable differences between the *flhDC* regulatory regions between EHEC and K-12, with K-12 having an IS inserted within this region, which alters *flhDC* transcription and consequently motility [297]. While we originally hypothesized that the genetic regulatory differences in the two *E. coli* backgrounds could be explained by the apparently lack of an HTH on the EHEC sQseD isoform, complementation studies

demonstrated that both isoforms of the protein were able to complement flagellar gene expression in K-12, where differences in the *flhDC* regulatory region may be the underlying reason of this differential regulation between EHEC and K-12.

This trend of both isoforms of QseD being able to rescue QseD-regulated phenotypes extends to regulation of *iraD* (Figure 4.3) and the LEE (Figure 4.5) in EHEC, arguing that although O157:H7 *E. coli* possess a truncated form of QseD, this regulatory protein is still regulating similar targets to full length QseD. However, one has to take into consideration that unlike the lQseD, the sQseD isoform lacks a DNA-binding HTH domain, and does not control gene expression by directly binding to its target genes (Figure 4.6). We have therefore proposed a model whereby selection for a truncated QseD isoform was compensated for by its ability to interact with other LTTRs. We first tested whether LEE gene regulation by sQseD occurred through its interaction with other known LEE-regulating LTTRs, such as QseA [86] and LhrA [300]. However, sQseD failed to interact with both of these LTTRs (Figure 4.7), suggesting that this regulation might occur through interactions with other yet unidentified LTTRs involved in LEE regulation.

Considering that LTTRs is one of the most widespread classes of transcriptional regulators in bacteria, and that *E. coli* has over 60 LTTRs [5, 301] encoded within its genome, it is plausible that sQseDs interacting partner has not been identified yet. As depicted in the QseD regulatory model in Figure 4.8, while lQseD is able to directly bind to the *ler* promoter, sQseD must interact with an additional positive *ler* regulatory protein (designated as LysR-X) in a dominant negative manner, thereby preventing transcription activation (Figure 4.8).

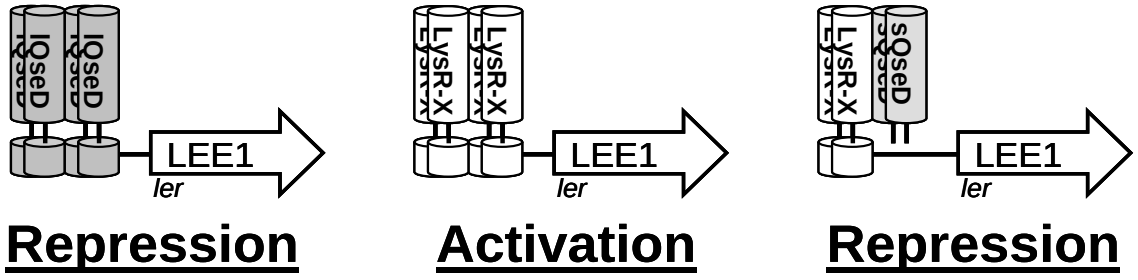


Figure 4.8. Model of the regulatory role of lQseD and sQseD in EHEC 86-24. In non-O157:H7 EHEC strains the presence of full-length "long" QseD (lQseD) represses *ler* and LEE transcription. In the absence of lQseD, an as yet unidentified LysR (LysR-X), activates LEE transcription. However in O157:H7 EHEC strains, the presence of truncated "short" QseD (sQseD) represses *ler* and LEE transcription presumably through dominant negative interactions with LysR-X and resulting in incomplete DNA remodeling and transcriptional activation.

While direct protein-protein interactions between multiple LTTR's has never been proven, hetero-dimerization with additional LTTRs or members of alternative regulatory protein family has been predicted [242-243, 302-303].

Evolutionary selection for a truncated LTTR is not unheard of, although to date such proteins have been reported to lack regulatory function [304]. As demonstrated by its prevalence almost exclusively in the O157:H7 serotype, sQseD must have been selected for late in an evolutionary branch close to the origin of this now highly prevalent and virulent EHEC serotype (Figure 4.9, Table 4.1). This evolutionary selection could afford QseD a higher plasticity concerning gene regulation by interacting with one or more LTTRs, and expanding the breath of its regulatory cascade. This hypothesis is compelling considering that sQseD was shown to regulate a higher number of genes than lQseD in our transcriptome studies (Tables 4.2, and 4.3). After compiling a collection of sequenced *E. coli* isolates, in which QseD isoforms were characterized (Table 4.1), it is suggested that QseD truncation occured in between the emergence of O157:H7 EHEC from its predicted ancestor O55:H7 atypical EPEC (aEPEC) (Figure 4.9).

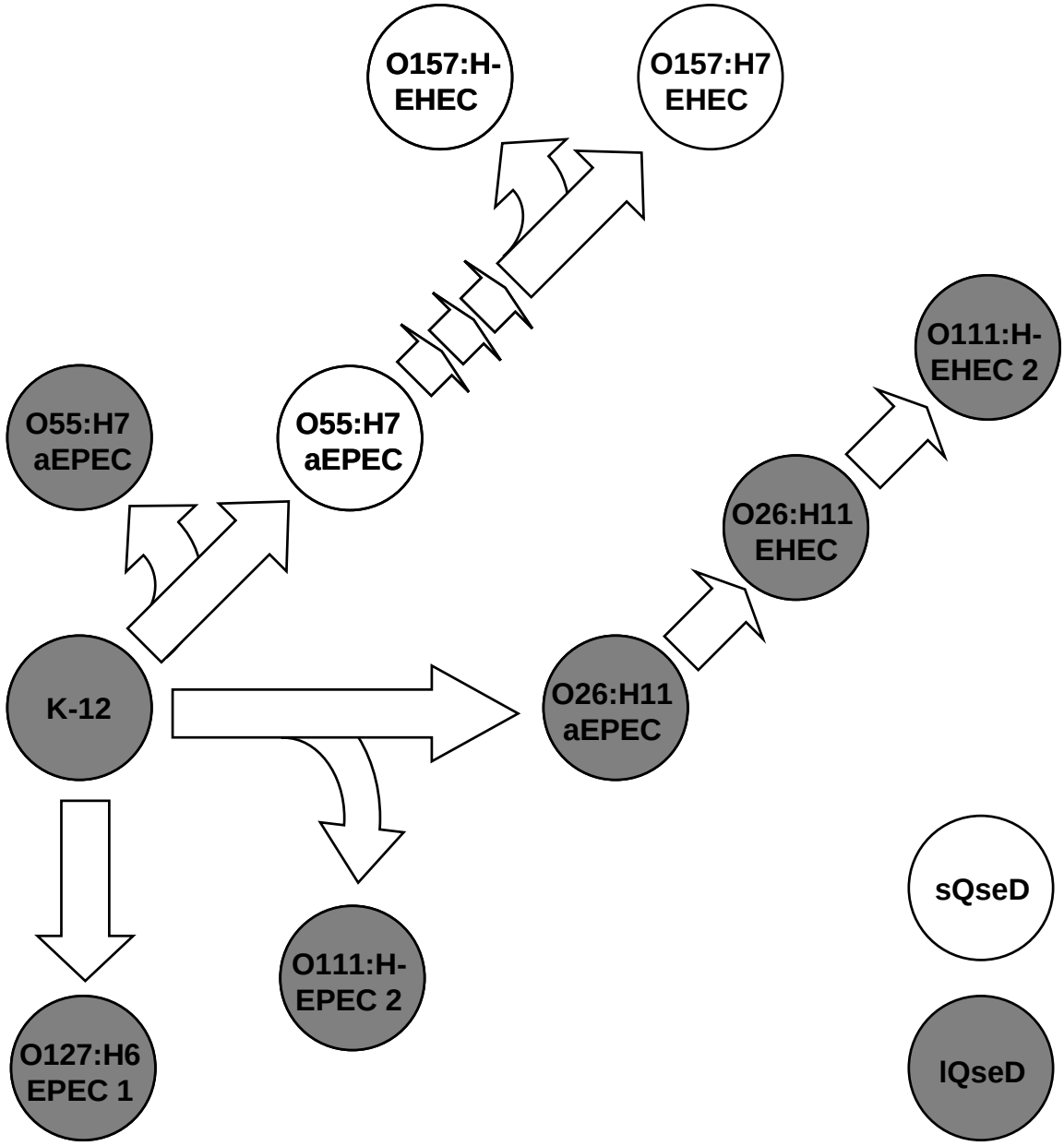


Figure 4.9. Evolution and prevalence of the various isoforms of QseD in *E. coli*. Cartoon representation of the evolution of EPEC and EHEC from their prototypical non-pathogenic K-12 ancestor. Solid (gray) strains represent the presence of lQseD and open (white) strains represent the presence of sQseD (adapted from [291].

The evolutionary advantage gained by O157:H7 isolates due to the truncation of QseD still remains to be determined. However, one possibility is that through the regulation of *iraD*, a positive regulator of RpoS stability that was identified in a screen for genes that increased the error rate of replication in *E. coli* [295], QseD differentially regulates EHECs ability to respond to cellular stress. A pathogen's ability to respond to stress is critical, and in EHEC it has been linked to pathogenesis through multiple pathways including: improving survival in the acidic environments such as those encountered in the host stomach [305], and an altered RpoS response [88, 306], which may lead to additional levels of regulation of LEE and hemolysin expression [307]. It should also be noted that *qse*D is located at the end of an operon containing four genes, including *yjiG* and *yjiH* that are predicted to form an inner membrane spanning channel, and *iadA*, which is an isoaspartyl dipeptidase involved in the sensing and cleavage of damaged, degraded, or mis-folded proteins, that is well suited for dealing with cellular stress (Figure 4.1A) [308-309].

The regulation of virulence in enteric pathogens such as EHEC, which often involves the integration of multiple signals, is extremely complex and hierarchical. While we have identified and added a new member to this regulatory family, the true complexity of QseDs regulatory function and network remains to be foreseen. Only through the elucidation of these virulence regulating networks will we gain the knowledge essential to understanding EHEC pathogenesis, and necessary to develop new tools and treatments against this deadly human pathogen.

CHAPTER FIVE

REGULATORY EFFECTS OF LsrR IN EHEC

INTRODUCTION

Enterohemorrhagic *Escherichia coli* O157:H7 (EHEC) causes outbreaks of hemorrhagic colitis and hemolytic uremic syndrome (HUS). EHEC is a member of a larger group of enteric pathogens that cause attaching and effacing (AE) lesions on intestinal epithelial cells due to a type three secretions system (TTSS) encoded by the locus of enterocyte effacement (LEE) pathogenicity island (PA) [60-64]. The LEE is composed of 41 genes, most of which arranged into five major operons named *LEE*1 through *LEE*5 [65]. Within the *LEE*1 operon lies the *ler* gene encoding a regulator that activates transcription of all LEE genes [65-66]. Regulation of the LEE is highly complex involving multiple regulatory proteins and pathways [4, 63, 66, 68-85]. EHEC produces several additional virulence factors including flagella, which allow for bacterial motility, an SOS induced phage encoded Shiga toxin (Stx), and several non-LEE encoded (Nle) secreted effectors [21, 136, 310].

Bacteria produce diffusible small hormone-like molecules known as autoinducers (AI) in order to communicate with each other, sense their surrounding population diversity, signal their cellular metabolic state, and regulate gene expression [207-208]. Pathogens not only utilize their own cellular signaling systems, but also take advantage of host cell signaling molecules, such as hormones, in order to sense their host environment and to coordinately regulate their virulence gene expression [78, 230, 294]. EHEC senses and responds to the human host produced catecholamines epinephrine and norepinephrine, through two two-component systems, QseBC and QseEF [1, 85]. It also senses the bacterial cell-cell signaling molecules AI-2 and AI-3 through LsrR, and QseBC respectively [230, 293]. Whereas the breadth of AI-3 signaling has not been as extensively characterized [232], AI-2, the enzymatic product of LuxS, is proposed to promote interspecies signaling in a broad range of bacterial species [9].

LuxS is a bacterial enzyme that is involved in S-adenosylmethionine (SAM) metabolism. SAM is the substrate for methylation of many cellular molecules. LuxS converts SAM into the toxic intermediate 5-S-adenosyl-homocysteine (SAH), which is hydrolyzed by the enzyme Pfs to 5-ribosyl-homocysteine (RH) [212]. RH is then cleaved by LuxS to form homocysteine and the AI-2 precursor, 4,5 dihydroxy-2,3-pentanedione (DPD). DPD is an unstable molecule that spontaneously cyclizes into several furanones, collectively referred to as AI-2. SAM itself is necessary for the methylation of proteins and nucleic acids to occur, and without its constant regeneration, cell division defects occur [213].

LuxS mutants have been used in multiple bacterial studies to explore the effect of AI-2 loss on cellular signaling and virulence [222-225]. Due to the integral involvement

of LuxS in central metabolism and the cellular methyl cycle, the lack of an AI-2 sensor in several *luxS* positive bacteria [226], and the apparent spontaneous formation of AI-2 in the absence of *luxS* [227], many studies are still necessary to fully address *luxS* function [211]. To uncouple AI-2 signaling from metabolic effects due to the *luxS* mutation, addition of exogenous AI-2 is necessary [228-229]. AI-2 is recognized by three periplasmic proteins LuxP in *Vibrio* species [209], LsrB and possibly LsrB orthologs in *Salmonella typhimurium*, *Escherichia coli*, and other enteric species [8, 215], and RbsB in *Actinobacillus actinomycetemcomitans* [214]. In *Vibrio* species, LuxP binds to the LuxQ membrane-bound histidine sensor kinase, modulating its activity in order to regulate luminescence [311]. Upon binding to LsrB and RbsB, AI-2 is imported through the ABC transporters LsrAC and RbsAC [8, 312]. Once within the cell, AI-2 is phosphorylated by the LsrK kinase and interacts with LsrR [313]. LsrR, a member of the SorC type transcriptional regulator (STTR) family, has been shown in *Salmonella typhimurium* and *E.* coli to repress the importation, phosphorylation, and degradation of AI-2, by the direct negative regulation of the *lsrACDB*, *lsrK*, and *lsrFG* genes when it is in the phospho-AI-2 unbound state [8-10].

The luxS/AI-2 signaling system has been implicated in biofilm formation in multiple bacterial species [8, 314-315]. The ability of EHEC to colonize multiple organic and inorganic surfaces and to form biofilms has been well documented [316-318]. Although multiple factors and conditions, conductive to biofilm formation have been identified and characterized [319-323], the exact extent and role of chemical signals during biofilm formation is still unknown. While some chemical cues, such as epinephrine and norepinephrine have been shown to increase biofilm production [324],

others such as indole reduce them [325]. In *E. coli*, LsrR has been implicated, along with LsrK, in AI-2 dependent regulation of biofilm architecture, and small-RNA (sRNA) expression [11]. Additionally AI-2 signaling has been suggested to affect pathogenesis and motilty in EHEC [12].

In this study we took an unbiased approach and generated all the single and combinatorial mutants of *luxS, lsrR,* and *lsrK* in an EHEC 86-24 WT background, and analyzed the effect on bacterial phenotypes that ranged from cellular attachment and biofilm production, to motility and TTSS expression. This allowed us to simultaneously, in one background, dissect the regulatory role of AI-2 from the metabolic defects in the *luxS* mutant strains, and analyze the breadth and diversity of LsrR regulated phenotypes. Herein we report that while the Δ*lsrR* and Δ*luxS* both exhibit reduced motility and biofilm production that the combinatorial Δ*lsrR*/Δ*luxS* strain displays an inverted phenotype where it exhibits increased motility and biofilm production. Additionally, while it has been reported that LEE expression is down-regulated in the Δ*luxS* EHEC strain [78], we demonstrate that in a Δ*lsrR* EHEC strain expression of the LEE is up-regulated. Together these results demonstrate that while the LsrR regulatory network overlaps with and utilizes LuxS-produced AI-2, that its function is not completely dependent upon AI-2.

RESULTS

The LsrR regulon in EHEC.

In *E. coli* and *Salmonella typhimurium* LsrR has been shown to repress the importation, phosphorylation, and degradation of AI-2 by directly inhibiting transcription of the *lsrACDB*, *lsrK*, and *lsrFG*, respectively (Figure 5.1) [8-10]. In *E. coli*, LsrR and LsrK have also been implicated in AI-2 dependent regulation of biofilm architecture, small-RNA (sRNA) expression [11], and pathogenesis [12]. To asses the effects of AI-2 signaling, and determine the breadth of the regulatory role of LsrR in EHEC gene expression and/or pathogenesis, we constructed non-polar mutants of *lsrR*, the gene *lsrK* encoding the AI-2 kinase, and the combinatorial mutant *lsrRK*, in both the WT EHEC strain 86-24 and its isogenic *luxS* mutant.

Affymetrix *E. coli* 2.0 microarrays were used to compare expression profiles of the WT EHEC to the corresponding isogenic *lsrR* mutant. These arrays contain ~10,000 probe sets, covering the genomes of two sequenced EHEC strains (EDL933 and Sakai), K-12 strain MG1655, uropathogenic *E. coli* (UPEC) strain CFT073, and 700 probes to intergenic regions that can encode non-annotated ORF's, or small reguatory RNAs. In EHEC during growth in DMEM, a condition known to induce virulence gene expression, 341 probe sets were up-regulated (66 EHEC specific), and 901 were down-regulated (214 EHEC specific) in the *lsrR* mutant (Table 5.1).

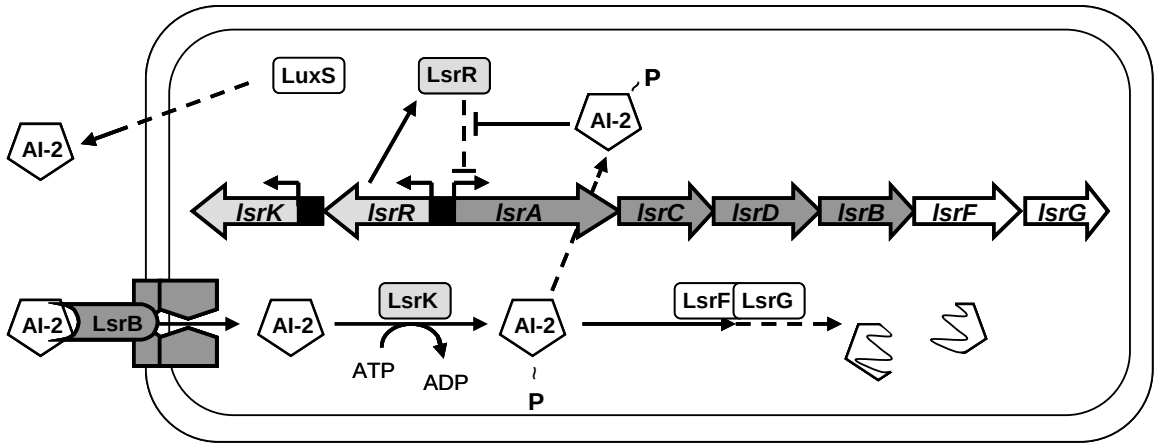


Figure 5.1. Cartoon representation of AI-2 dependent regulation of the LsrR regulon. LsrR negatively regulates transcription of the *lsrACDBFG* operon (*lsrR* and *lsrK*). Upon binding to LsrB, AI-2 is imported through the ABC transporter LsrACD complex where it is phosphorylated by LsrK and binds to LsrR. LsrR-phospho-AI-2 is unable to bind to DNA, thereby derepressing the expression of the LsrR regulon. Increased expression of the AI-2 binding protein LsrB, and the AI-2 ABC transporter LsrACD complex then deplete AI-2 from the environment. Phospho-AI-2 is degraded by LsrF and LsrG, removing inhibition of LsrR binding and repression of the LsrR regulon resumes.

Table 5.1. Comparison of WT EHEC 86-24 to the Δ*lsrR* under various growth conditions.

	Increased	**Marg. Incr.**	**Decreased**	**Marg. Decr.**	**No Change**
EHEC-	341	388	901	340	8238
EHEC-LB	317	557	2419	125	6790

Increased and decreased are at least two fold changes in expression levels. Marginally increased or decreased are changes that are either less than two fold or designated as such by the Affymetrix anaylsis software GCOSv1.4. EHEC was grown to an OD_{600} of 1.0 in either low glucose DMEM or LB media.

Table 5.2. Pathovar distribution of altered gene expression in the EHEC Δ*lsrR*.

	MG1655	**EDL933**	**Sakai**	**CTF073**	**Intergenic**
EHEC "86-24"-					
Decreased	279	214	52	183	147
Marg. Decreased	150	69	19	52	49
Increased	190	66	13	59	13
Marg. Increased	279	70	9	17	9
No Change	3172	1787	280	2175	1079
EHEC "86-24"-LB					
Decreased	756	820	123	252	435
Marg. Decreased	55	27	3	11	27
Increased	174	51	14	61	15
Marg. Increased	391	101	18	32	15
No Change	2694	788	215	2130	805

Increased and decreased are at least two fold changes in expression levels. Marginally increased or decreased are changes that are either less than two fold or designated as such by the Affymetrix anaylsis software GCOSv1.4. EHEC was grown to an OD_{600} of 1.0 in either low glucose DMEM or LB media.

The largest number of genes with altered expression were found in the *E. coli* K-12 strain MG1655 genome (46%), which represent a common *E. coli* backbone conserved among *E. coli* pathovars (Table 5.2) [296]. In comparison, when EHEC was grown in LB, there were considerably more genes with altered expression levels, 317 up-regulated (51 EHEC specific), and 2419 down-regulated (820 EHEC specific) in the *lsrR* mutant. Additionally, while the largest number of genes with altered expression was still found in the *E. coli* K-12 strain MG1655 genome (40%), a much greater percentage of EHEC specific genes were altered in LB (30%) versus DMEM (22%). Taken together these data suggest that in EHEC, LsrR has multiple additional regulatory targets outside of *lsrK* and the *lsrACDBFG* operon, and that a greater percentage of this regulatory repertoire is affected when EHEC is grown in a nutrient rich environment such as LB.

In *E. coli*, the LsrR mutant was expected to have enhanced expression of *lsrK* and the *lsrACDBFG* operon [3], and to exhibit increased AI-2 uptake [10], due to the lack of LsrR repression of the AI-2 LsrACB transporter [8]. Indeed in the EHEC *lsrR* mutant gene arrays, expression of *lsrK* and the *lsrACDBFG* operon were up-regulated (Figure 5.2A). Consistent with the array data we observed that expression of *lsrA* was up-regulated in the *lsrR* mutant by real-time RT-PCR (Figure 5.2B), and that the *lsrR* mutant exhibited increased uptake of AI-2 from the environment (Figure 5.2C), thus confirming that phenotypes previously reported to be under LsrR control, were reproduced in our mutant.

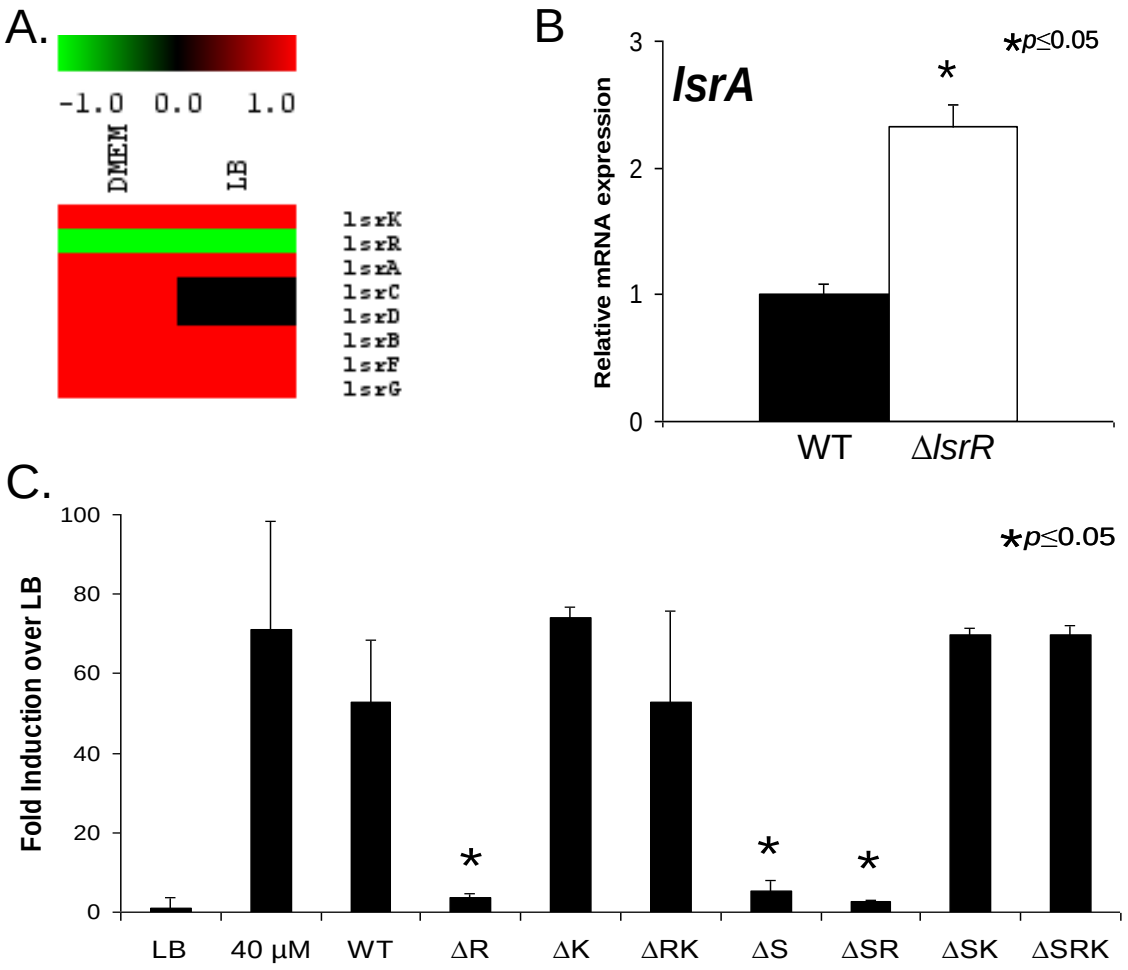


Figure 5.2. In EHEC LsrR down-regulates expression of *lsrK* and the *lsrACDBFG* operon and regulates AI-2 import. (A) Heat maps generated from the Δ*lsrR* microarray analysis depicting the up-regulation of the LsrR regulon in the absence of LsrR repression. **(B)** QRT-PCR of *lsrA* in WT EHEC 86-24, Δ*R*, Δ*K*, Δ*RK*, Δ*S*, Δ*SR*, Δ*SK*, and Δ*SRK* grown in DMEM (OD_{600} of 1.0). **(C)** AI-2 remaining in the supernatant after one-hour incubation with the EHEC WT and mutant strains was quantitated by addition to the *Vibrio* AI-2 reporter strain BB170. Results were measured in triplicat, and represent the fold increase over LB addition. Strains represented: WT = EHEC 86-24, Δ*R* = Δ*lsrR*, Δ*K* = Δ*lsrK*, Δ*RK* = Δ*lsrRK*, Δ*S* = Δ*luxS*, Δ*SR* = Δ*luxS* Δ*lsrR*, Δ*SK* = Δ*luxS* Δ*lsrK*, and Δ*SRK* = Δ*luxS* Δ*lsrRK*.

LsrR, LsrK, and LuxS individually and combinatorially regulate motility, biofilm formation and pathogenesis in EHEC.

In order to better assess LsrR and AI-2 regulation in EHEC, several other mutants in this pathway were generated. Expression of the *lsrACDBFG* operon is repressed by LsrR [8-10]. Upon binding of phospho-AI-2 to LsrR, this repression is relieved, because LsrR complexed with phospho-AI-2 can no longer bind to the regulatory region of *lsrACDBFG* [220, 313]. Hence, an *lsrK* mutant, which can no longer phosphorylate AI-2, cannot promote de-repression of *lsrACDBFG* expression, due to the absence of phosphorylated AI-2 binding to LsrR (Figure 5.2C). Consequently, the *lsrK* mutant should uptake less AI-2 than the WT strain (Figure 5.2D) according to the model proposed for AI-2 uptake in *E. coli* [3]. Additionally, one would expect that a *lsrRK* double mutant would have similar phenotypes to the *lsrR* mutant, given that in the absence of LsrR, whether or not AI-2 is phosphorylated, transcription of the *lsrACDBFG* operon should be derepressed increasing AI-2 uptake. Surprisingly, the EHEC *lsrR lsrK* double mutant had similar levels of AI-2 uptake to WT (Figure 5.2D). These data suggest that repression of *lsrACDBFG* transcription, by phospho-AI-2, also occurs in a LsrR-independent fashion. AI-2 uptake was enhanced in the *luxS* mutant compared to WT because it can not produce endogenous AI-2; hence it depletes almost all of the synthetic AI-2 provided in this assay (Figure 5.2D). The double *luxS lsrR* mutant also shows enhanced AI-2 uptake, consistent with the lack of LsrR-phospho-AI-2 repression of *lsrACDBFG* expression. The *luxS lsrK* double mutant is also defective in AI-2 uptake, which is consistent with lack of AI-2 phosphorylation, and consequently lack of LsrR-phospho-AI-2 to derepression the expression of the LsrACB transporter. The triple *luxS*

lsrR lsrK mutant has a similar phenotype to the *luxS lsrK* double mutant suggesting again that repression of *lsrACDBFG* expression also occurs in a LsrR-independent fashion.

LsrR mutant arrays demonstrated regulation of multiple genetic pathways in addition to the LsrACB transporter. One of these pathways was the flagella regulon (Figure 5.3A) [12, 294, 326]. Expression of the flagella regulon in K-12 *E. coli* has been reported to be unaffected by LsrR and LsrK [11]. In contrast our assay data suggested that transcription of the flagella regulon in EHEC is activated by LsrR. This difference in regulation of flagella expression by LsrR can be explained by differences between the regulatory region of *flhDC*, the master regulator of the flagella regulon, between K-12 and EHEC. In K-12 *E. coli* there is an insertion sequence (IS) in the regulatory region of *flhDC* that is absent in EHEC [297]. The presence of this IS has been reported to alter *flhDC* expression, and consequently motility. In agreement with our transcriptome data, motility of the *lsrR* mutant is decreased at both 37º and 30º C (Figure 5.3B,C). Motility is also decreased in the *luxS*, *lsrK*, and *lsrRK* mutants. These data suggest that LsrR complexed with phospho-AI-2 activates expression of the flagella regulon, and consequently motility. However, the observation that motility is not decreased, and in some instances (at 37º C) enhanced in the *luxS lsrR*, *luxS lsrK*, and *luxS lsrR lsrK* mutants was surprising. In these three mutants AI-2 is completely absent, while it is present in the *lsrR*, *lsrK*, and *lsrR lsrK* mutants. One potential explanation for these results is that non-phosphorylated AI-2, in an LsrR-independent manner, represses motility, suggesting that non-phospho-AI-2 may interact with another transcription factor in EHEC.

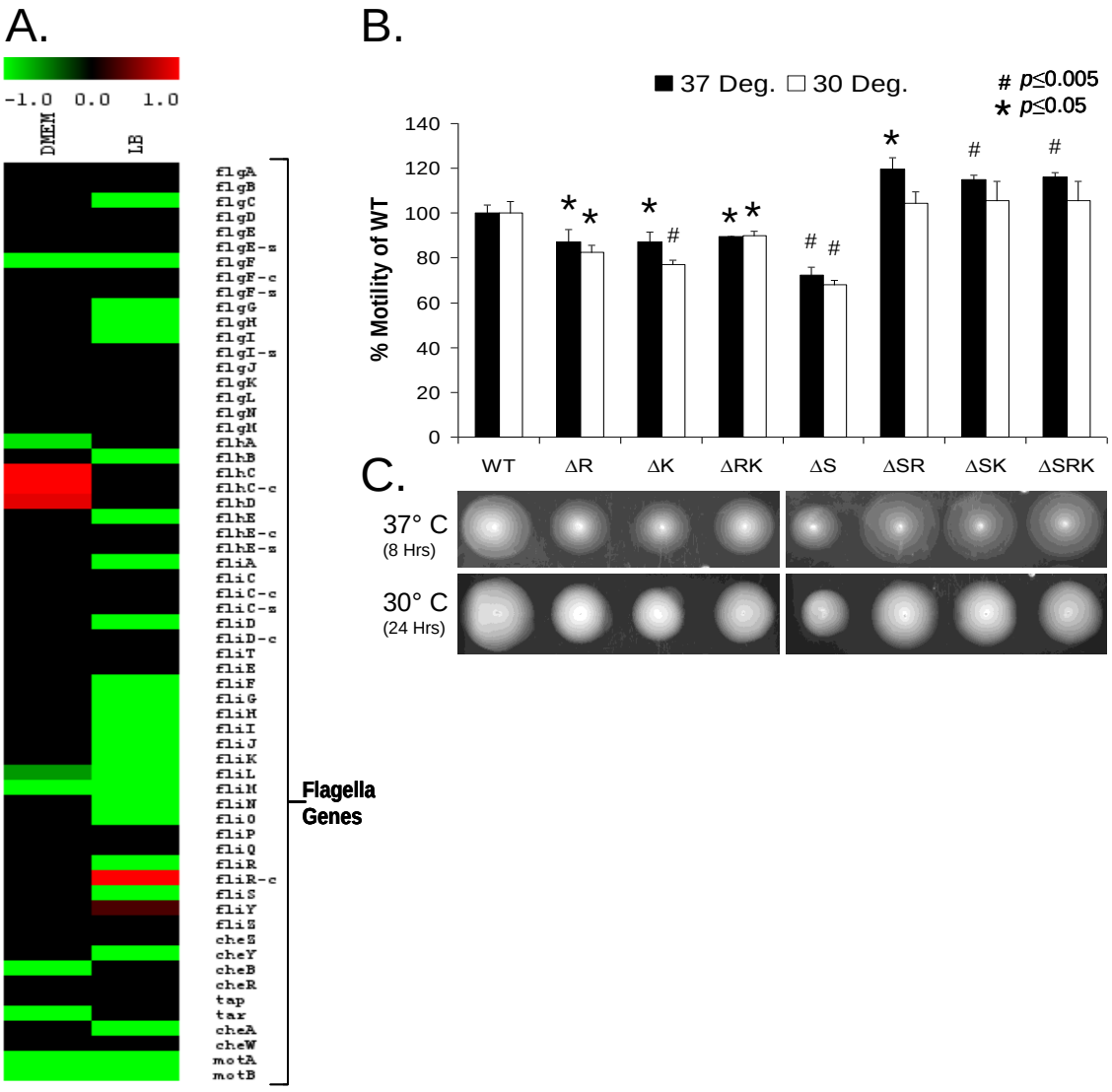


Figure 5.3. LsrR, LsrK and LuxS regulate motility in EHEC. (A) Heat maps generated from the Δ*lsrR* microarray analysis depicting the down-regulation of the flagella regulon in the absence of LsrR activation. **(B)** Quantification of motility plates **(C)** comparing WT EHEC 86-24 to its corresponding Δ*lsrR*, Δ*lsrK*, Δ*lsrRK*, Δ*luxS*, Δ*luxS* and Δ*lsrR*, Δ*luxS* and Δ*lsrK*, and Δ*luxS* and Δ*lsrRK*. Strains represented: WT = EHEC 86-24, Δ*R* = Δ*lsrR*, Δ*K* = Δ*lsrK*, Δ*RK* = Δ*lsrRK*, Δ*S* = Δ*luxS*, Δ*SR* = Δ*luxS* Δ*lsrR*, Δ*SK* = Δ*luxS* Δ*lsrK*, and Δ*SRK* = Δ*luxS* Δ*lsrRK*.

It has been reported that in E. coli *K-12*, *lsrR* and the *lsrK* mutants showed repression of biofilm formation [11]. We demonstrate that indeed the EHEC *lsrR* mutant display reduced biofilm formation, but unlike in K-12 the *lsrK* mutant only exhibited a minor defect in biofilm formation. In EHEC, the Δ*R*, Δ*RK*, and Δ*S* strains exhibited decreased biofilm formation at both 37º and 30º C, while the Δ*K* strain exhibited decreased biofilm formation only at 30º C (Figures 5.4A,B). These results mirror the motility phenotypes previously demonstrated for these mutant strains (Figure 5.3B,C). It has been reported that the addition of AI-2 increases biofilm formation in K-12 *E. coli* [326]. Indeed while we did observe an increase in biofilm formation in the *luxS* mutant (4 fold) upon AI-2 addition, we observed no effect on biofilm formation in any of the additional mutants or WT EHEC upon the addition of AI-2 (Figure 5.5). Complementation *in trans* with *sahH* from *Pseudomonas aeruginosa*, which compensates for the metabolic defects but not AI-2 production in the Δ*luxS* strain (Figure 5.5A), also increased biofilm production in the *luxS* mutant suggesting that decreased biofilm formation in the Δ*luxS* mutant results from signaling and metabolic effects. Interestingly, complementation with *sahH* reduced biofilm formation in *luxS* positive strains (Figure 5.5B). This suggests that in LuxS positive strains, there may be diminished AI-2 production, due to the removal of the LuxS substrate SAH by SahH, reducing biofilm formation.

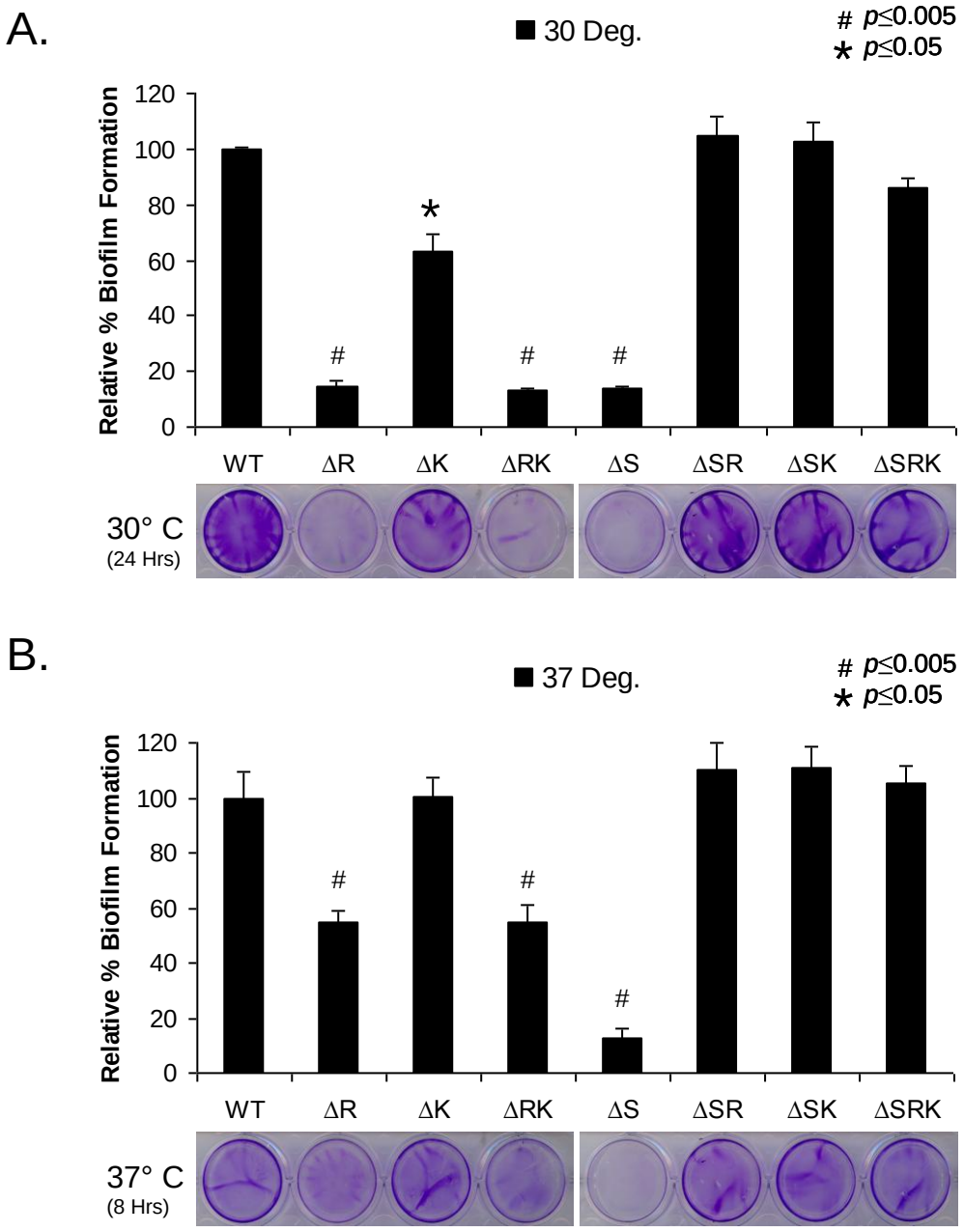
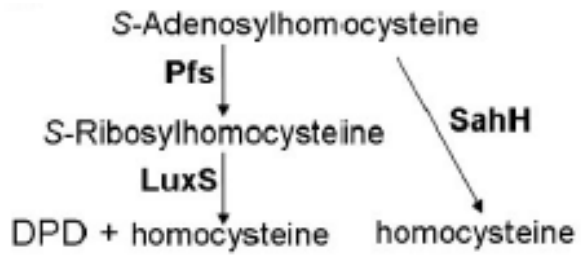


Figure 5.4. LsrR's and LsrK's regulatory affect on biofilm formation in EHEC is dependent upon LuxS. Quantification of crystal violet biofilm plate assays comparing the *in vitro* biofilm forming ability of WT EHEC 86-24 to its corresponding isogenic mutants at **(A)** 30º C and **(B)** 37º C. Strains represented: WT = EHEC 86-24, Δ*R* = Δ*lsrR*, Δ*K* = Δ*lsrK*, Δ*RK* = Δ*lsrRK*, Δ*S* = Δ*luxS*, Δ*SR* = Δ*luxS* Δ*lsrR*, Δ*SK* = Δ*luxS* Δ*lsrK*, and Δ*SRK* = Δ*luxS* Δ*lsrRK*.

A.



B.

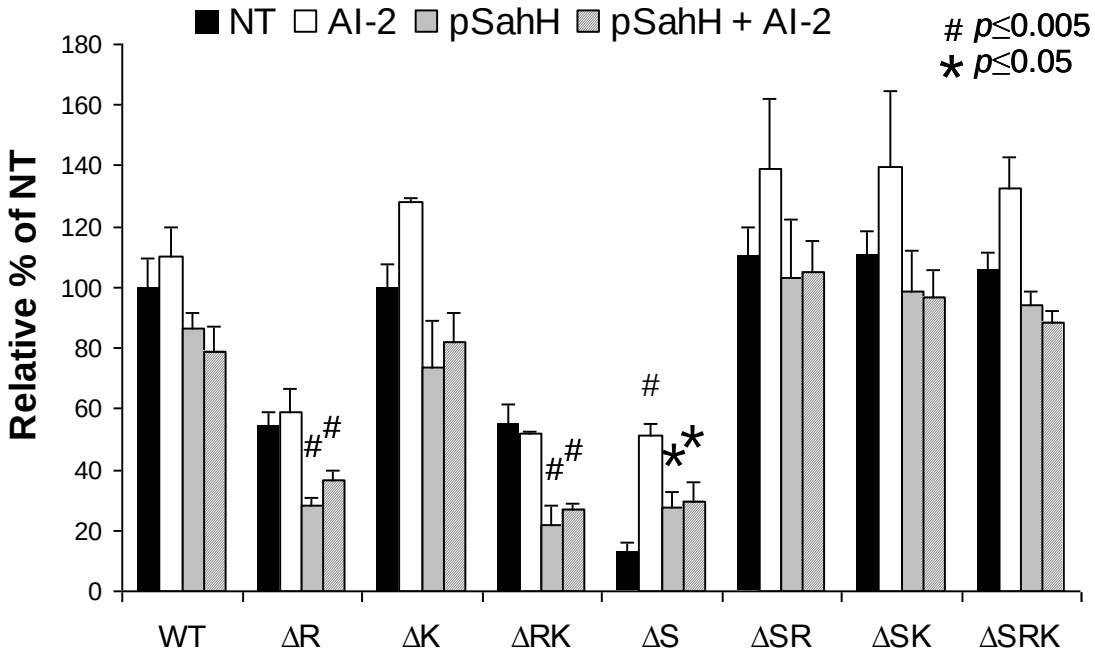


Figure 5.5. Biofilm production in EHEC is dependent upon AI-2. (A) The SahH SAM metabolism pathway does not produce AI-2. **(B)** Quantification of crystal violet biofilm plate assays comparing the *in vitro* biofilm forming ability of WT EHEC 86-24 and its isogenic mutants (black bars), upon the addition of 40 µM AI-2 (white bars), complementation with pSahH (gray bars), and complementation with pSahH and the addition of 40 µM AI-2 (striped bars). Strains represented: WT = EHEC 86-24, Δ*R* = Δ*lsrR*, Δ*K* = Δ*lsrK*, Δ*RK* = Δ*lsrRK*, Δ*S* = Δ*luxS*, ΔS*R* = Δ*luxS* Δ*lsrR*, ΔS*K* = Δ*luxS* Δ*lsrK*, and ΔS*RK* = Δ*luxS* Δ*lsrRK*.

Microarrays demonstrated that in the EHEC Δ*lsrR* strain, the LEE PAI expression was up-regulated in DMEM (Figure 5.6A). Expression of the LEE genes is regulated at multiple transcriptional and post-transcriptional levels. The first gene within the *LEE1* operon encodes for Ler, the master activator of LEE transcription. We have previously shown that transcription of *ler* (*LEE1*), and the *LEE2*, and *LEE3* operons are not enhanced by AI-2. However, expression of the *LEE4* and *LEE5* operons is enhanced by AI-2 [284]. Our array data shows that genes within *LEE4* and *LEE5* operons were up regulated in the *lsrR* mutant (Figure 5.6A), which may suggest that LsrR represses transcription of these genes, and that upon complexing with AI-2 that this repression is relieved. In addition to the LEE genes, expression of genes encoding non-LEE encoded effectors (Nles) were differentially regulated in the *lsrR* mutant (Figure 5.6B). Expression of *stx* was unaffected by LsrR (Figure 5.6C). Additionally while AI-2 has been reported to increase HeLa cell adherence [12], we find no evidence for such a phenotype in any of the mutants that we tested (Figure 5.7).

We therefore concluded that the regulatory role of LsrR in EHEC, which is not consistant with its characterized role in commensal K-12 *E. coli*, is to induce biofilm formation and motility, and that it also plays a role in regulating pathogenicity through alterations of the TTSS expression. Additionally while the regulatory role of LsrR seems to overlap with that of the AI-2/LuxS system, all the data can not be easily explained simply based upon the ability of phospho-AI-2 to negate DNA binding by LsrR.

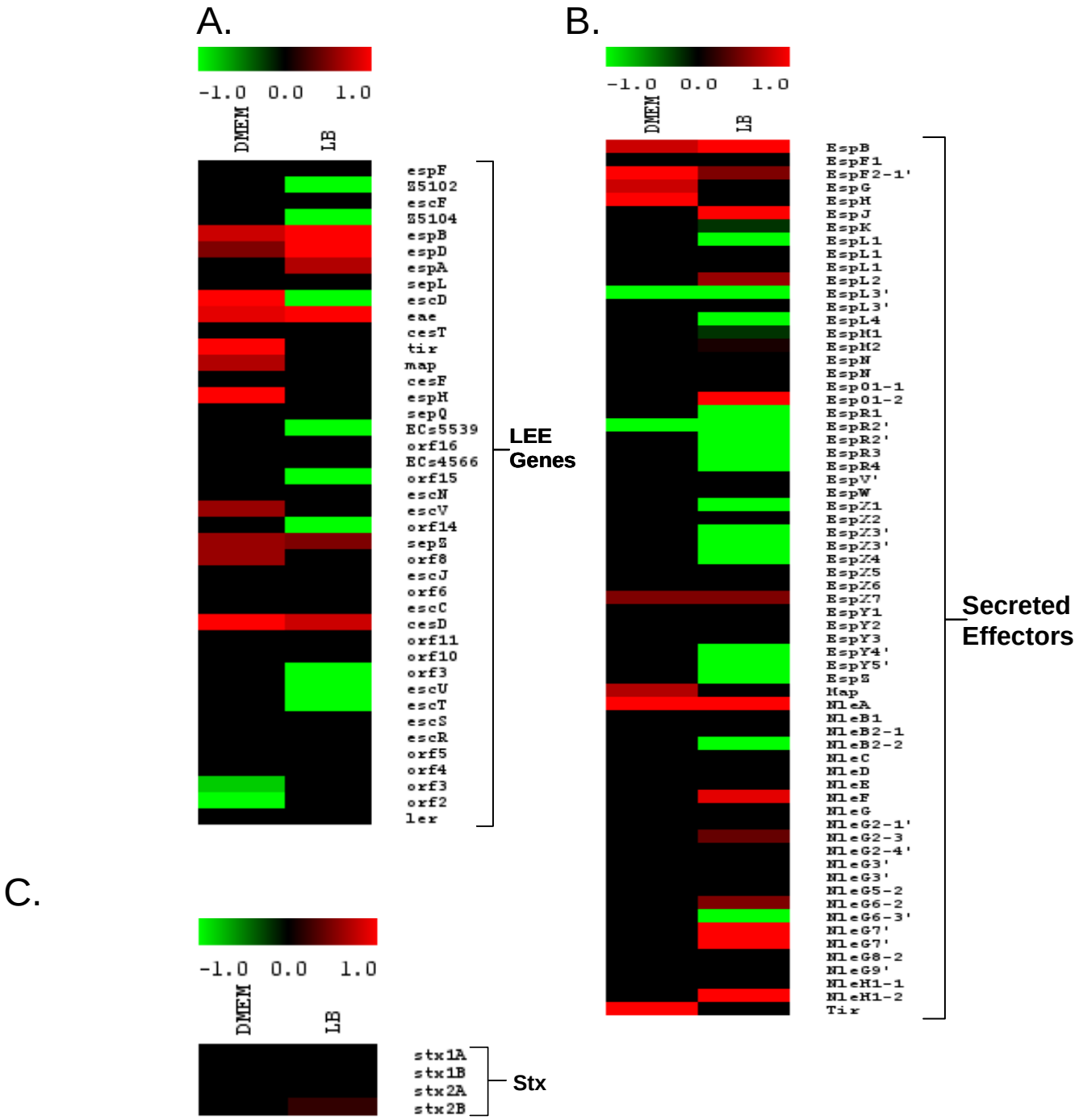


Figure 5.6. LsrR regulates the LEE pathogenicity island and the Nle's but not Stx in EHEC. Heat maps generated from the Δ*lsrR* microarray analysis depicting the **(A)** up-regulation of the LEE PAI, **(B)** the differential regulation of the Nle's, and **(C)** the lack of Stx regulation in the absence of LsrR signaling.

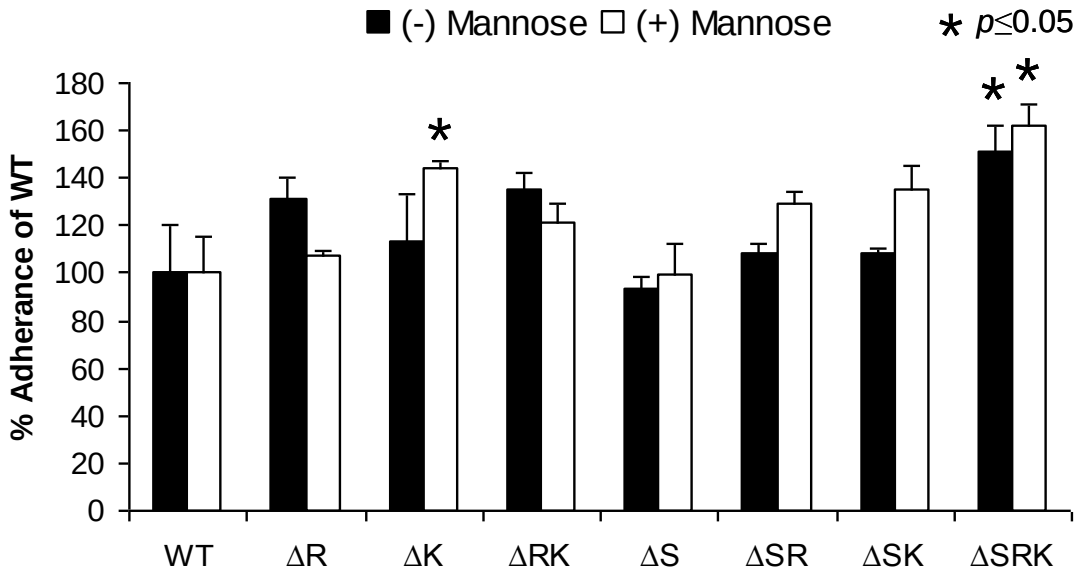


Figure 5.7. LsrR/AI-2 does not regulate cellular adherance in EHEC. Quantification and comparison of HeLa cell adherence between the WT EHEC and its isogenic mutants. Strains represented: WT = EHEC 86-24, Δ*R* = Δ*lsrR*, Δ*K* = Δ*lsrK*, Δ*RK* = Δ*lsrRK*, Δ*S* = Δ*luxS*, Δ*SR* = Δ*luxS* Δ*lsrR*, Δ*SK* = Δ*luxS* Δ*lsrK*, and Δ*SRK* = Δ*luxS* Δ*lsrRK*.

DISCUSSION

Bacteria have evolved complex regulatory mechanisms and networks that allow them to regulate their transcriptome in response to environmental cues. Cell-cell signaling, otherwise known as QS is one such mechanism. QS allows unicellular bacteria to sense and respond to each other and behave in a synchronized manner similar to the cells in a multicellular organism. AI-2, commonly referred to as the bacterial universal language, is a QS signaling molecule that is produced, secreted, and sensed by a variety of bacterial species. Differential genetic regulation by AI-2 signaling has been demonstrated to regulate bacterial phenotypes ranging from pathogenesis to biofilm formation [11-12].

In enterobacteria, such as *E. coli* and *Salmonella,* AI-2 is bound, internalized, phosphorylated and degraded by LsrB, the LsrACD ABC transporter complex, LsrK, and LsrFG, respectively (Figure 5.1) [10]. This system is under direct negative regulatory control by the transcriptional regulator LsrR. Upon binding of phospho-AI-2 to LsrR, DNA binding and hence repression of the *lsrACDBFG* operon by LsrR is relieved [220, 313]. Recently LsrR has been demonstrated to putatively function as a global QS regulator. In *E. coli*, LsrR has been implicated, along with LsrK, in AI-2 dependent regulation of biofilm architecture, and small-RNA (sRNA) expression [11, 327]. Additionally previous research has demonstrated that AI-2 stimulates biofilm production and motility in K-12 *E. coli*, principle through the transcriptional regulator MqsR [326], its toxin/antitoxin partner MqsA [328-330], and LsrR [11]. In this report we verify that

in EHEC, which lacks the toxin/antitoxin MqsR/A pair, that the transcriptional regulator LsrR/AI-2 has *lsrACDBFG* operon dependent and independent effects.

We verified that LsrR regulates the expression of *lsrK*, the *lsrACDBFG* operon, AI-2 uptake (Figure 5.2), and has global regulator functions (Tables 5.1, 5.2) in EHEC. Additionally, while LsrR, LsrK, and LuxS were demonstrated to positively regulate EHEC motility independently of MsqR regulation of QseBC, the combinatorial mutants of LsrR or LsrK in the Δ*luxS* background displayed increased motility beyond that of WT EHEC (Figure 5.3A,B,C). Interesting it was observed that either an *lsrR* or *lsrK* mutation in the Δ*luxS* background was able to rescue its motility defect. This mostly likely suggest that either LsrR has additional AI-2 dependent motility regulatory targets, or that non-phosphorylated AI-2, in a LsrR independent manner, represses motility, through interactions with another transcription factor in EHEC. The lack of the phosphorylation target AI-2 in the Δ*luxS* Δ*lsrK* double mutant suggests the potential for additional motility inhibitory phosphorylation targets of LsrK.

In agreement with previous K-12 studies, we demonstrated that in EHEC, LsrR increases biofilm formation (Figure 5.4A,B). However we found little role for LsrK in biofilm formation other than its ability to reverse the *luxS* mutants biofilm defect. It was also demonstrated that biofilm formation phenotypes mirrored the Δ*luxS* motility rescue phenotypes previously demonstrated for the Δ*luxS* Δ*lsrR*, and Δ*luxS* Δ*lsrK* double mutant strains.

The LuxS enzyme plays an important metabolic role in addition to generating AI-2. LuxS together with Pfs catalize the conversion of SAM to homocysteine and DPD (AI-2) in *E. coli*. *Pseudomonas aeruginosa* achieves this catalysis in one enzymatic step

through the SahH enzyme, and does not produce AI-2. Complementation *in trans* with SahH from *P. aeruginosa*, which rescues the *luxS* metabolic defects, increased biofilm production in the *luxS* mutant, presumably by compensating for the metabolic defects created by alterations to the SAM cycle (Figure 5.5). We also observed that complementation with SahH reduced biofilm formation in several *luxS* positive strains. Due to the biofilm increasing potential of AI-2, we hypothesize that perhaps this suggests a loss of AI-2 production, due to a reduction in the LuxS AI-2 substrate SAH by competition with SahH to generate homocysteine. However, while we observed an increase in biofilm production upon the addition of AI-2 to the *luxS* mutant we saw no effect in any of the other mutants under any conditions tested.

In EHEC virulence factor expression is regulated by epinephrine, norepinephrine and AI-3 through the two two-component sensor kinases QseC and QseE [1, 85]. Pathogenicity defects have been demonstrated in the EHEC *luxS* mutant [78]. However this result was shown to be the indirect result of reduced AI-3 production in the *luxS* mutant due to cellular metabolic alterations [232]. Additionally, AI-2 has been reported to increase the expression of *LEE4* and *LEE5*, and maybe virulence in EHEC [12, 284]. In agreement with these previous results we found that expression of LEE4 and LEE5, in addition to several non-LEE encoded effectors, was increase in the *lsrR* mutant (Figure 5.6). However, in contrast to reports demonstrating increased HeLa cell attachment by the addition of AI-2 [12], we did not observe any evidence for such a phenotype in any of the mutants that we tested (Figure 5.7).

The regulation of EHEC virulence is extremely complex, involves multiple biotic and abiotic signals that are interpreted and integrated through large regulatory networks.

While in K-12 *E. coli* AI-2 has been reported to regulate motility, biofilm formation, and colonic acid production through the regulatory protein MsqR [327]. EHEC lacks MsqR and so must have adapted additional regulatory pathways to utilize AI-2 signaling. Herein, we have provided evidence that in EHEC LsrR/AI-2 signaling has *lsrACDBFG* operon dependent and independent effects, including the regulation of biofilm formation, motility, and TTSS expression. Additionally, by generating and analyzing all the single and combinatorial mutants of *luxS*, *lsrR*, and *lsrK* in an EHEC 86-24 background, we were able to dissect the regulatory role of AI-2 from the metabolic defects in the *luxS* mutant and analyze the breadth and diversity of LsrR regulated phenotypes. Together these results demonstrate that LsrR, independently of the toxin/antitoxin MqsR/A pair, is a global regulator in EHEC, and that LsrK, AI-2, and phospho-AI-2 have alternative regulatory roles outside of the *lsrACDBFG* operon.

CHAPTER SIX

DISCUSSION AND FUTURE DIRECTIONS

EHEC is a human enteric pathogen that colonizes the human colon and causes outbreaks of hemorrhagic colitis and hemolytic uremic syndrome (HUS) throughout the world. EHEC causes approximately 73,000 illnesses, 2,000 hospitalizations, and 69 deaths in the U.S. annually [17]. EHEC enters the colonic lumen through ingestion of contaminated food or water, once there it coordinately regulates its virulence expression. In addition to being motile, EHEC produces multiple virulence factors including a type III secretion system (TTSS) encoded by the locus of enterocyte effacement (LEE) PAI, and a phage encoded Stx. Flagellation allows the bacteria to swim into close proximity to the colonic epithelium, where expression of the LEE PAI TTSS allows for intimate AE lesion formation. Eventually through host or pathogen mediated cell stress Stx is produced and released leading to host morbidity and mortality [59].

EHEC is able to sense both biotic cues, such as the human host produced catecholamines epinephrine and norepinephrine, and abiotic cues, such as sulfate and phosphate, through the use of two two-component sensor kinases, QseBC and QseEF [1,

85]. In addition EHEC is able to sense several cell-cell signaling hormone-like molecules referred to as autoinducers (AI), which allows the bacteria to sense both the microbial population density and diversity in its surrounding environment [230, 293]. Through the interpretation and integration of these multiple regulatory pathways and networks, EHEC coordinately regulates its virulence gene armamentarium.

The overall objective of this body of work was to increase the understanding of EHEC virulence regulation through the characterization of the previously undescribed LysR-type transcriptional regulator (LTTR) QseD (encoded by the *yjiE* gene), and by exploring the global LsrR/AI-2 regulatory network. We focus our efforts on these goals for three major reasons: (1) LTTRs, such as QseA, have been previously shown to be integrally involved in regulation of the LEE in EHEC [83, 86], (2) the role of AI-2/LuxS signaling in EHEC pathogenesis has never been completely characterized [12, 284], and (3) recently LsrR, whose regulatory function is controlled by the cellular concentration and phosphorylation state of AI-2, has been demonstrated to have a global regulatory role outside of the expression of the *lsrACDBFG* operon [11, 220-221].

The QseD (*yjiE*) project was started for two reasons: (1) genomic profiling of the EHEC *luxS* mutant demonstrated that *yjiE* expression was up-regulated 20-fold in the *luxS* mutant versus WT EHEC, and (2) upon closer examination of *yjiE*, we noticed that it was both predicted to encode a putative transcriptional regulator, and more interestingly, that while it is present in all enterobacteria, due to a point mutation generating a stop codon, it seemingly existed almost exclusively in O157:H7 EHEC isolates in a helix-turn-helix (HTH) truncated form. Due to the EHEC *luxS* mutants inability to produce AI-2 and AI-3, and the presumed loss of DNA-binding functionality

in the *yjiE* translated protein, we were presented with the stimulating prospect of characterizing a novel cell-cell signaling regulated transcription factor that was uniquely modified only in the most prolific and pathogenic EHEC serovar [140, 290-291].

To test our hypothesis we first validated that *yjiE* was regulated in a cell density dependent manner, and that its presumed operon structure was not disrupted due to the stop codon generating point mutation. Next we generated an isogenic *yjiE* mutant in EHEC and characterized its effect on the EHEC transcriptome and virulence expression. Affimetrix E. coli 2.0 DNA microarrays demonstrated the *yjiE* did appear to have a regulatory role in EHEC, and that one of its presumed targets was the LEE TTSS. We therefore renamed *yjiE* quorum sensing *E. coli* regulator D (*qseD*).

Once we determined that QseD was a functional EHEC transcriptional regulator, we began to compare the regulatory effects of both the *E. coli* K-12 full length "long" (lQseD) and the EHEC "short" (sQseD) isoforms in EHEC, and to determine the function of the endogenous lQseD in K-12. DNA microarrays demonstrated that in K-12 the main functional role of lQseD was in repressing flagella expression. We verified this result as well as demonstrated a lack of flagella regulon regulation by the sQseD in EHEC using tryptone soft agar motility plates, quantitative RT-PCR (qRT-PCR), and flagella Western blots. We also verified using qRT-PCR that in EHEC sQseD was repressing the LEE, and that in both EHEC and K-12 *E. coli* that QseD was regulating the adjacent genes *yjiC* and *iraD*, as their transcripts were up-regulated two-fold and 13-fold in the *qseD* mutant, respectively. While YjiC has no predicted homology to any known protein, IraD has recently been demonstrated to prevent degradation of the stress induced alternative sigma

factor RpoS, by RssB sequestration, leading to an altered bacterial stress response and increased mutation rates [7, 295].

We speculated that in EHEC the HTH truncated sQseD was interacting with additional regulatory proteins in order to bind to DNA in the absence of its own binding domain. We believed that the most likely candidates were the LTTRs QseA and LrhA as they were previously demonstrated to regulated LEE transcription [86, 300], and were members of the LTTR family, which have been demonstrated to be promiscuous in their protein-protein interactions [242-243, 302-303]. However, sQseD failed to interact with both of these LTTRs in a targeted yeast-two-hybrid (Y2H) experiment, suggesting that this regulation might occur through interactions with other yet unidentified LTTRs involved in LEE regulation. Additionally, while we showed in EMSA competitions that sQseD lacked the ability to interact with QseA and thus prevent its binding to its target *LEE1* promoter, lQseD was able to independently bind to the *LEE1* promoter.

Based on our data we therefore presented a model where while lQseD is able to directly bind to the *ler* promoter, sQseD must interact with an additional positive *ler* regulatory protein (designated as LysR-X) in a dominant negative manner, thereby preventing transcription activation. Additionally through regulation of *iraD*, QseD could be altering the cellular response to stress, and therefore affecting global transcriptome regulation.

While our results allow us to hypothesize that O157:H7 EHEC isolates evolved a truncated sQseD isoform specifically to increase their pathogenicity, additional experimentation is necessary to validate this conjecture, and to determine the actual regulatory mode of action of sQseD. Follow up studies will include replacing the sQseD

in the EHEC chromosome with the lQseD isoform, and the generation of non-O157 EHEC lQseD mutants, followed by transcriptome and virulence characterization. Additionally, repeating the Y2H to screen an EHEC genomic library should identify sQseD interaction partners. We believe another interesting approach would be to perform chip on chip experiments with lQseD in order to identify additional lQseD DNA binding targets in either K-12 *E. coli* or non-O157:H7 EHEC's.

Our work also focused on unraveling the regulator diversity and breadth of the SorC type transcriptional regulator (STTR) LsrR. The LsrR/AI-2 project was started to for two reasons: (1) while AI-2 signaling has been implicated in LEE regulation, presumably through modulation of LsrR DNA binding [12, 78], an adequate model to explain these results had not been presented, (2) the regulatory breadth of LsrR was expanding as it was being demonstrated to function on the global scale [11, 220-221]. We therefore speculated that LsrR had a role in regulation of the LEE and possibly additional virulence factors and that its activity was being regulated at the level of AI-2 binding and DNA interaction modification [220, 313].

To test our hypothesis we took an unbiased approach and generated all the single and combinatorial mutants of *lsrR*, and *lsrK* in an EHEC 86-24 WT and Δ*luxS* background, and analyzed the effect on bacterial phenotypes that ranged from cellular attachment and biofilm production, to motility and TTSS expression. In *E. coli*, LsrR has been demonstrated to repress the importation, phosphorylation, and degradation of AI-2, by the direct negative regulation of the *lsrACDB*, *lsrK*, and *lsrFG* genes when it is in the phospho-AI-2 unbound state [8-10]. We first verified that in the *lsrR* mutant, the LsrR regulon (*lsrACDBFG*, and *lsrK*) and *lsrA* expression was down regulated using DNA *E.*

coli 2.0 microarrays and qRT-PCR, respectively. AI-2 uptake was then quantitated in all the mutants. The *lsrR* and *lsrK* mutants, in either the WT or the Δ*lsrR* background, exhibited the predicted phenotype of increased and decrease AI-2 uptake, respectively. Interestingly, while we would have expected the *lsrRK* double mutant, in either background, to have increased AI-2 uptake similarly to the *lsrR* single mutant, this was not observed. Additionally, the *luxS* mutant was able to remove all the added AI-2 from the external environment suggesting that the exogenously added substrate concentration was sufficiently great enough to inhibit LsrR repression.

While expression of the flagella regulon in K-12 *E. coli* has been reported to be unaffected by LsrR and LsrK, our *lsrR* mutant arrays suggested otherwise [11]. In fact, we verified that transcription of the flagella regulon in EHEC is activated by LsrR. This difference in regulation of flagella expression by LsrR can be explained by differences between the regulatory region of *flhDC*, the master regulator of the flagella regulon, between K-12 and EHEC. In K-12 *E. coli*, there is an insertion sequence (IS) in the regulatory region of *flhDC* that is absent in EHEC [297]. The presence of this IS has been reported to alter *flhDC* expression, and consequently motility. Interestingly while we verified that the *luxS* mutant had reduced swimming motility [294], additional mutations, in the Δ*luxS* background, in either, or both *lsrR* and *lsrK* restored motility to WT levels. We believe this most likely suggests that either LsrR has additional AI-2 dependent motility regulatory targets, or that non-phosphorylated AI-2, in a LsrR independent manner, represses motility, through interactions with additional transcription regulators in EHEC. The lack of the phosphorylation target AI-2 in the Δ*luxS* Δ*lsrK*

double mutant suggests the potential for additional motility inhibitory phosphorylation targets of LsrK.

It has been reported that in E. coli *K-12*, *lsrR* and the *lsrK* mutants have reduced biofilm formation [11]. We also verified that in EHEC LsrR, LuxS, and to some extent LsrK increased biofilm production. These results mirror the motility phenotypes previously demonstrated for these mutant strains. AI-2 has been reported to increase biofilm formation in K-12 *E. coli* [326]. Indeed, while we were able to reproduce this effect in the *luxS* mutant, we observed no effect on biofilm formation in any of the additional mutants or WT EHEC upon the addition of AI-2. Complementation *in trans* with *sahH* from *Pseudomonas aeruginosa*, which compensates for the metabolic defects but not AI-2 production in the Δ*luxS* strain, increased biofilm production in the *luxS* mutant. Interestingly, complementation with SahH reduced biofilm formation in *luxS* positive strains suggesting that loss of AI-2 production, due to the removal of the LuxS substrate SAH by SahH, is sufficient to reduce biofilm formation.

In EHEC, while defects in pathogenicity were demonstrated in the EHEC *luxS* mutant [78], these results have been shown to be the indirect result of reduced AI-3 production in the *luxS* mutant [232]. AI-2 has also been reported to increase the expression of *LEE4* and *LEE5*, and virulence in EHEC [12, 284]. In agreement with these previous results we found that LEE PAI expression, in addition to several non-LEE encoded effectors, were increased in the *lsrR* mutant. However, in contrast to reports demonstrating increased HeLa cell attachment by the addition of AI-2 [12], we did not observe any evidence for such a phenotype in any of the mutants that we tested.

Based on our data we agree with the speculation that LsrR has additional regulatory roles, and we add that some of these roles may be AI-2 independent. Additionally, we provide evidence, using combinatorial mutants of *lsrR*, and *lsrK* in both WT EHEC 86-24 and the Δ*luxS* backgrounds, that LsrR and LsrK are able to regulate motility and biofilm production independently of AI-2.

While our results have enhanced our understanding of the combinatorial roles of *lsrR*, *lsrK* and *luxS* in EHEC 86-24, additional experimentation is necessary to validate our conjectures, and to complete this study. Several unanswered questions still remain to be tested, including what effect the addition of exogenous AI-2, and/or pSahH complementation has on motility, and LEE PAI expression in all the single and combinatorial mutants. Additionally, recently studies have demonstrated the involvement of MsqR [326], which is absent in EHEC, LsrR, and LsrK in regulation of McbR/McbA. The McbR/McbA pathway has been implicated in the regulation of biofilm formation [327]. Therefore additional combinatorial mutants in the genes encoding for these two proteins should be assessed in our mutants, for additional alterations to motility, biofilm formation, and TTSS regulation.

By characterizing the previously unknown LTTR QseD, and broadening the understanding of the LsrR/AI-2 regulatory network, we have increased the overall understanding of QS and transcriptome regulation in EHEC. We hope that these, and future studies of this kind, will allow for the design of more effective antimicrobials targeted at disrupting virulence gene regulation, and therefore EHEC pathogenesis [221, 331].

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