THE ROLE OF HOST HORMONES AND METABOLITES IN THE REGULATION OF VIRULENCE IN ENTEROHEMORRHAGIC *Escherichia coli* (EHEC)

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DEDICATION

To my wonderful family, my parents Stephen and Margaret Njoroge and my siblings Eric, Nelly and Janet. For always believing that nothing was beyond my reach.

And to my sweetie Mark Barfield. For being my rock these last four years, for pushing me when I needed it and for sharing your family with me.

THE ROLE OF HOST HORMONES AND METABOLITES IN THE REGULATION OF VIRULENCE IN ENTEROHEMORRHAGIC *ESCHERICHIA COLI* (EHEC)

BY

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DISSERTATION

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

February 2012

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Publication No. _____

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The University of Texas Southwestern Medical Center at Dallas, 2012

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Gastrointestinal (GI) bacteria sense diverse environmental signals, including host hormones and carbon nutrients, as cues for differential gene regulation and niche adaptation. These GI bacteria include the enteric pathogen enterohemorrhagic *Escherichia coli* O157:H7 (EHEC) that causes hemorrhagic colitis, hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (142). EHEC has been shown to sense mammalian hormones epinephrine and norepinephrine, autoinducer 3 (AI-3), a hormone-like compound produced by intestinal bacteria, as well as simple molecules such as phosphates and sulfates in order to discern its arrival to its colonization niche, the colon (41, 120, 247). This consequently allows for a temporal and energy efficient up-regulation of its virulence factors. These virulence factors include the flagella and motility genes that allow the bacteria to swim closer to the colon's epithelium, the potent Shiga toxin which is responsible for the HUS complication, and the genes necessary for the formation of attaching and effacing (AE) lesions on epithelial cells (142). AE lesion formation is characterized by the attachment of bacteria to colonic epithelial cells

followed by an induction of extensive actin rearrangement underneath the bacteria and effacement of surrounding microvilli (159, 204, 304). Most of the genes necessary for the formation of AE lesions are contained within a pathogenicity island (PI) known as the locus of enterocyte effacement (LEE) (190). The LEE PI encodes for the structural components of a type three secretion system (TTSS), some of its regulators as well as some effectors that are translocated through this TTSS into the host epithelial cell (130, 133). The TTSS also translocates some non-LEE encoded effectors such as EspFu/TccP (35, 89) and NleA/EspI (102, 208), which mimic mammalian signaling proteins and hijack host cell signal transduction.

The precise and efficient regulation of these virulence traits is essential for EHEC to successfully infect the host. One of the signals sensed by EHEC to activate virulence is epinephrine. Here we investigated the extent of epinephrine regulation in EHEC through transcriptome studies. The bacterial adrenergic kinases QseC and QseE both respond to epinephrine to regulate the LEE pathogenicity island (PI) positively and negatively respectively. QseC through phosphotransfer to one of its response regulators (RR) KdpE, activates the transcription of the LEE PI and *nleA*, while QseE through its inhibition of the transcription of the RR RcsB, inhibits their transcription. We also demonstrated for the first time that co-incubation with epinephrine increases the formation of AE lesions, and that QseC and QseE are the only sensors of epinephrine in EHEC (224). Epinephrine is not the only host hormone sensed by EHEC. We showed that another human hormone, serotonin is sensed by EHEC, *Citrobacter rodentium* and uropathogenic *E.coli* (UPEC). In EHEC and *C.rodentium* we showed that serotonin inhibits the transcription of the LEE PI. We also determined that the mechanism of

LEE PI inhibition by serotonin is through the reduction of autophosphorylation of the bacterial sensor kinase CpxA, which is itself an activator of the LEE PI.

In addition to chemical signaling, nutrient availability plays an important role in bacterial gene regulation. Hence, we investigated the role that carbon nutrition plays in the regulation of EHEC virulence. We showed that the LEE PI is activated under gluconeogenic conditions, which has been shown to be important for the maintenance of colonization *in vivo,* and inhibited under glycolytic conditions. We also identified a novel regulator of the LEE PI, Cra, which interacts with KdpE to regulate EHEC virulence. This regulation of the LEE PI was shown to be glucose concentration dependent. This study also allowed us to identify other targets of Cra and KdpE, including known and putative virulence factors. These findings enhanced our understanding of the role that epinephrine plays in virulence, and introduced two other signals, serotonin and glucose which are both important for the regulation of EHEC virulence genes.

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LIST OF DEFINITIONS

5HT	5-hydroxytryptamine (serotonin)
86-24	Enterohemorrhagic *E.coli* wild-type strain 86-24
AE	Attaching and effacing
AI	Autoinducer
Bp	Base pair
BSA	Bovine serum albumin
CDC	Centers for Disease Control and Prevention
CFU	Colony forming unit
CNS	Central nervous system
DAEC	diffusely adherent *E.coli*
DMEM	Dulbeco's modified Eagle's medium
DNA	Deoxyribonucleic acid
dNTP	dideoxynucleotide triphosphate
DTT	Dithiothreitol

EAEC	Enteroaggregative *E.coli*
E.coli	*Escherichia coli*
EDTA	Ethylenediamine-tetraacetic acid
EHEC	Enterohemorrhagic *E.coli*
EIEC	Enteroinvasive *E.coli*
EMSA	Electrophoretic mobility shift assay (gel shift)
ENS	Enteric nervous system
EPEC	Enteropathogenic *E.coli*
Epi	Epinephrine
Epi/NE	Epinephrine/ norepinephrine
ETEC	Enterotoxigenic *E.coli*
FITC	Fluorescein Isothiocyanate
GI	Gastrointestinal
GPCR	G-protein coupled receptor
HK	Histidine kinase
HUS	Hemolytic uremic syndrome

IL	Interleukin
IPTG	β-D-thiogalactopyranoside
kDa	kilo Dalton
LB	Luria-Bertani
LEE	Locus of enterocyte effacement
Ler	LEE encoded regulator
NCBI	National Center for Biotechnology Information
NE	Norepinephrine
NEB	New England Biochemicals
NEN	New England Nuclear
Nle	Non-LEE encoded
OD	Optical density
ONPG	o-nitrophenyl-β-D-galactopyranoside
PAGE	Polyacrylamide gel electrophoresis
PI	Pathogenicity island
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
PMSF	Phenylmethylsulfonyl fluoride
PNK	Polynucleotide kinase
PVDF	Polyvinylidene difluoride
qRT-PCR	quantitative real-time PCR
QS	Quorum sensing
Qse	Quorum sensing *E.coli* regulator
RNA	Ribonucleic acid
RpoA	RNA polymerase subunit A
RT	Room temperature
RR	Response regulator
SDS	Sodium dodecyl sulfate
SERT	serotonin transporter
Stx	Shiga toxin
TBST	Tris buffered saline with Tween
TF	Transcription factor

Tir	Translocated intimin receptor
Tph	Tryptophan hydroxylase
TTP	Thrombotic thrombocytopenic purpura
TTSS/T3SS	Type III secretion system
UPEC	Uropathogenic *E.coli*
WB	Western blot
wt	wild type
X-gal	5-bromo-4-chloro-3-indolyl β-galactopyranoside

CHAPTER ONE

LITERATURE REVIEW

TAXONOMY AND HISTORICAL PERSPECTIVE OF EHEC

Escherichia coli, classified as a member of the Enterobacteriaceae family of gamma proteobacteria, is named after Theodor Escherich, the German pediatrician and bacteriologist who first isolated it in 1885. *E.coli* is a Gram-negative, facultative anaerobe and is a major part of the flora and fauna of human orifices including the lower gastrointestinal (GI) tract. Most strains of *E.coli* found in the human gut are commensals and typically colonize the mammalian GI tract within hours of birth. However, certain strains of *E.coli* have evolved to acquire virulence traits that allow them, upon colonization of their host's GI, to cause a broad spectrum of diseases (142) ranging from mild diarrhea to bloody diarrhea to death. These highly adapted pathogenic strains acquired these virulence genes through mobile elements like plasmids, pathogenicity island (PI), and phages. There are six well described categories of enteric *E.coli* that cause disease: diffusely adherent *E.coli* (DAEC), enteroaggregative *E.coli* (EAEC), enterohemorrhagic *E.coli* (EHEC), enteroinvasive *E.coli* (EIEC), enteropathogenic *E.coli* (EPEC) and enterotoxigenic *E.coli* (ETEC) (30, 142).

This dissertation focuses on EHEC, an emerging global health issue, and in particular the strain EHEC O157:H7 86-24, which was isolated from a patient with hemorrhagic colitis in 1986 (100); the designation O157:H7 denotes the surface antigen (O) and the flagellin antigen (H) of

the strain. EHEC O157:H7, henceforth referred to as EHEC, was first isolated in the US from beef patties, which were attributed to cases of hemorrhagic colitis (251). Since the first reported US case in 1983, approximately 73,000 illnesses, 2,000 hospitalizations and 69 deaths have been attributed to EHEC annually (251)(Lynch, et al., 2006). These EHEC infections are characterized by hemorrhagic colitis with possible complications that include hemolytic uremic syndrome (HUS), which is characterized by hemolytic anemia, renal failure and thrombotic thrombocytopenic purpura (TTP) (146, 232, 251, 259). HUS has been shown to contribute significantly to the morbidity and mortality observed with EHEC infections, especially in the very young and elderly. Another characteristic of EHEC that makes it an effective pathogen, is its relatively low infectious dose of 50 colony forming units (cfu). EHEC causes numerous outbreaks every year worldwide.

EPIDEMIOLOGY AND CLINICAL PRESENTATION

EHEC is a food borne pathogen. Since the first case reported in the US in 1983, there have been several EHEC outbreaks every year. These outbreaks have been linked to numerous food sources including apple cider, lettuce, radishes, sprouts, spinach, cheese, cookie dough and bologna (26, 31, 198, 305). Ruminants, which include cattle, sheep and goats, are asymptomatic natural reservoirs of EHEC, and a significant incidence of EHEC outbreaks are associated with petting zoos (167). In 1994, the Centers for Disease Control and Prevention (CDC) deemed EHEC a nationally important pathogen, and by 2000 required reporting of all cases in the continental US (22). There were three EHEC O157:H7 outbreaks reported in the US

in 2011 (Fig.1.1). The latest US EHEC outbreak reported was a multistate outbreak in the Fall/Winter of 2011 that was linked to the consumption of contaminated romaine lettuce. In this outbreak, 60 people in 10 states were infected, 67% of whom were hospitalized, with two people developing HUS, however, no deaths were reported. There was also one international outbreak reported in 2011 whose source was finally determined to be contaminated brussel sprouts from a German farm. The cause of this outbreak was found to not be the O157:H7 strain normally associated with EHEC outbreaks but the strain O104:H4 (74). In Germany alone, 3816 cases (including 54 deaths) were reported and 22% of these cases developed HUS. Reporting of outbreaks has led to an improvement of regulation of the fast food industry, which had been linked to several outbreaks (USFDA Administration, 1993). It has also led to the prevention of increased human infections and fatalities due to timely recalls of contaminated food products, and the implementation of prevention strategies such as adequate hand washing facilities at petting zoos (11, 181).

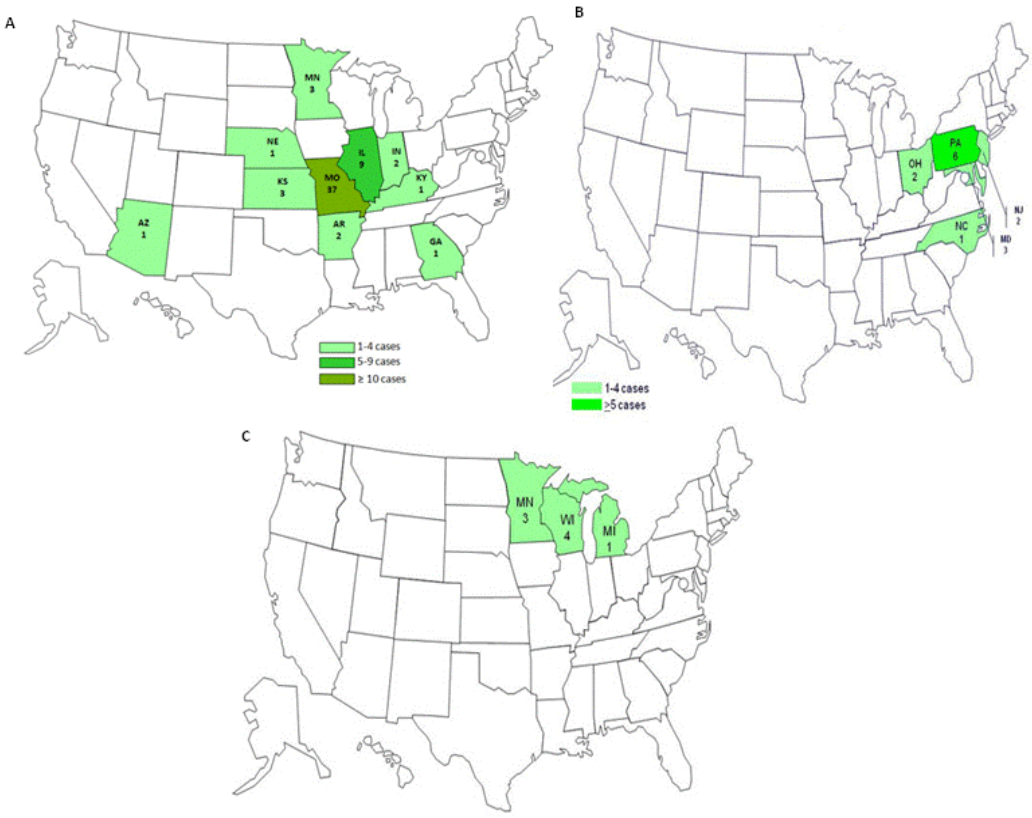


Fig.1.1 . Epidemiology of EHEC infection in the United States (2011). The maps show states (in green) where cases were reported in 2011 and linked to contaminated **(A)** hazelnuts, **(B)** Lebanon bologna and **(C)** romaine lettuce. Images courtesy of the CDC website.

Symptoms of EHEC infection normally present between two and five days after infection with a mean incubation period of three days (251) (Fig.1.2). An EHEC infection initially causes severe abdominal cramps and watery (non-bloody) diarrhea. The colitis shifts to hemorrhagic colitis on the second to third day of illness for 35-90% of infected patients with severity ranging from streaks of blood in the stool to completely bloody stool (22). Fifty percent of patients have nausea and vomiting, but unlike most other infections there is no fever (3). Symptoms normally resolve within seven to ten days after the initial presentation. However, two to 14 days after

resolution of the diarrhea, 6-10% of patients develop HUS, which is characterized by hemolytic anemia, thrombocytopenia and renal failure (39, 237). Detection and diagnosis involves streaking stool samples on sorbitol-MacConkey agar; EHEC, unlike other enteric *E.coli,* cannot ferment sorbitol (187). This test can be confirmed with commercially available Shiga toxin ELISA, and PCRs or qRT-PCRs kits for the gene encoding the toxin (234). Treatment of EHEC is supportive, consisting only of fluid and electrolyte replacement. Antimicrobial therapy has been linked to an increased rate of HUS and is currently discouraged (233). Treatment of HUS requires dialysis, control of hypertension and treatment of anemia. Patients are monitored for a recovery in platelet counts and creatinine levels, a negative sorbitol-MacConkey test, and lack of diarrheal symptoms.

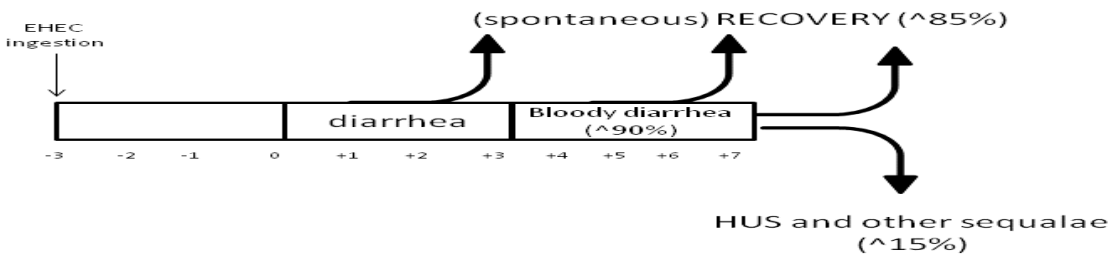


Fig.1.2. Timeline for the progression of EHEC infection (251)

VIRULENCE FACTORS OF EHEC

The Locus of Enterocyte Effacement (LEE)

EHEC infection is characterized by bacterial attachment to host enterocytes, leading to rearrangement of the host actin cytoskeleton into a pedestal-like structure and the effacement

of microvilli (Fig.1.3). These morphological changes are referred to as attaching and effacing (AE) lesions (157, 204, 304). Most of the genes required for the formation of AE lesions are encoded within a 35 kb pathogenicity island (PI) known as the locus of enterocyte effacement (LEE) (190) (Fig.1.4a). The LEE contains 41 genes that are grouped into five major operons *LEE1-5* (33, 67, 194), and encode for a type three secretion system (TTSS or T3SS) that translocates effectors from the bacteria to the host cells. The effectors manipulate host processes and promote pathogenesis (119).

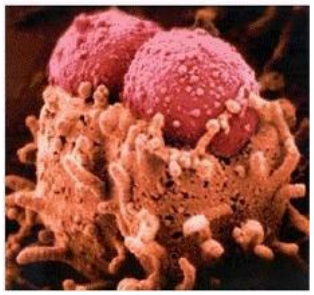


Fig.1.3. Attaching and effacing (AE) lesion formation. EHEC bacteria (pink) cupped by pedestal-like structures formed by the rearrangement of host actin underneath the colonic epithelium (142).

Assembly of the TTSS occurs sequentially using the Sec machinery. EscRSTUV encoded within the *LEE1* and *LEE3* operons, compose the inner membrane complex, EscC encoded within the *LEE2* operon is part of the outer membrane pore and EscJ (*LEE2*) is the periplasmic component of this TTSS (Fig.1.4b) (49, 91). EscF, which is encoded within the *LEE4* operon forms the TTSS needle (*LEE4*) (331). EspA forms the filament through which effectors, both LEE encoded and non-LEE encoded, are translocated into host cells (50, 160). EspB and EspD (*LEE4*) form the 3-5 nm translocation pore in the host cell membrane (125, 165). EscN (*LEE3*) is the

ATPase (10), and SepD and SepL are the gating system (*LEE4*). The LEE PI also encodes for the chaperones CesF for EspF (65), CesT for Tir and Map (1, 47, 64) as well as CesD, CesD2 and CesAB for EspD and EspB (223, 317). CesAB is required for EspA filament biogenesis (48).

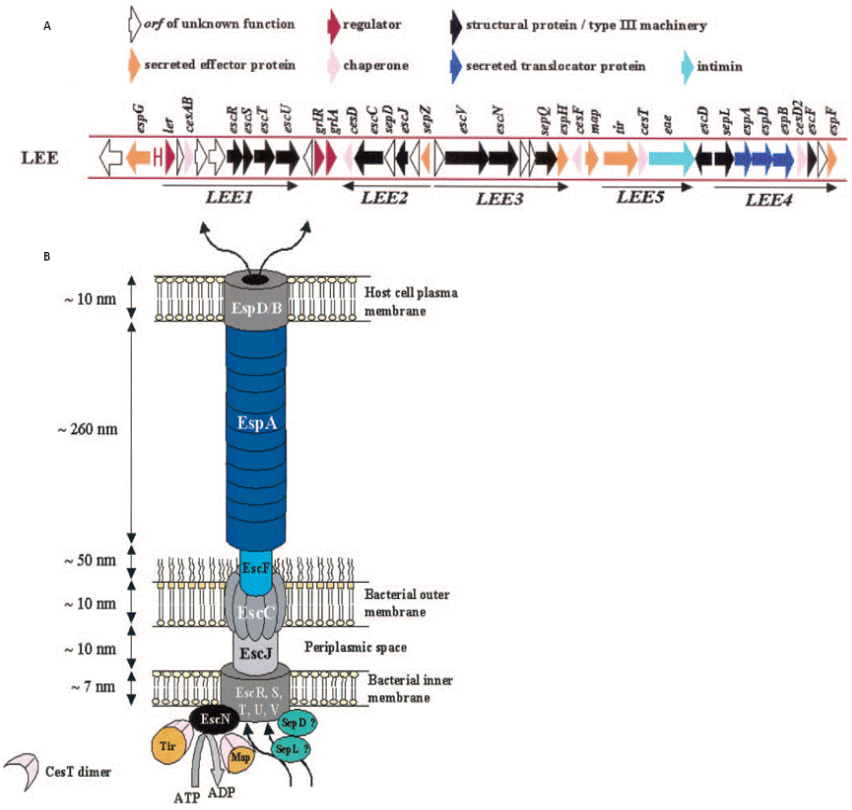


Fig.1.4. The locus of enterocyte effacement (LEE) pathogenicity island (PI). (A) Genetic organization of the LEE PI that encodes for the structural and regulatory components of the type three secretion system (TTSS). **(B)** A cartoon representation of EHEC's TTSS.(88)

Another important pair of LEE genes are e*ae* and *tir* encoded within the *LEE5* operon (67). The gene *eae* encodes for the adhesin intimin, which localizes to the outer membrane of the bacterium, and has been shown to be important for AE lesion formation, intestinal colonization and general pathogenesis (58, 59, 111). The receptor for Intimin, the translocated

intimin receptor (Tir), is translocated through the TTSS into the host epithelial cell where it localizes to the cell membrane (150). Its interaction with Intimin allows for the intimate attachment between the bacterium and the host epithelium. Tir interacts with the host protein, insulin receptor tyrosine kinase substrate (IRTKS), and IRTKS interacts with another effector, EspFu to form a complex that recruits N-WASP (35, 89). N-WASP activates actin assembly by stimulating the actin nucleating complex Arp2/3, culminating in the actin polymerization underneath the bacteria, characteristic of the pedestal-like structure that cups the bacterium (312).

There are five other secreted effectors encoded by the LEE PI. Map has been reported to localize to the mitochondria and inhibits mitochondrial function, to disrupt intestinal junctions, to be involved in actin rearrangement and may also be involved in ion channel regulation (54, 150, 151). EspF has been shown to interfere with intestinal barrier function (192). Map and EspF are part of the WxxE family whose members mimic the GTP-active form of Rho-family GTPases (9, 229). EspG has been shown to disrupt golgi structure and function, and modifies the host cytoskeleton (44, 106, 273, 301). The functions of EspH and EspZ are not currently known but the former has been shown to localize to the host membrane and may modulate the actin cytoskeleton (302).

The regulation of the LEE PI is very complex. Ler which is encoded by the first gene of *LEE1* is a master regulator of the LEE genes (67, 194, 281). Transcription of Ler has been shown to be under the control of many regulators including Hha (275), Fis (97), GrlA and GrlR (18, 56, 134, 135, 324), Pch (2, 129, 211), GadE (140), EtrA and EivF (336). Transcription of *ler* is

positively and directly regulated by the integration host factor (IHF) (77), QseA (149, 276, 280) and indirectly by ClpXP (128). H-NS is a negative regulator of all the LEE operons including *LEE1* (33, 168, 306). This negative regulation is overcome when the Ler protein binds to promoters and displaces H-NS (105, 194, 258, 286). Self regulation of Ler is controversial, with one report showing that Ler may act in a negative autoregulatory manner (24), although previous work had shown that Ler does not regulate the *LEE1* operon (66). It has also recently been shown that *ler* transcription can be indirectly down-regulated by the multicopy expression of the RNA binding protein CsrA (27). The high level of control of *ler* ensures that, in response to diverse environmental signals, EHEC is able to tightly regulate the spatiotemporal expression of the *LEE* genes and consequently virulence.

In addition to the LEE-encoded effectors, there are over 40 non-LEE encoded effectors in the EHEC genome. These effectors have been confirmed to be secreted but for the majority of them, their function is still unknown. Many of these effectors are encoded within λ phages indicating recent acquisition through horizontal gene transfer (299). Some of the effectors that have been characterized include EspFu, which together with Tir and IRTKS forms a complex that rearranges host actin. The recently-identified effector NleA is an important virulence factor that has been shown to be required for virulence in the *Citrobacter rodentium* murine model (102, 208). It has recently been reported to disrupt intestinal tight junctions (296), and to localize to the Golgi apparatus where it inhibits cellular protein secretion (156).

Shiga toxin

EHEC produces a potent toxin known as Shiga toxin (Stx) which is the causative agent of HUS, the leading cause of renal failure in children and EHEC-related mortality (144). When directly injected into a rabbit model this toxin was shown to cause non-bloody diarrhea and death (250). The *stx* family of genes is prophage encoded. Stx toxins can be divided into two subtypes: Stx1 and Stx2 (289). The Stx2 toxin cannot be neutralized with an Stx1 antibody (75, 288, 289). Stx2 has been shown to be 1000x more cytotoxic than Stx1 and patients infected with strains encoding this toxin are seven times more likely to develop HUS (177, 230).

Stx is a classic AB-cytotoxin that contains a single A polypeptide and five B polypeptides. Stx is internalized through clathrin-dependent endocytosis which is mediated by the interaction of the B subunit with the glycolipid receptor globotriaosylceramide (Gb3) (154, 174). Once inside the host cell, the A subunit, an N-glycosidase inhibits host protein translation by removing a single adenine from the 28S host rRNA (68, 267). Shiga toxin expression is regulated by the SOS response. The *stxAB* genes are contained within the late genes of λ prophages and their expression is repressed by the prophage encoded (cI) repressor when the the phage is lysogenized into the EHEC chromosome (220, 238, 239). Upon the induction of an SOS response, DNA damage produces ssDNA which activates RecA. RecA activates the autocleavage of LexA, a repressor of DNA repair proteins (175). RecA also cleaves cI leading to the induction of the prophage (252). The induced prophages enter into the lytic cycle leading to the expression and release of Shiga toxin (303). Multiple antibiotics including ciproflaxin induce the SOS response leading to prophage induction and Shiga toxin expression. Consequently,

antibiotic use during EHEC infections increases the likelihood of the development of HUS (147, 262).

Additional virulence factors

EHEC carries a large 90 kb plasmid (pO157) that encodes among other things, a hemolysin, a type II secretion system, a serine protease, EspP, and some outer membrane proteins (32, 76, 268, 269).

ANIMAL MODELS

EHEC animal models can be used to either study colonization/pathogenesis of EHEC or the effects of Shiga toxin. Animal species that have been used to study EHEC infections include mice, rabbits, chickens, dogs, pigs, cows, ferrets, macaques and baboons (195). One of the best currently used animal model is the infant rabbit model. When inoculated with EHEC, 3-day old rabbits experience diarrhea, colonic inflammation and death (232).

Current mouse models for EHEC infection (conventional, germ-free, streptomycin-treated and mitomycin C-treated mice) are useful for studying the effects of Shiga toxin but cannot be used to study AE lesion formation or hemorrhagic colitis (61, 78, 316). An alternative method of doing *in vivo* work involves the use of the natural mouse enteric pathogen *Citrobacter rodentium. C. rodentium* shares the LEE PI with EHEC and causes AE lesions in the colon of mice. C3H/HeJ mice are highly susceptible to this murine pathogen, and will succumb to death approximately 10 days after an oral challenge with wild type *C.rodentium* (330). Two to three days post oral challenge, *C.rodentium* can be detected in the distal colon and a couple

of days after colonic hyperplasia is observed. Because mice are genetically tractable, the *C. rodentium* murine model has been extensively used to study the effect of AE pathogens on host innate immunity.

CELL-TO-CELL SIGNALING

Cell-to-cell signaling in bacteria has been shown to involve both bacteria-produced molecules, as well as mammalian-produced signals. Upon reaching a certain threshold concentration, these signals, termed autoinducers (AIs), can differentially regulate gene transcription either by altering histidine kinase autophosphorylation levels or by interacting with transcription factors. Cell-to-cell signaling was first reported in *Vibrio fischeri* and *V. harveyi* where it was shown to regulate bioluminescence (218, 219). This signaling or quorum sensing was shown to be a general mechanism of regulation in many bacteria. These signaling systems are divided into three main groups according to the AI signal: AI-1, AI-2 and AI-3.

The AI-1 system, first identified in *V. fischeri,* is controlled by two proteins, LuxI and LuxR (218). LuxI is the AI synthase, which produces the acyl homoserine lactone (AHL) autoinducer. LuxR is the transcription factor that binds to the AI and directly regulates the target genes, which in the case of *V. fischeri,* is the luciferase operon (69). Since then, many homologues of the LuxRI luciferase regulatory system have been identified and characterized, including EsaRI from *Pantoea sterwartii* and YpsRI from *Yersinia pseudotuberculosis* (14, 52, 53, 199). Different bacterial species incorporate unique acyl chains onto the homoserine chain in order to create an AHL that is specific to their LuxR receptor (329). The LuxRI system has been intensively studied in the human opportunistic pathogen *Pseudomonas aeruginosa* where it has been

shown to have three LuxRI homologs namely, LasRI, RhlRI and QscR (80, 81, 172, 225, 278). These systems have been shown to be important for the regulation of adhesion, biofilm formation, and virulence (257, 278). EHEC and *Salmonella enterica* contain a LuxR homolog, SdiA, but these enteric pathogens do not encode for a LuxI homolog and do not produce AHLs (5, 123, 197). Such orphan LuxR homologs have been shown to be involved in interspecies signaling (60, 104, 197).

The AI-2 system was first discovered in *V.harveyi* as an alternative to the classic LuxRI system in the regulation of luminescence (19). AI-2 is a by-product of the activated methyl cycle which generates S-adenosyl-L-methionine a molecule used in the methylation of proteins, RNA, DNA and certain metabolites (311). LuxS, which is an enzyme of this methyl cycle, is important for AI-2 production, and has been shown to be involved in quorum sensing (293). In *Salmonella typhimurium* and *E.coli,* AI-2 controls the regulation of the *lsr* operon, which encodes for an ABC transporter that is required for the uptake of AI-2 (295). Deletion analyses involving *luxS* have shown that the enzyme it encodes is important for carbon and nitrogen metabolism (318).

The AI-3 system involves a molecule that is chemically distinct from either AI-1 or AI-2. Although not well characterized, work done primarily in EHEC has demonstrated that AI-3 regulates the LEE genes and the flagellar regulon (284, 319). AI-3 is produced by many Gram-negative bacteria including human commensals such as *Enterobacter cloacae,* as well as human pathogens such as *Klebsiella,* EHEC, *Shigella a*nd *Salmonella* species (318). The receptor for AI-3 is the sensor kinase QseC (41). Upon sensing AI-3, QseC activates virulence in EHEC (149, 276, 284).

Epinephrine sensing by the bacterial sensor kinases QseC and QseE

The GI tract is the largest endocrine organ in the body and its cells release numerous biologically active compounds such as gastrin, epinephrine, norepinephrine and serotonin (5HT) (92, 179). These hormones are important enteric signaling molecules that influence the motor and secretory functions of the gut. They have also been suggested to play roles in immune activation and inflammation (153). In mammals, the adrenergic hormones epinephrine and norepinephrine are an integral part of the stress response (95). Norepinephrine is produced by the adrenergic neurons of the enteric nervous system, with gut concentrations approximated in the micromolar range (83). During the stress response, epinephrine is released from the adrenal medulla and the central nervous system into the blood stream, and acts systematically (23). These hormones in mammalian cells are sensed by membrane bound G protein-coupled receptors (GPCRs) initiating a regulatory cascade that culminates in the "fight or flight" response in higher animals that enhances their survival. In microorganisms, the hormones epinephrine and norepinephrine (Fig.1.5) have been shown to be sensed by a variety of disease causing organisms including enterohemorrhagic *Escherichia coli* O157:H7 (EHEC O157) (41, 85, 120, 243), enterotoxigenic *E.coli* (ETEC) (183), *Salmonella enterica* serovar *Typhimurium* (15, 20, 21, 205), *Vibrio parahaemolyticus* (212) and recently in the fish and human pathogen *Edwardsiella tarda* (326). These enteric pathogens use epinephrine as a signal for differential regulation of virulence factors including motility (20, 41, 326), and attaching and effacing (AE)

lesion formation, which are typical of EHEC O157 and enteropathogenic *E.coli* (EPEC) infections (204, 287).

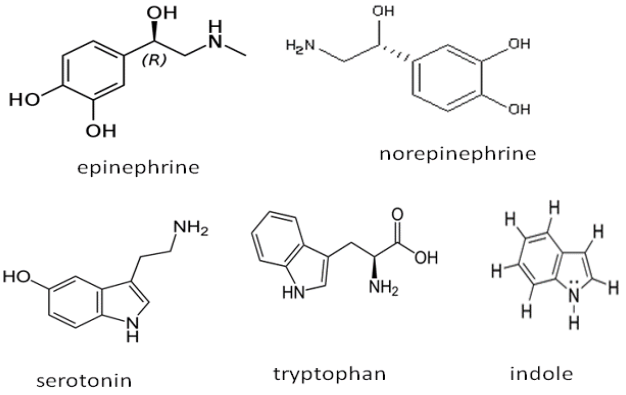


Fig.1.5. Chemical structures of Epinephrine, Norepinephrine, Tryptophan, Serotonin and Indole

In EHEC, it has been shown that the histidine kinases QseC and QseE respond to epinephrine by increasing their autophosphorylation (41, 247). QseC is also able to sense AI-3 and norepinephrine (284), while QseE also senses sulfate and phosphate (247) (Fig.1.6). QseC transfers its phosphate to three response regulators (RR) namely QseB, QseF and KdpE (120). Depending on its phosphorylation state, QseB acts as an activator (phosphorylated form) or an inhibitor (unphosphorylated form) of motility and flagella genes (43, 120). KdpE has been shown to be an activator of the transcription of *ler*, the master regulator of the LEE PI. QseF has been shown to be involved in the activation of *espFu* and *stx2A* gene expression (120, 248).

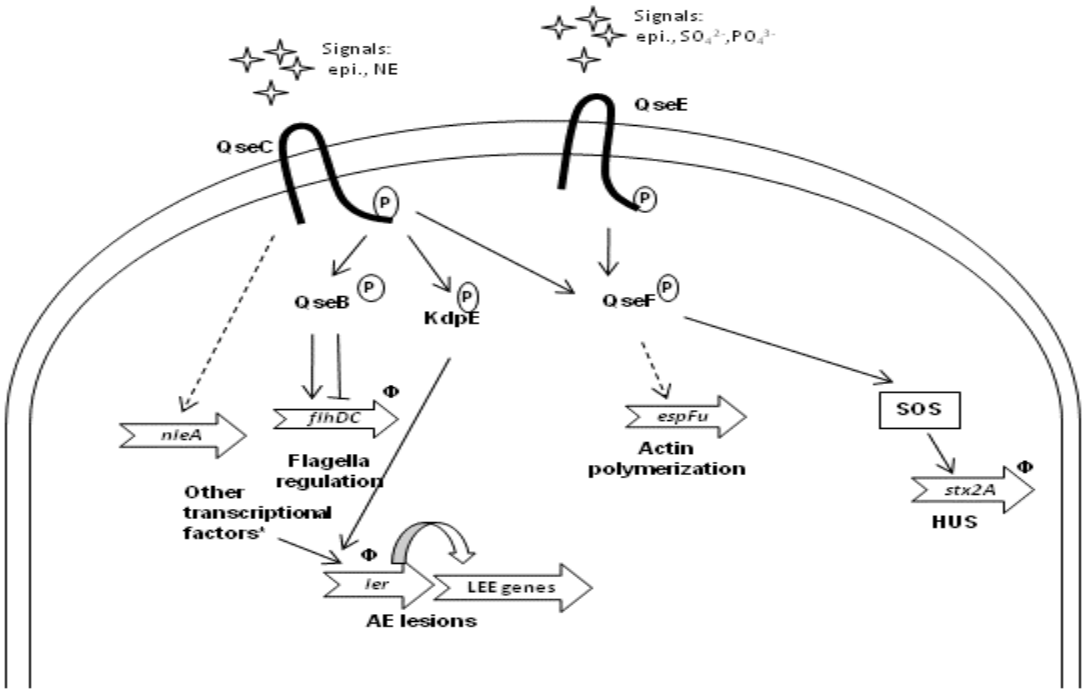


Fig.1.6. Summary of signaling sensing in EHEC. The histidine kinases QseC and QseE sense epinephrine. QseC activates transcription of *nleA*, *flhDC, ler, espFu* and *stx* via the response regulators QseB, KdpE and QseF. The dotted arrows indicate that direct interaction (with their targets had not been shown.

Sensing of tryptophan derivatives' by EHEC

Tryptophan is an aromatic amino acid that is essential in the human diet (Fig1.7). The amount of tryptophan in the gut is thought to be in the micromolar range. This was approximated from mouse studies that showed that an average of 1µM tryptophan is found in feces of mice on a normal (non-tryptophan spiked) diet (29). Tryptophan is not only essential for protein synthesis, but its levels have also been linked to disease diagnosis and prognosis. Decreased tryptophan has been observed in patients with HIV (96), neuroborreliosis (90) and

Streptococcus pyogenes infections (210). Poor prognosis has also been reported for cancer and rheumatoid arthritis patients (231, 271, 328). In Crohn's disease the expression of a tryptophan-metabolizing enzyme, indoleamine 2,3 dioxygenase (IDO), has been shown to be significantly increased (40). High levels of IDO was shown to lead to more activated T cells infiltrating colonic mucosa leading to the characteristic tissue damage observed in irritable bowel disease (IBD). In enteric bacterial pathogen studies, tryptophan spiking of the food/media of *Caenorhabditis elegans* was shown to be essential for the paralysis and killing of these worms by EHEC and EPEC (12). Deletion of the gene encoding the tryptophan-metabolizing enzyme, TnaA, in EHEC and EPEC prevented these pathogens from being able to paralyze or kill *C.elegans,* suggesting that it was a tryptophan derivative that was responsible for virulence regulation. TnaA is an enzyme only found in some bacteria and it has been shown to metabolize tryptophan to indole, ammonia and pyruvic acid (55) (Fig.1.7). This enzyme has been linked to virulence in other bacterial pathogens including *Haemophilus influenza* (188, 209).

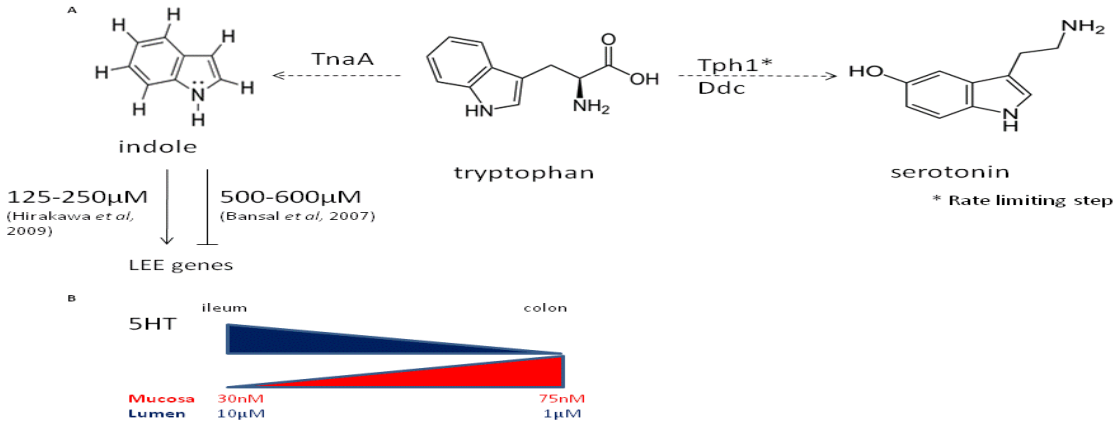


Fig.1.7. Conversion of Tryptophan to Indole and Serotonin. (A) Tryptophan is converted to indole by TnaA , an enzyme expressed by bacteria. In mammals, tryptophan is converted to serotonin through the work of several enzymes including Tph1 which is involved in the rate limiting step of the reaction. **(B)** The concentration of serotonin in the human gut is not known, but is approximated to be highest in the lumen of the ileum.

The main product of TnaA metabolism, indole, is found in large quantities in the environment due to its production and secretion by 85 species of Gram-positive and Gram-negative bacteria (171). Indole positive pathogens include EHEC, EPEC, some *Shigella* strains, *V. cholerae* and *Enterococcus faecalis. Salmonella, Pseudomonas* species and the mouse pathogen *C. rodentium* are negative for indole. Indole has been reported to act as an intercellular signal which controls different aspects of bacterial physiology such as plasmid stability, drug resistance, biofilm formation and virulence (Fig.1.8) (171, 322). In EHEC, it has been reported to act as a signal for the expression of TTSS genes (113). There is also evidence that indole reduces biofilm formation, motility and epithelial cell attachment while increasing epithelial cell tight-junction resistance and the expression of metabolism genes (16, 17). Bansal *et al.* suggested that SdiA, a LuxR homologue, mediated indole sensing (170). However, their *sdiA* mutant was still responsive to indole in all phenotypic tests they performed. Other regulators and regulatory pathways that have been linked to the indole signaling cascade include BaeS-BaeR, CpxA-CpxR, RpoS, Hfq and GadY-GadX (112, 161).

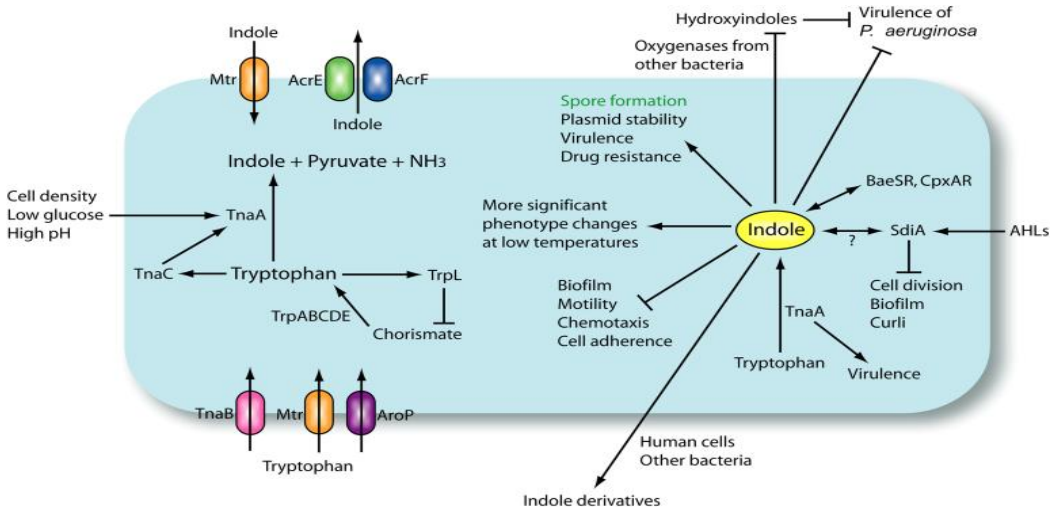


Fig.1.8.Indole biosynthesis and signaling in *E.coli* (171)

Another tryptophan derivative, serotonin is a mammalian neurotransmitter (Fig.1.7). The GI tract contains approximately 90% of the body's serotonin and most of this is produced and stored in enterochromaffin (EC) cells (155, 196, 300). EC cells synthesize serotonin from L-tryptophan in a process that involves a rate limiting step catalyzed by the enzyme tryptophan hydroxylase 1 (Tph1) (73, 320) (Fig.1.7, Fig1.9). Although there are two isoforms of the Tph enzymes, Tph1 and Tph2, the former is present mainly in the GI tract and spleen while the latter is predominant in the brain stem (321). Serotonin released by EC cells activates serotonin receptors (both GPCR and ligand Ca^{2+} channel gates) in order to influence various biological and neurological processes including appetite, mood and nausea (118). 5HT receptors found in the gut include 5HT-2A, 2B, 2C, 3, 4 and 7. Serotonin activity is terminated by its rapid uptake via a highly selective sodium and chloride coupled 5HT transporter (SERT) (93). Serotonin has been shown to play a significant role in a number of GI diseases including irritable bowel disease (IBD), Crohn's disease and ulcerative colitis (6, 153). In these diseases, it is thought that a

positive feedback loop of increased 5HT signaling leads to increased gut inflammation (45). Serotonin is found in most higher eukaryotes including all animals, fungi and plants (141), and is not naturally produced by bacteria. However one lower eukaryote, the pathogenic amoeba *Entamoeba histolytica* has been reported to not only produce serotonin, but also increase its virulence in response to this neurotransmitter (191).

Most bacterial pathogen-serotonin studies had focused on the effect that pathogens have on serotonin signaling. A number of bacteria and viruses including EPEC, *S. typhimurium, V. cholerae, C. rodentium* and rotavirus have been show to influence 5HT signaling (70, 101, 162, 227). Infection with these pathogens has been shown to increase mucosal 5HT either due to a decrease in SERT-dependent 5HT uptake and/or an increase in the release of luminal 5HT.

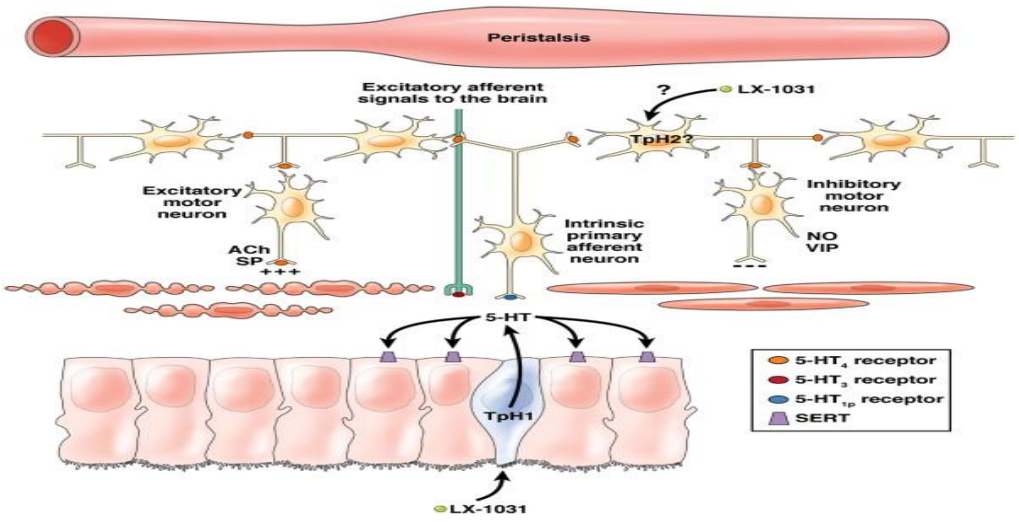


Fig.1.9. Cartoon representation of 5HT synthesis in the human gut (294).

CHAPTER TWO

OVERALL OBJECTIVE AND SYNOPSIS

The human enteric pathogen EHEC causes hemorrhagic colitis and hemolytic uremic syndrome. Given that it has a very low infectious dose (50 cfu), and the use of antimicrobial therapy is counter-indicated, understanding the regulation of virulence in EHEC may lead to better treatments and control of this pathogen. EHEC uses cell-to-cell signaling to regulate virulence expression. This signaling allows bacteria to sense their environment and respond in a spatiotemporally efficient manner.

Previously, it had been shown that the mammalian hormone epinephrine is sensed by many pathogens including EHEC, *Salmonella enterica* serovar *Typhimurium* and *Franscisella tularensis.* Two bacterial histidine kinases, QseC and QseE, were identified as sensors of epinephrine. QseC was further shown to activate its targets (i.e. the LEE genes, Shiga toxin and motility genes,) in an epinephrine dependent manner. *In vivo* studies in mice and infant rabbits had shown that deleting *qseC* decreases virulence while deleting *qseE* increases it. The fact that two kinase proteins that both increased their basal phosphorylation in response to epinephrine had converse influences on virulence led us to hypothesize a very complex epinephrine gene regulatory cascade. We also speculated that these two kinases were the only sensors of epinephrine. In order to better map the epinephrine dependent regulation of both sensors, and confirm whether QseC and QseE were the only sensors of epinephrine, we constructed a double kinase mutant *ΔqseCΔqseE* then performed phenotypic analyses. Using qualitative real-time PCR (qRT-PCR) and microarrays, we showed that QseC is an activator and QseE is an

inhibitor of the expression of the LEE genes and non-LEE effectors. We also showed that although both kinases sense epinephrine in order to regulate downstream genes, epinephrine-dependent regulation of the LEE genes is mostly dependent on QseC, while epinephrine-dependent regulation of the non-LEE effector *nleA* is mostly dependent on QseE. Using qRT-PCR, microarrays and AE lesion formation assays, we confirmed that QseC and QseE are the only sensors of epinephrine in EHEC. This was also the first time that epinephrine was shown to increase the formation of AE lesions in EHEC infected epithelial cells.

Another host hormone, serotonin, also plays a role in EHEC virulence regulation. Serotonin is synthesized from tryptophan. Approximately 90% of all serotonin found in a human body is synthesized in the gut. We speculated that this relatively high concentration of serotonin would have an impact on the virulence of enteric pathogens. To test this hypothesis, we performed phenotypic analyses on EHEC grown in the presence of micromolar amounts of serotonin. We showed that serotonin represses the transcription of the LEE genes and does so through its ability to inhibit the autophosphorylation and the transcription of CpxA, a known activator of the LEE pathogenicity island (PI).

Hormones are not the only small molecules that EHEC is exposed to while in the host's gut. Another set of small molecules that EHEC responds to is carbon nutrients. EHEC's ability to initiate growth and maintain colonization *in vivo* depends on whether the carbon source is glycolytic or gluconeogenic, and glucose polymers in particular have been shown to be important cues for colonization. We speculated that EHEC uses carbon nutrients as colonization cues and as signals to regulate virulence expression. To test this hypothesis, we grew EHEC with

different glycolytic or gluconeogenic carbon sources, and evaluated their impact on known and putative virulence factors. We found that glycolytic conditions inhibited expression of the LEE PI, while gluconeogenic conditions activated it. We were able to identify Cra and KdpE as two transcription regulators that interact with each other to activate the LEE genes in a glucose concentration-dependent manner. We were also able to identify other putative virulence factors that were commonly regulated by these two transcription factors.

With these studies, we were able to genetically and biochemically map regulatory cascades for several small molecules that are present in the gut. Understanding the mechanisms involved in EHEC sensing of signals in the environment, and the subsequent regulation of virulence genes will give insights into general bacteria gene regulation, and development of novel antimicrobial strategies to treat EHEC and other enteric infections.

MATERIALS AND METHODS

STRAINS, PLASMIDS AND GROWTH CONDITIONS

All bacterial strains, plasmids and oligonucleotides used in this study are listed in Table 3.1 and 3.2. Unless otherwise stated, strains were grown in Luria Bertani (LB) medium or low glucose Dulbecco's Modified Eagle's Medium (DMEM) at 37^{o}C and 250rpms. Unless otherwise stated, the DMEM used contains low salt (0.1M) and 0.001M Sodium pyruvate. Media was supplemented, when necessary, with 50μg ml^{-1} streptomycin, 50μg ml^{-1} kanamycin 50 μg ml^{-1} chloramphenicol or 100μg ml^{-1} ampicillin. For protein expression, media was also supplemented with 0.2% arabinose or 0.5mM Isopropyl β-D-1-thiogalactopyranoside (IPTG).

For epinephrine studies, strains were grown in light protected conditions after addition of epinephrine to a final concentration of 50μM. For tryptophan derivative studies, strains were grown with serotonin and/or indole to a final concentration of 1μM and 500μM respectively. For carbon sources studies, 'no glucose, no pyruvate' media was used as the base media and final concentrations of 0.1% glucose, 0.4% of glucose, 0.4% glycerol, 0.4% pyruvate or 0.4% succinate were added to the media.

RECOMBINANT DNA TECHNIQUES

Methods used for PCR amplification, plasmid purification, restriction enzyme digestion, ligation and transformation were performed according to standard protocols (264). IDT and Primer Express v1.5 (AB) were used to design the oligonucleotide primers (Table 3.2) used in this work. Construction of the *ΔqseC, ΔqseE* and *ΔkdpE* mutants has been described previously (120, 248, 285). The non-polar mutants *ΔqseCΔqseE*, *ΔrcsB, Δcra, ΔkdpEΔcra, ΔcpxA, ΔtnaA* and

ΔtnaAΔcpxA were constructed using a lambda Red mediated recombination method (51). In brief, using the helper plasmid pKD4 as a template, primer pairs YfhKP1 and YfhKP2 for *qseE,* JrcsB redF and JrcsB redR for *rcsB,* JcraredF and JcraredR for *cra,* JtnaAredF and JtnaAredR for *tnaA* and JcpxAredF and JcpxAredR for *cpxA* were used to amplify PCR products that were then gel-purified (Qiagen). *ΔqseC, ΔkdpE, ΔtnaA* and wild type (wt) transformed with the helper plasmid pKD46 were prepared for electroporation and transformed with the PCR products. The electroporated cells were then recovered in SOC media for 6 hours at 30^{o}C, plated on kanamycin supplemented LB plates and incubated overnight at 42^{o}C. Resultant colonies were screened for ampicillin sensitivity and kanamycin resistance. The kanamycin cassette was then resolved by electroporating deletion candidates with the resolvase plasmid pCP20, heat shocking at 42^{o}C then screening resulting colonies for sensitivity for both ampicillin and kanamycin. Final verification of gene deletion was performed by PCR amplification and sequencing.

Plasmids for mutant complementation, β-galactosidase assays and protein expression were constructed by amplifying the coding regions from the EHEC strain 86-24 using phusion polymerase, digesting with appropriate restriction enzymes and ligating with T4-ligase (NEB) as previously described (285). Briefly, primer pairs JqseEbad33F/ JqseEbad33R were used to amplify the *qseE* gene, the primer pairs JkdpE33_2F/JkdpE33_2R and Jcra33F/Jcra33R were used to amplify the *kdpE* and *cra* genes respectively, and primer pairs JcpxAbad33F/ JcpxAbad33R were used to amplify the *cpxA* gene. The resulting PCR product was ligated into the pBAD33 vector predigested with *Xba* I and *Hin*d III (103). To construct flag tagged versions of the above plasmids the reverse primers were replaced with Jkdpe33flagR and Jcra33flagR

and the cloning process repeated to create pJN45 and pJN46 respectively. The primers pairs JrcsBmycF/ JrcsBmycR were used to amplify the *rcsB* gene, the primer pairs JcramycF and JcramycR were used to amplify the *cra* gene, and the primers pairs JcpxAmycF/ JcpxAmycR and JtnaAmycF/ JtnaAmycR were used to amplify the *cpxA* and *tnaA* genes respectively. The resulting PCR product was ligated into the pBADmycHis vector predigested with *Kpn* I and *Eco* RI. The protein expression plasmid pJN55 was constructed by amplifying the *cra* gene using primers Jcra21F and Jcra21R and cloning the resulting PCR product into the BamHI and NotI cloning site of vector pET21 (EMBD Biosciences). Proper cloning of the plasmids was confirmed by sequencing. The construction of the plasmids pVS155 (QseC), pKH49-2 (CpxA), pKH39-1 (QseB) and pKH4-28 (KdpE) has been described previously (42, 333).

RNA PURIFICATION AND QUANTITATIVE REAL TIME POLYMERASE CHAIN REACTION (qRT-PCR)

Cultures grown overnight aerobically at 37^{o}C in LB were diluted 1:100 into DMEM and grown in triplicate to an OD_{600} 1.0. Trizol (Invitrogen) and the Ribopure Bacteria isolation kit (Ambion) were then used to extract RNA from these biological replicates according to manufacturer's protocols.

Quantification of RNA transcription was performed as described previously (319). Briefly, diluted extracted RNA was mixed with Sybr Green, validated primers (table 3.2), RNase inhibitor and reverse transciptase (AB). The mix was used in a one step reaction utilizing an ABI 7500 sequence detection system. Data was collected using ABI Sequence Detection 1.2 software, normalized to endogenous *rpoA* levels and analyzed using the comparative critical

threshold (C_T) method. Unless otherwise indicated, analyzed data was presented as fold changes over wt levels. The Student's unpaired t test was used to determine statistical significance. A P value of ≤0.05 was considered significant.

NESTED DELETION AND β-GALACTOSIDASE ASSAYS

Reporter plasmids were constructed as previously described (248). For the construction of pYN01 and pYN02, the *ler* promoter region was amplified from the 86-24 strain, using the primer pairs Y2/R1 and Y1/R2 respectively. The resulting PCR products were then cloned into the BamHI and EcoRI cloning site of pRS551 (277). The reporter plasmid pCG50 was constructed by amplifying the *espFu* promoter region using primers EspFulacZF2 and EspFulacZR and cloning the PCR product into the BamHI and EcoRI/MfeI cloning site of pRS551. Construction of pVS224, pVS175 and pVS182 has been previously published (276, 285).

The beta-galactosidase assays were performed as described previously (276). Briefly, appropriate strains containing different *lacZ* fusion expressing plasmids (table 3.1) were grown overnight aerobically at 37^{o}C in LB. 1:100 dilutions were grown in triplicate in clear DMEM (low glucose, 0.1M salt, 0.001M pyruvate) and appropriate antibiotics to mid exponential (OD_{600} of 0.5). For the hyperosmotic stress tests the DMEM was supplemented with NaCl to a final concentration of 0.5M. Cells were diluted in Z buffer and lysed with chloroform and 0.1%SDS. After addition of o-nitrophenyl-β-D-galactopyranoside (ONPG), the reaction was timed and stopped using 1M Na_2CO_3. The OD_{420} was measured and used to calculate the Miller units as previously described (178). The Student's unpaired t test was used to determine statistical significance. A P value of ≤0.05 was considered significant.

PROTEIN PURIFICATION

The pET21 based plasmids encoding for Cra, KdpE, CpxA and QseB were transformed into the *E.coli* strain BL-21(DE3) (Invitrogen). Resulting transformants were grown to OD_{600} 0.5 at 37^{o}C in LB, then induced by adding IPTG to a final concentration of 0.5mM and growing overnight at 25^{o}C. Nickel columns (Qiagen) were used to purify the proteins. Purity was verified by Coomasie and Western blot, while the protein concentration was quantified by Nanodrop and the Biorad protein assay.

WESTERN BLOTTING (WB) AND FAR WESTERN BLOTTING (FWB)

Whole cell lysates (wcl) and secreted proteins (SP) were isolated from wt and the mutants grown in the appropriate media to OD_{600} of 1.0 as previously described (130). Cultures were spun down and separated into pellet and supernatant. To the supernatant (SP) was added EDTA, PMSF, aproptinin and 100µg BSA, with the latter acting as a loading control. Super lysis buffer was added to the pellet (wcl) and incubated at RT overnight. The samples were then spun down and the supernatant used as the wcl for the WB. Ponceau staining was used to check uniformity of loading. Samples were run on gels, transferred to PVDF membranes and probed using antisera against EspA or RpoA.

A modified protocol from Wu *et al* was used to perform FWB assays (332). Equimolar amounts of purified His-tagged protein was separated on a 12% SDS gel, transferred and blocked with 10% milk in Tris buffered saline containing 0.05% Tween (TBST). Replicate membranes were then probed with wcl of the *ΔkdpEΔcra* double mutant (negative control) or

the double mutant overexpressing either Flag-tagged KdpE or Cra. As a further (negative) control a replicate membrane was left unprobed by the wcl. All membranes were then probed with either anti-His or anti-Flag primary antibodies then incubated with a secondary antibody conjugated to streptavidin-HRP. ECL reagent (GE) was added and membranes exposed to film to detect interacting proteins.

ELECTROPHORETIC MOBILITY SHIFT ASSAYS (EMSA)

EMSAs were used to determine direct interaction between Cra and/or KdpE proteins and the promoter regions of their potential targets. The assays were performed as previously described (149). Briefly, defined regions of the promoter (see text, table 3.2) were amplified by PCR, purified, quantified and end-labeled using radiolabeled ATP [γ-^{32}P] (Perkin Elmer) and T4 polynucleotide kinase (NEB) according to manufacturer's instructions. The radiolabeled probes were then repurified to remove unincorporated ATP. EMSAs were performed by adding increasing amounts of purified recombinant protein to 2ng labeled probe in binding buffer [60nM HEPES pH 7.5, 5mM EDTA, 3mM DTT, 300mM KCl, 25mM $MgCl_2$, 50ng polydIdC, 500µg/ml BSA (NEB)](42). In relevant experiments metabolites were added to the indicated final concentrations. The reactions were incubated for 20 min at room temperature then loaded on a 6% polyacrylamide gel after addition of a 5% ficol DNA loading buffer. The gel was run at 180V for 6hrs or 50V overnight, dried and exposed on a phosphoimager.

DNase I FOOTPRINTING

The footprints were performed as previously described(286). Briefly radiolabeled probes were made as with the EMSA assays. The binding reactions were also performed as described for the EMSA assays. After the 20 min incubation, a 1:5 dilution of DNase I (Invitrogen) was added and allowed to digest unprotected DNA at room temperature for a set amount of time. The digestion was the stopped by adding 100µl stop buffer (200mM NaCl, 2mM EDTA and 1%SDS). Protein was then removed using isoamyl-phenol-chloroform and the DNA precipitated using 3M potassium acetate, 100% ethanol and 1µl glycogen. The concentrated samples and a sequencing reaction (Epicentre) were then run on an 8% polyacrylamide gel, dried and exposed on a phoshoimager. To generate the sequencing reaction the initial PCR products were used as template and amplified with end-labeled reverse primers according to manufacturer's instructions.

MICROARRAY GLOBAL ANALYSIS

Microarray global analysis was performed on extracted RNA according to manufacturer's instructions outlined in the Affymetrix Gene Expression Technical Manual (http://www.affymetrix.com). Briefly, RNA extracted as described above was used as a template for reverse transcription to cDNA. The cDNA was then processed and hybridized to the *E.coli* Genome GeneChip 2.0. The Genechips contain over 10,000 probe sets directed towards 20366 genes from four different strains of *E.coli*: the K-12 laboratory strain MG1655, the O157:H7 EHEC strain EDL933, the O157:H7 EHEC strain Sakai and the uropathogenic strain CFT073.

To analyze the results, output from scanning replicates was collected using GCOS v1.4 according to manufacturer's instructions. The data was then normalized using Robust Multiarray analysis (28, 126) and analyzed for differences in gene expression due to the addition of signals (50μM epinephrine, 10μM serotonin creatinine sulphate) and/or the deletion of *qseC, qseE, kdpE* and *cra.*

MOTILITY ASSAYS

Assays were performed as described previously (120). Briefly, overnight cultures grown shaking at 37^{o}C were used to stab motility agar plates (0.3% agar, 1% tryptone and 0.25% NaCl). These plates were then incubated at 37^{o}C for 8 hours after which the motility halo diameters were measured and images taken.

KINASE ASSAYS

Liposomes were reconstituted as previously described (41). Briefly, *E.coli* phospholipids (20mg/ml in chloroform; Avanti Polar Lipids) were evaporated and dissolved in potassium phosphate buffer containing N-octyl-β-D-glucopyranoside. The solution was dialyzed overnight against potassium phosphate buffer. Using liquid N_2, the resulting liposome suspension was subjected to freeze/thaw.

QseC and CpxA were loaded into the liposomes as described previously (41). Liposomes were destabilized using dodecylmaltoside. The kinase proteins and biobeads were added to the destabilized liposomes at a 1:40 ratio, incubated at room temperature for 10 minutes then at 4^{o}C overnight. The supernatant was then added to fresh biobeads for 1 hour to remove the

detergent. The resulting proteoliposomes were spun down, washed to remove unincorporated protein, subjected to freeze/thaw then stored at -80^{o}C until used. Loading was confirmed by running the proteoliposomes on an SDS gel and staining with Coomasie dye.

For autophosphorylation assays, proteoliposomes were adjusted to 10mM $MgCl_2$ and 1mM DTT. After addition of signal to the final concentration indicated in the text (or water as the negative control), samples were either first subjected to freeze/thaw and 1 hour recovery at room temperature or used as is in the following step. [γ32P] dATP(110TBq/mmol) was added to each reaction and at each indicated time point, aliquots were removed and added to 20% SDS loading buffer. Samples were run on SDS/PAGE without boiling and visualized via phosphoimager. The bands were quantified using ImageQuant v.5.0 software (Amersham).

FLUORESCENT ACTIN STAINING (FAS)

To examine pedestal formation, FAS assays were performed as previously described (158, 248). HeLa cells were grown on coverslips in plates in DMEM supplemented with 10% Fetal Bovine Serum (FBS) (Invitrogen) and 1% PSG antibiotic mix at 37^{o}C, 5% CO_2 overnight to about 80% confluency. For signal studies, the FBS used was dialyzed to remove all molecules with a molecular weight less than 10,000 including any epinephrine, tryptophan, indole or serotonin (5HT) that may be present. The wells containing the coverslips were then washed three times with phosphate buffered saline (PBS) and replaced with fresh media lacking antibiotics. For epinephrine studies, the drug was added to a final concentration of 50μM. Overnight static bacterial cultures were then used to infect the washed cells. The plates were light protected and incubated for 6 hours at 37^{o}C, 5% CO_2. The coverslips were then washed,

fixed and permealized. Fluorescein Isothiocyanate (FITC)-labeled phalloidin was used to stain actin green and propidium iodide (PI) was utilized in the staining of bacteria and HeLa nuclei red. The coverslips were then mounted on slides and visualized with a Zeiss Axiovert microscope. To quantify infected cells, at least 100 cells were counted per coverslip and the number of bacteria infecting them counted. Serially diluted samples of the original bacterial cultures were also plated to confirm similar colony forming units (cfus) were used for the infection.

INDOLE TEST

Strains were grown in LB in tubes overnight in the absence or presence of arabinose. Q-tips were dipped into the overnight cultures and once saturated the Q-tips were removed. 10µl Indole spot reagent (p Dimethylaminocinnamaldehyde, DMACA) was spotted on the Q-tip and images taken after 2 min. Pink or orange indicated that the strain is indole negative while blue or green indicated that the strain is indole positive.

EXPERIMENTS USING SIGNALS

In all assays requiring growth of cultures with signals (RNA extraction, wcl and SP assays, FAS), samples were primed with the signals i.e grown overnight with the appropriate signal. Water was used as a negative control. During actual assays' growth, overnight cultures were diluted 1:50 to reduce the amount of time to OD 1.0 consequently reducing possible signal degradation. For all epinephrine studies, samples were light protected.

STASTICAL ANALYSIS

To analyze significance of the results obtained from the assays in this work, all experiments were performed at least twice with at least triplicate samples each time. The student's unpaired t test was used to determine statistical significance. A P value of <0.05 was considered significant.

Table 3.1.Strains and plasmids used in this work

strain	**Genotype**	**reference**
8624	wt O157:H7 EDL933	Griffin et al 1988 Ann. Intern. Med
DH11	Δ*kdpE* non polar mutant	Hughes et al 2009 PLoS
JN01	*Δcra*	this study
JN02	*ΔkdpEΔcra*	this study
JN03	Δcra complemented with pCra in pBAD33	this study
JN04	ΔkdpEΔcra complemented with pKdpE and pCra	this study
JN07	*ΔqseCΔqseE*	This study
JN071	*ΔqseCΔqseE* complemented with *pqseC* and *pqseE*	This study
JN075	DH11 complemented with pJN49	this study
JN078	JN15 complemented with pJN59	this study
JN080	*ΔqseE* complemented with *pqseE* (pJN62)	This study
JN081	*ΔrcsB* complemented with *prcsB* (pJN63)	This study
JN15	*ΔcpxA*	this study
JN18	*ΔrcsB*	This study
JN19	*ΔcpxA* in *C.rodentium*	this study
JN20	*ΔtnaA*	this study
JN21	*ΔtnaAΔcpxA*	this study
NR01	*ΔqseE*	(Reading *et al.*, 2007)
VS138	*ΔqseC*	(Sperandio *et al.*, 2002)
VS179	*ΔqseC* complemented with *pqseC* (pVS155)	(Sperandio et al., 2002)
plasmid		
pBAD33	cloning vector	Guzman *et al* 1995 Jbac
pBAD-myc-hisA	C-terminal Myc-His tag cloning vector	Invitrogen
pCG61	*espFu*regulatory region in pRS551	this study
pCP20	λ red helper plasmid	Datsenko *et al* 2000 PNAS
pET21	expression vector	Invitrogen
pJN45	Kdpe in flag tagged pBAD33	this study
pJN46	Cra in flag tagged pBAD33	this study
pJN49	Kdpe in pBAD33	this study
pJN53	EspA in pET28	this study
pJN55	Cra in pET21	this study
pJN56	Cra in pBADmyc His	this study
pJN57	Cra in pBAD33	this study
pJN59	CpxA in pBAD-myc-hisA	this study
pJN62	*qseE* in pBAD33	This study
pJN62	CpxA in pBAD33	this study
pJN63	*rcsB* in pBADmyc His	This study
pJN64	TnaA in pBAD-myc hisA	this study
pKD4	λ red template plasmid	Datsenko *et al* 2000 PNAS
pKD46	λ red helper plasmid	Datsenko *et al* 2000 PNAS
pKH39-1	QseB in pET21	Yamamoto *et al* 2005 JBC
pKH4-28	KdpE in pET21	Yamamoto *et al* 2005 JBC
pRS551	*lacZ* reporter gene fusion vector	Simons *et al* 1987 Gene
pVS155	*qseC* in pBADmyc His	(Sperandio et al., 2002)
pVS175	*fliC::lacZ* in pRS551	(Sperandio *et al.*, 2001)
pVS182	*flhD::lacZ* in pRS551	(Sperandio et al., 2001)
pVS224	*ler* regulatory region (-173 to +86) in pRS551	Sharp *et al 2007* Infection and immunity
pYN01	*ler* regulatory region (-173 to -42) in pRS551	this study
pYN02	*ler* regulatory region (-42 to +86) in pRS551	this study

Table3.2. Oligonucleotides used in this study

Name	Sequence
Jsepl gsF	CAC CTT CCT CAC GTA TAT CAA GTA AAA ACT
eae RTF	GCTGGCCTTGGTTTGATCA
eae RTR	GCGGAGATGACTTCAGCACTT
espA RTF	TCAGAATCGCAGCCTGAAAA
espA RTR	CGAAGGATGAGGTGGTTAAGCT
EspFulacZF2	CAT CAATTG CTGTCGGCTCTCTTCTAGAT
EspFulacZR	GTA GGATCC ATATTGCGGTTGACGGTTGG
glmY RTF	TCATTCACCGACTTATGTCAGCCC
glmY RTR	ATATCAGAAGTTGGACGGCAGGCA
Jcitro cpxacheckF	CGC AGC ATC TCG GTC AGG TGG TAT
Jcitro cpxacheckR	TAA TTT ACC TGA TGG CGC TGT GCT TAT CAG GC
Jcitro cpxaredF	gcTGA ACT CGC GAA CGA TCC GCC AAA CGA TTT AAT GT GTGTAGGCTGGAGCTGCTTC
Jcitro cpxaredR	gcAAT AGC GCA GCG CGT TGC GGA CGA TAT TTT C CATATGAATATCCTCCTT
JcpxA 21 F	ctcGGATCC ATG ATA GGC AGC TTA ACC GCG
JcpxA 21R	ctcGCGGCCGC ACT CCG CTT ATA CAG CGG C
JcpxA inF	CGCAGCGAAATGCAGATCATTCGT
JcpxA inR	TTGCGCTTCGGTTTCAATACGCTC
JcpxA myc3R	cgtggtacc aactccgcttatacagcgg
Jcpxa red2F	TGG ATA GCG AAC AGC GTC AGG GTC TGA TGA TTG AGC AGC ATG TTG AAG CGG AGC TGG CGA ACG ATC CGC CCA ACG ATT TAA T GTGTAGGCTGGAGCTGCTTC
Jcpxa red2R	CTAACGCCAGGACCATCGTCGTCCACCGTAATGGTGATACCGTCTTTATCTACCGCAAAGC CCA CTTCAATCTTCGTA CATATGAATATCCTCCTT
JcpxAexF	TGC ACA TTT CCA ACC TGC GTC GTA
JcpxAexR	GGA GTG TAG GCC TGA TAA GAC GCT ATC A
JcpxAmhis2F	cgcCTCGAGataggcagcttaaccgcgcgc
JcpxR exF	TCTGCTGACGCTGATGTTCGGTTA
JcpxR exR	TCTTCTGCGGATGATCGGCGTTAT
JcpxR inF	AGACACACCAGACGCCTGTCATTA
JcpxR inR	TGTGCCAGCAAATAGAGCAGGGTA
JcpxR mhisF	ctcGGTACC AAT AAA ATC CTG TTA GTT GAT GAT GAC CGA GAG C
JcpxR mhisR	ctcGAATTC TGAAGCAGAAACCATCAGGTAGCC
JcpxR redF	cttccctattaaaggagctgctcgagatggaaggcttcaacgtgattgttgcccacgatggggaacaggcgcttg GTGTAGGCTGGAGCTGCTTC
JcpxR redR	aaggttttaaaccacgggtgaccatctttacgatccggcagtttacgacgcaggttggaaatgtgcatatcgatagcgcggt CATATGAATATCCTCCTT
Jcra exF	CGT GAA TTT AAC CCA TAC CAG TAC AAT GGC TA
Jcra exR	GTG AAA TTC ACC TGG CGC GTA TTT TTG TTC
Jcra21F	ctggatcc GTG AAA CTG GAT GAA ATC GCT CGG CT
Jcra21R	ctgcggccgc GCT ACG GCT GAG CAC ACC G
Jcra33F	taTCTAGA ccataccagt acaatggcta tggtttttac
Jcra33flagR	tcAAGCTTttaCTTGTCGTCATCGTCTTTGTAGTC GCT ACG GCT GAG CAC ACC
Jcra33R	taAAGCTT TTA GCT ACG GCT GAG CAC AC
JcramycF	ctcGGTACC AAA CTG GAT GAA ATC GCT CGG CTG
JcramycR	ctcGAATTC GCT ACG GCT GAG CAC ACC
JcraredF	cagtcatggct gtggtgcgtg agcacaatta ccacccgaac gccgtggcag GTGTAGGCTGGAGCTGCTTC
JcraredR	cggtgacgttgagccactgccagcaccgggcactgtaagaagtcgagcagtCATATGAATATCCTCCTT
JespA p28F	cagGGATCCgaa aat ctg tat ttt cag ggc GAT ACA TCA AAT GCA ACA TCC GTT GTT AAT GTG AGT
JespA p28R	ccGCGGCCGC TTATTT ACC AAG GGA TAT TGC TGA AAT AGT TCT ATA TTG TAG AGA TTG
JespAF	Cgacatcgacgatctatgacttaggtaata
JespARNB	TAATACGACTCACTATAGGGCAA GGG ATA TTG CTG AAA TAG TTC TAT ATT GTA GAG A
JespGrtF	ATGTCGAGGACTCGGCAATGCAAA
JespGrtR	TGCTATTTGCTCTGCATCATGGCG
JkdpA pr F	tgatgaaaat agtaccgcct tttgtgtaat
JkdpA prR	CTA TCA CGC CTG CAC TCA CAG
Jkdpe mycF	ATggtacc ACA AAC GTT CTG ATT GTT GAA GAT GAA CAG

Jkdpe mycR	ATaagctt AAG CAT AAA CCG ATA ACC AAT ACC GGT TTC
JkdpE33_2F	ATtctaga GTG TTA CAC TTC CCC AGC AAA CT
JkdpE33_2R	ATaagctt tcaCTTGTCGTCATCGTCTTTGTAGTC AAG CAT AAA CCG ATA ACC AAT ACC GGT TTC
JkdpE33flagR	tcaagctt tcaCTTGTCGTCATCGTCTTTGTAGTC AAG CAT AAA CCG ATA ACC AAT ACC G
JkdpeRT1 F	TGGGATTGAGTTTATCCGCGACCT
JkdpeRT1 R	CGCCAATGCCAAACGGCTTACTTA
Jler _173F	CGGGATCCCGATGATTTTCTTCTATATCATTG
Jler _42R	CGGAATTCCGCGACCTTATCAGGAAGGACC
Jler aF	tcGGATCC CAT GTG CTG CGA CTG CGT TCg
Jler aR	tcGAATTC GCA TTT CCA TTT AGT TAA GCG TTT CTC TTT ATA AGA TAG ATC TCA TTG C
Jler bF	tcGGATCC tgcaattattaaagtcgtttgttaacgagatgattttcttct
Jler bR	tcGAATTC AGC GAC CTT ATC AGG AAG GAC CAA CAA TTA A
Jler cF	tcGGATCC ATT CAC TCG CTT GCC GCC TTC
Jler gsF	AGAGAAACGCTTAACTAAATGGAAATGC
Jler gsR	AGCGACCTTATCAGGAAGGACCAA
Jler R1	CGGGATCCTCTATCAAATTAGGACACAT
JLerDistal_F2	CGGAATTCCTGGGGATTCACTCGCTTGC
JLerDistal_R1	TCTAATGTGTAAAATACAT
JNCpxa RT1 F	CAACCAAATCACCAGCCGTAA
JNCpxA RT1 R	CATCGTGGCTGGGTGAAAG
JnleAGS F	cgAAG AGC ATA AAG CTG CCA AGC ATT ATA TGT CTT A
JnleAGS R	cg GTC ACA TAT CCG ATG TGG ACA GCT TTA ATA TAT AAC T
Jorf1 F	CGGAATTCATGTGCTGCGACTGCGTTCG
JqseE bad33F	gctctaga GGC TAT TCG CGT CTG ACG AGA GTA
JqseE bad33R	cccaagctt TTATTTCGTGTTTTTCGACGACGGTAATTCAATG
JrcsB 33F	ATtctaga ggaatagaaa aatacatcag cgacattgac
JrcsB 33R	at AAGCTT TTA GTC TTT ATC TGC CGG ACT TAA CG
JrcsB checkF	cg AAC CAG TGA CTT TGC TGC GTT AGC
JrcsB checkR	cg CTG TTG AAA TAA TGG GAA TCG TAG GAC GGA
JrcsB mycF	ctcGGTACC AAC AAT ATG AAC GTA ATT ATT GCC GAT GAC CA
JrcsB mycR	ctcGAATTC GTC TTT ATC TGC CGG ACT TAA CGT TAC TG
JrcsB redF	cgTGA ACG TAA TTA TTG CCG ATG ACC ATC CGA TAG TCT TGT TCG GTA TTC GTGTAGGCTGGAGCTGCTTC
JrcsB redR	cg CCA GCT TCA TCA TCG CAG ATT TCT TCT GGC TAC TGA TGG TTT TAA TAC TG CATATGAATATCCTCCTT
JsepLGS F	gcgtaatggttt atctgcttca tagggcgtgg
JsepLGS R	cgATG TAA GTT CAC CAT ATT TTT TCT CAT TGT TCA ACC A
JtnaA33F	ATtctaga TCT CTC GTT TAT TTA CTT GTG TTA GTA AAT GAT GGT GC
JtnaA33R	ATgcatgc TTA AAC TTC TTT CAG TTT TGC GGT GAA GTG AC
JtnaAcheckF	TGT GAC CTC AAA ATG GTT CAA TAT TGA CAA CAA GAT
JtnaAcheckR	AAT TTA AAT TTG CTT CTA ATA ACA ATA ACC CGG AAT GAA GCA TT
JtnaAmycF	ctcGGTACC AAG GAT TAT GTA ATG GAA AAC TTT AAA CAT CTC CCT GAA C
JtnaAmycR	ctcGAATTC AAC TTC TTT CAG TTT TGC GGT GAA GTG AC
JyfhKP1	GGCAAAGCCTGAATGCGCCTTAGCGACCAGGCGGCGCTGGTCAACCGCACCACGCTTATCGATGCCCGGCGCAGCGAAGCAATGACCAA CGCGGCGCTGGATGTAGGCTGGAGCTGCTTC
JyfhKP2	TTGCCCGCTCTCGTCGACCAGATACAGTTCCCCTTGCATACGGCGAATACAATCCCTGGCAATGCTTAATCCCAGACCGCTGCCCTTCACC GCCCCTTTTATATGAATATCCTCCTTA
Jz0639rtF	ATGAATGCGCTGACAACCGATGTG
Jz0639rtR	AACTGTTGGTGCGTTTGGGTTACG
Jz0640rtF	TGCCTCTGCCATGTCGCTGATTAT
Jz0640rtR	TTGCGTATACACCCACCCTTTCCA
Jz2077gsF	tgggagggga gagagttaga gtttcttatt
Jz2077gsR	GTT TTT TCT GTA ATA CAA GTC GAT TGT TTG TGA TTT CGC
Jz2077rtF	GCAACCTGGAACAGCAGATCAACA
Jz2077rtR	GGGCACTTAAGAAATTGTGTGTCGC
Jz3388gsF	ttgaataatt cccctgatat tgcaagggct
Jz3388gsR	GGC GCG TCT TAC AAG GAC GTT T
Jz3388rtF	CGG GGA ACG CTT CAG CGA TT
Jz3388rtR	CTA GAT ATT TTG TGT ACT TGA TTT GCA AAC AGC TCC G
Jz39_40gsF	TCA TTT TCT CTT GTT CAA AAT AAG TCG TAT TAA TGT TTC
Jz39_40gsR	TTA AAT TTT CCT GCC TGG CGT AAA CC
Jz4267gsF	GCA AAT CGT CCG GGG AAA CCT TAC

Jz4267gsR	TGG AGT ACT CCG AAA CTC GGA CG
Jz4267rtF	TGGTGAGCATCTTCATCTCTGCGT
Jz4267rtR	TCAAGGCTACCGATCACCAGTTCA
ler RTF	CGACCAGGTCTGCCCTTCT
ler RTR	GCGCGGAACTCATCGAAA
micF RTF	GCTATCATCATTAACTTTATTTATTACCGTC
micF RTR	AAACCGGATGCGAGGCA
nleArt549F	AGCCACTACTTCGACGGTAACC
nleArt624R	ACGAACCACTTGAGCTGTTAATCC
omrB RTF	CCAGAGGTATTGATAGGTGAAGTCAACTTCGGG
omrB RTR	GCGCATCTGCGCAGGCTGGTGTAATTCAT
qseC RT1 443F	AATGGGAATACCGTGAAGACATG
qseC RT1 505R	CCAACCACGGGATCAATTG
QseE RTF	CCC TTC ACC GCC CCT TT
QseE RTR	CGC GCC ATG ATC TTC GA
R1	CGGGATCCTCTATCAAATTAGGACACA
R2	CGGGATCCGTATGGACTTGTTGTATGTG
rcsBF	TCTCTCGCCAAAAGAGAGTGAAG
rcsBR	CGATCTCGGTCACCAGGAA
rpoA RTF	GCGCTCATCTTCTTCCGAAT
rpoA RTR	CGCGGTCGTGGTTATGTG
ryjA RTF	ACGTGCTCGAATGAGGTGTGTTGA
ryjA RTR	GCCACAAGGGCGCTTTAGTTTGTT
tir RTF	CCATGGAGAGCAGACGTAGCT
tir RTR	CGGTGATCCTGGATTTAACCTT
UcsgE RTF	TTATTTACGCTGGATTGTGGCGGC
UcsgE RTR	CATCGTGCACTGGGCCTTTCATTA
UgadX RTF	ATTATGGGATGACGCCCACAGAGT
UgadX RTR	AAATCCCTTGCGCAGCCATACTTG
UhlyA RTF	CGCAATGCGGGAAACAGACTCATT
UhlyA RTR	TTCTCTGCTGTGCCGAATACCTGT
UrpoA RTF	GGCAACCATTCTGGCTGAACAACT
UrpoA RTR	AGCGGACAGTCAATTCCAGATCGT
Y1	CGGAATTCATAAACGTTATCTCACATAATTTATATCATTTGATTAATTGTTG
Y2	CGGAATTCTAAATGGATTTTAAAAATATATGATTTTTTTGTTGAC
yfhKP1	GGCAAAGCCTGAATGCGCCTTAGCGACCAGGCGGCGCTGGTCAACCGCACCACGCTTATCGATGCCCGGCGCAGCGAAGCAATGACCAA CGCGGCGCTGGATGTAGGCTGGAGCTGCTTC
yfhKP2	TTGCCCGCTCTCGTCGACCAGATACAGTTCCCCTTGCATACGGCGAATACAATCCCTGGCAATGCTTAATCCCAGACCGCTGCCCTTCACC GCCCCTTTTATATGAATATCCTCCTTA

CHAPTER FOUR

Enterohemorrhagic Escherichia coli Virulence Regulation by the Two Bacterial Adrenergic Kinases QseC and QseE

INTRODUCTION

In mammals, the adrenergic hormones epinephrine and norepinephrine are an integral part of the stress response (95). These hormones initiate a regulatory cascade by binding to membrane bound G protein-coupled receptors (GPCRs) on mammalian cells. In microorganisms, the hormones epinephrine and norepinephrine are sensed by a variety of pathogens including enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 (41, 85, 120, 243), enterotoxigenic *E.coli* (ETEC) (183), *Salmonella enterica* serovar *Typhimurium* (15, 20, 21, 205), *Vibrio parahaemolyticus* (212) and recently in the fish and human pathogen *Edwardsiella tarda* (326). Detection of epinephrine triggers differential regulation of virulence pathways including motility (20, 41, 326), invasion (205, 243) and attaching and effacing (AE) lesion formation, which are typical of EHEC and enteropathogenic *E.coli* (EPEC) infections (204, 287).

EHEC is an enteric bacterium that causes hemorrhagic colitis (142). In some cases, complications may arise including hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) (100). In a similar fashion to other pathogens, EHEC controls virulence gene expression aiming for maximal energy efficiency. It senses signals from both the mammalian and intestinal microbial flora to discern its arrival to its colonization niche, the

colon. EHEC has been shown to sense the autoinducer 3 (AI-3) signal produced by the intestinal microbial flora, as well as the aforementioned host produced hormones epinephrine and norepinephrine (41, 120, 243).

Two histidine sensor kinases have been identified as sensors of epinephrine and norepinephrine in EHEC. The first, histidine kinase QseC, has been reported to increase its autophosphorylation in response to epinephrine, norepinephrine or AI-3 (41). QseC then transfers its phosphate not only to its cognate response regulator (RR) QseB, but also to two other RRs: QseF and KdpE (120). QseC via QseB regulates flagellar and motility genes through the direct binding of QseB to the promoter region of *flhDC,* the master regulator of the flagellar regulon (43, 120). Through QseF, QseC activates Shiga toxin production (120, 182, 183).

AE lesion formation, which is characterized by the attachment of bacteria to colonic epithelial cells followed by an induction of extensive actin rearrangement underneath the bacteria and effacement of surrounding microvilli (133, 158, 204, 287), has been shown to be regulated by QseC through the KdpE RR (120, 243)(Njoroge *et al,* submitted). We have shown that KdpE directly binds to the promoter region of *ler*, which encodes for the master regulator of the locus of enterocyte effacement (LEE) genes that are required for AE lesion formation (190). The LEE genes are mostly organized into five operons (*LEE1-5*) with the first operon encoding for the LEE transcriptional activator Ler (67, 190, 194, 286). Most of the genes in the LEE are necessary for AE lesion formation, and include genes that encode for the structural components of a type three secretion system (TTSS), as well as some effectors that are translocated through this TTSS into the host epithelial cell (130, 190). EspA, a *LEE4*-encoded

secreted protein, forms part of the translocon of the TTSS, providing a structural direct link between the bacteria and the infected host cell (131, 152, 222). The *LEE5* gene *tir* encodes an effector which gets translocated through the TTSS into the host cell where it serves as a receptor for another *LEE5* encoded protein, the adhesin Intimin (encoded by the *eae* gene) (132, 150). The interaction of these two proteins allows for the intimate attachment of EHEC to the host epithelial cell. The TTSS also translocates non-LEE encoded effectors such as EspFu/TccP (35, 89) and NleA/EspI (102, 207, 208, 253), which mimic mammalian signaling proteins and hijack host cell signal transduction. The NleA effector is an important virulence factor that has been shown to be required for virulence in the *Citrobacter rodentium* murine model (102, 208). It has been reported to disrupt intestinal tight junctions (296) and to localize to the Golgi apparatus where it inhibits cellular protein secretion (156). The positive control of the LEE genes, Shiga toxin production and motility by QseC culminates in the activation of the EHEC virulence repertoire. Deletion of *qseC* has been shown to attenuate virulence of not only EHEC but also *Salmonela enterica* Typhimurium*, Francisella tularensis,* uropathogenic *E.coli* (UPEC) and *Edwarsiella tarda* (20, 41, 163, 243, 326) *in vitro* and *in vivo*.

The second epinephrine sensor, the histidine kinase QseE, responds to epinephrine, phosphate and sulfate by increasing its autophosphorylation level, and then transfers its phosphate to its cognate RR QseF (247). Importantly, QseC acts upstream of *qseEF*, given that QseC activates expression of *qseEF* (248). The QseEF two component system has been characterized as being important for *espFu* transcription (248). The fact that both QseC and QseE increase their phosphorylation in an epinephrine-dependent manner, and that QseC has

been shown to initiate a signal transduction cascade in response to this hormone, posed an interesting question of how this intricate control of epinephrine-dependent pathogenesis is maintained. To answer this, we performed transcriptional and phenotypical analyses on strains lacking one or both of the genes encoding for these kinases in the absence or presence of the hormone epinephrine. Although the influence of epinephrine on QseC-dependent regulation of the LEE genes, motility and Shiga toxin production has been previously reported (41, 243), the effect of this hormone on QseE-dependent regulation of downstream genes has not been carried out. In this work, we show that the adrenergic kinases QseC and QseE act in an antagonistic manner to regulate both LEE encoded and non-LEE encoded genes in order to control overall virulence of the enteric pathogen EHEC. We also report the role of epinephrine-dependent increase in AE lesion formation, and the important role that these two adrenergic kinases play in the formation of these lesions.

RESULTS

Global assessment of QseC and QseE gene regulation in EHEC

Previous microarray data comparing the single mutants *ΔqseC* and *ΔqseE* to wild-type EHEC O157 (wt) in Dulbeco's modified eagle medium (DMEM) have shown divergences in global gene regulation by these two adrenergic receptors (120, 246). We have previously reported the role of QseC in the activation of the expression of genes involved in motility (42, 43), Shiga toxin production (120) and the LEE pathogenicity island (120) (Njoroge *et al,* submitted). We have also reported the role of QseE in the regulation of *espFu* (248). The regulation of virulence factors by these two sensor kinases as had been identified before this work is summarized in

Fig.4.1a (41, 120, 247, 248) (Njoroge *et al,* submitted). As the summary indicates, both QseC and QseE have been shown to sense epinephrine (41, 247). Epinephrine dependent gene expression had only been reported for genes downstream of QseC, but not for targets downstream of QseE. Another open question was whether QseC and QseE are the only sensors of epinephrine in EHEC O157. To address these issues, we first needed to define genes that were regulated by both kinases, and then test their expression in response to the presence of this adrenergic hormone. Additionally, we hypothesized that if these two kinases were the only epinephrine sensors, deletion of both QseC and QseE would make the resultant double mutant unable to respond to epinephrine. We therefore constructed a non-polar double deletion of the *qseC* and *qseE* genes (*ΔqseCΔqseE*). Using quantitative real-time reverse transcriptase PCR (qRT-PCR), we confirmed the deletion of both genes as well as the efficacy of plasmid encoded QseC and QseE to rescue gene transcription (Fig.4.1b).

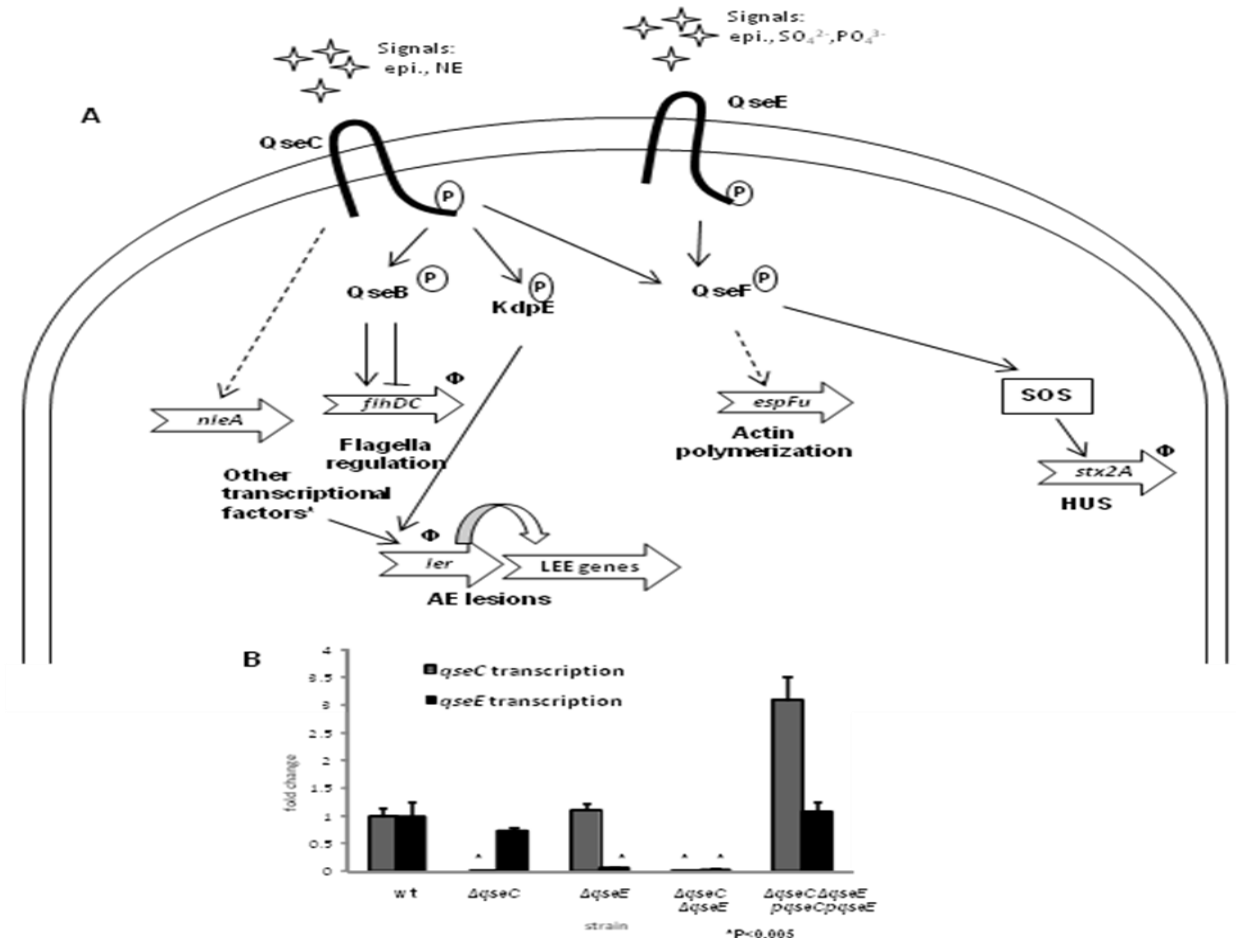


Fig.4.1. Confirmation of non-polar deletion and rescue of expression of the genes encoding for the adrenergic kinases *qseC* and *qseE.* (A) A summary of QseC and QseE's cascade of virulence factor regulation as reported before this work. Genes whose expression had been shown to be affected by epinephrine have Φ next to them. * The *ler* promoter is highly regulated by many transcription factors including GrlA, Pch, GadE, QseA and H-NS (18, 33, 129, 140, 276). Epi, epinephrine. NE, norepinephrine. AE, attaching and effacing. **(B)** qRT-PCR analysis examining q*seC* and *qseE* expression in wt, *ΔqseC, ΔqseE, ΔqseCΔqseE* and the complemented double mutant strains grown to an OD_{600} of 1.0 in low glucose DMEM. The genes' transcript levels were quantified as fold differences normalized to wt gene transcription levels. The samples' *rpoA* transcript levels were used as internal controls to normalize the output C_T values. The data is from at least three independently grown replicates.

Next, using Affymetrix *E.coli* 2.0 microarrays we performed a global transcriptomics analysis of wt, *ΔqseC, ΔqseE* and the double mutant *ΔqseCΔqseE* grown in DMEM, which is

optimal for the expression of TTSS genes and other EHEC virulence factors. These growth conditions were performed in the presence of AI-3, which is endogenously produced by EHEC O157 and is sensed by QseC to differentially regulate its targets (41, 85, 319). The arrays contain over 10,000 probe sets that cover genes in the genomes of the two sequenced EHEC strains (EDL933 and Sakai), the K-12 strain MG1655, the UPEC strain CFT073 as well as intergenic regions that can encode for sRNAs or non-annotated small open reading frames (ORFs).

The microarray analysis revealed that although a majority of the probe sets in the double kinase mutant were unchanged compared to wt, 510 probe sets showed increased expression, with 47% of these being pathogen specific (Table 4.1). Additionally, a total of 300 probe sets in the double mutant had decreased expression with 65% of the genes being pathogen specific. This percentage of pathogen specific genes that were differentially regulated in the double mutant was similar to *ΔqseC*'s. The *ΔqseC* strain had 149 probe sets increased and decreased with the pathogen specific ones representing 52% of both the increases and the decreases. On the other hand, the *ΔqseE* global gene regulation profile revealed more differential expression than is seen in the double mutant, with twice as many probe sets increased in *ΔqseE* than in *ΔqseCΔqseE* (1282 vs. 510). Additionally, more than four times as many probe sets in *ΔqseE* were decreased than in *ΔqseCΔqseE* (1294 vs. 300). The mostly up-regulated probe sets in the double kinase mutant, as indicated by the microarray, included many hypothetical genes, metabolism genes, and a few (putative) sensor kinase genes such as *yedV* and *zraS.* These genes' expression remained unchanged in the single mutants' profile suggesting that QseC and QseE's regulatory effect on them may be redundant, and only the

deletion of both sensors could make a difference in their expression. The most highly down-regulated probe sets in the double kinase mutant, which included pathogen specific genes such as *Z4320, c1516* and *c4309,* had differential regulation in the single mutants that did not follow a distinct pattern.

Next we investigated whether there were any commonly regulated genes in the arrays. *ΔqseCΔqseE* has more down-regulated genes in common with *ΔqseC* (49 genes) than with *ΔqseE* (29 genes) (Fig4.2a). Additionally *ΔqseCΔqseE* shares more up-regulated genes with *ΔqseE* (91 genes) than with *ΔqseC* (43 genes) (Fig.4.2b). These data suggest that the double kinase mutant has the plasticity to regulate gene expression to mimic either one of the single mutants depending on the set of genes being evaluated. Of the 300 genes decreased in *ΔqseCΔqseE,* only 4 genes were commonly regulated with *ΔqseC* and *ΔqseE* (Fig.4.2a), while of the 510 genes increased in the double kinase mutant, only 8 genes were commonly regulated with the single kinase mutants (Fig.4.2b). These commonly regulated genes included four that were metabolism related (*fruA, rbsD, ais, srlA*), and four that were involved in metal sensing (*ygiW, ais, arnF, basR*). The others were hypothetical genes. This leaves a total of 610 genes (226 decreased and 384 increased) that are differentially regulated in the *ΔqseCΔqseE* strain that are not shared with the single mutants. This indicates that the double kinase mutant transcriptome does not fully overlap with the single kinase mutants, suggesting that deletion of one or both kinases promotes extensive rewiring of downstream signaling.

Another possible explanation for the paucity of commonly regulated targets may be that the two kinases conversely regulate similar target genes. Indeed in the single mutant arrays we identified a total of 95 genes conversely regulated by these two kinases. Expression of 78 genes

was decreased in *ΔqseC,* and increased in *ΔqseE*, while expression of 17 genes was increased in *ΔqseC* and decreased in *ΔqseE* (Fig.4.2c and Fig.4.2d). These conversely regulated genes included the LEE genes and *nleA* encoding a non-LEE effector. Altogether, these data indicated that although there may be convergent regulation of some genes by QseC and QseE, other genes may be regulated by only one of these adrenergic kinases. (Array data has been deposited on the NCBI GEO database, GEO number 33895).

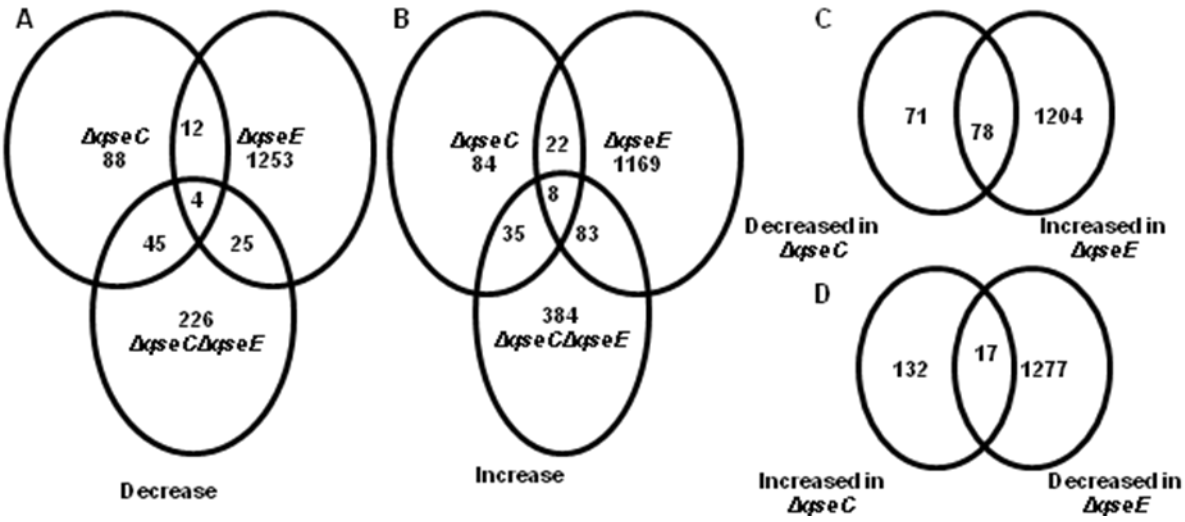


Fig.4.2. Global analysis of QseC and QseE's effect on EHEC O157 gene transcription. Venn diagrams showing the number of overlapping **(A)** down-regulated genes and **(B)** up-regulated genes between the *qseC,* the qseE *a*nd the *qseCqseE* mutant strains compared to wt. **(C)** Venn diagram indicating genes that are decreased in *ΔqseC* and increased in *ΔqseE*. **(D)** Venn diagram indicating genes that are increased in *ΔqseC* and decreased in *ΔqseE*. Strains for the microarrays were grown to an OD_{600} of 1.0 in low glucose DMEM.

QseC and QseE conversely regulate transcription of the LEE and *nleA*

Global transcriptome analysis of the single and double kinase mutants indicated that there was differential regulation of some targets (Fig.4.2). These included the LEE genes,

previously reported to be activated by QseC in DMEM (120, 244), and *nleA* which had also been previously reported to be mildly activated by QseC in DMEM (120). However, whether QseE played any role in the regulation of the LEE or *nleA* was still an open question, as well as if and how QseC and QseE may interact in this regulation. We first performed qRT-PCR to compare the differences in mRNA levels of genes in the *LEE4* an *LEE5* operons. RNA was extracted from wt, *ΔqseC, ΔqseE* and *ΔqseCΔqseE* grown in low glucose DMEM to OD_{600} 1.0, and assessed for differences in transcription of the *tir* and *eae* genes (both within *LEE5*) and the *espA* gene (*LEE4*). The mRNA levels of all these three genes were significantly decreased in *ΔqseC* compared to wt (Fig.4.3a-c) with *tir, eae* and *espA* transcription decreasing two-fold for all three. On the other hand, the same genes had a significant increase in transcription in *ΔqseE* relative to wt, with mRNA levels of *tir, eae* and *espA* being augmented 12-fold, four-fold and six-fold respectively. When the mRNA levels of the three LEE genes in *ΔqseCΔqseE* were evaluated, their levels were comparable to those of *ΔqseE* (*tir* up nine-fold, *eae* and *espA* up six-fold). Transcription of all genes was rescued upon complementation.

Next we evaluated whether this converse gene regulation by QseC and QseE extended beyond those encoded by the LEE pathogenicity island. NleA is a non-LEE encoded effector translocated by the LEE TTSS into host cells, and has been shown to play an important role in virulence (102, 156, 296). It has been shown to be mildly activated by QseC in DMEM (120). The microarray data indicated that *nleA's* expression was decreased in *ΔqseC,* increased in *ΔqseE* and also elevated in *ΔqseCΔqseE*. This differential *nleA* regulation by these two kinases mirrored the LEE regulation. Therefore we assessed whether *nleA* transcriptional analysis using the more sensitive qRT-PCR method would also mirror these previous observations. Compared

to wt, *nleA* mRNA levels were decreased two-fold in *ΔqseC,* while we observed over 10-fold increases in both *ΔqseE* and *ΔqseCΔqseE* (Fig.4.3d). These findings support a positive and negative role for QseC and QseE respectively in the regulation of both LEE genes and the gene encoding the non-LEE effector, NleA. Although both kinases regulated *LEE4*, *LEE5* and *nleA* (Fig.4.4c), QseE is epistatic to QseC as observed by the fact that the double mutant has a phenotype comparable to a *qseE* deletion.

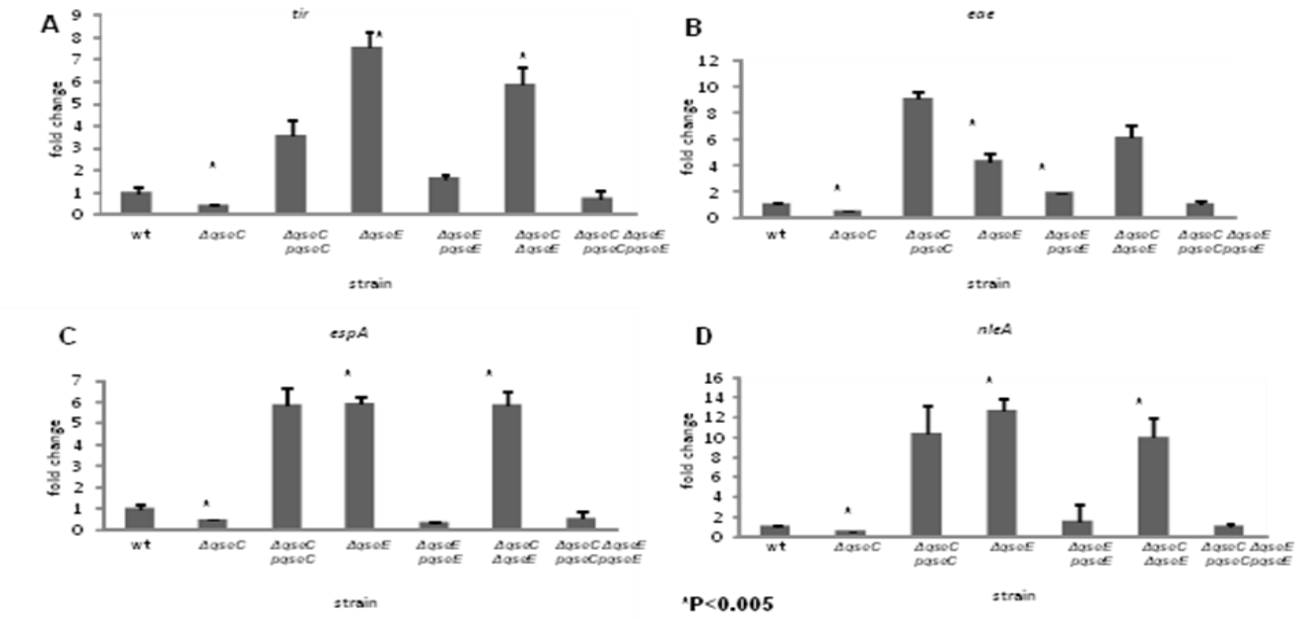
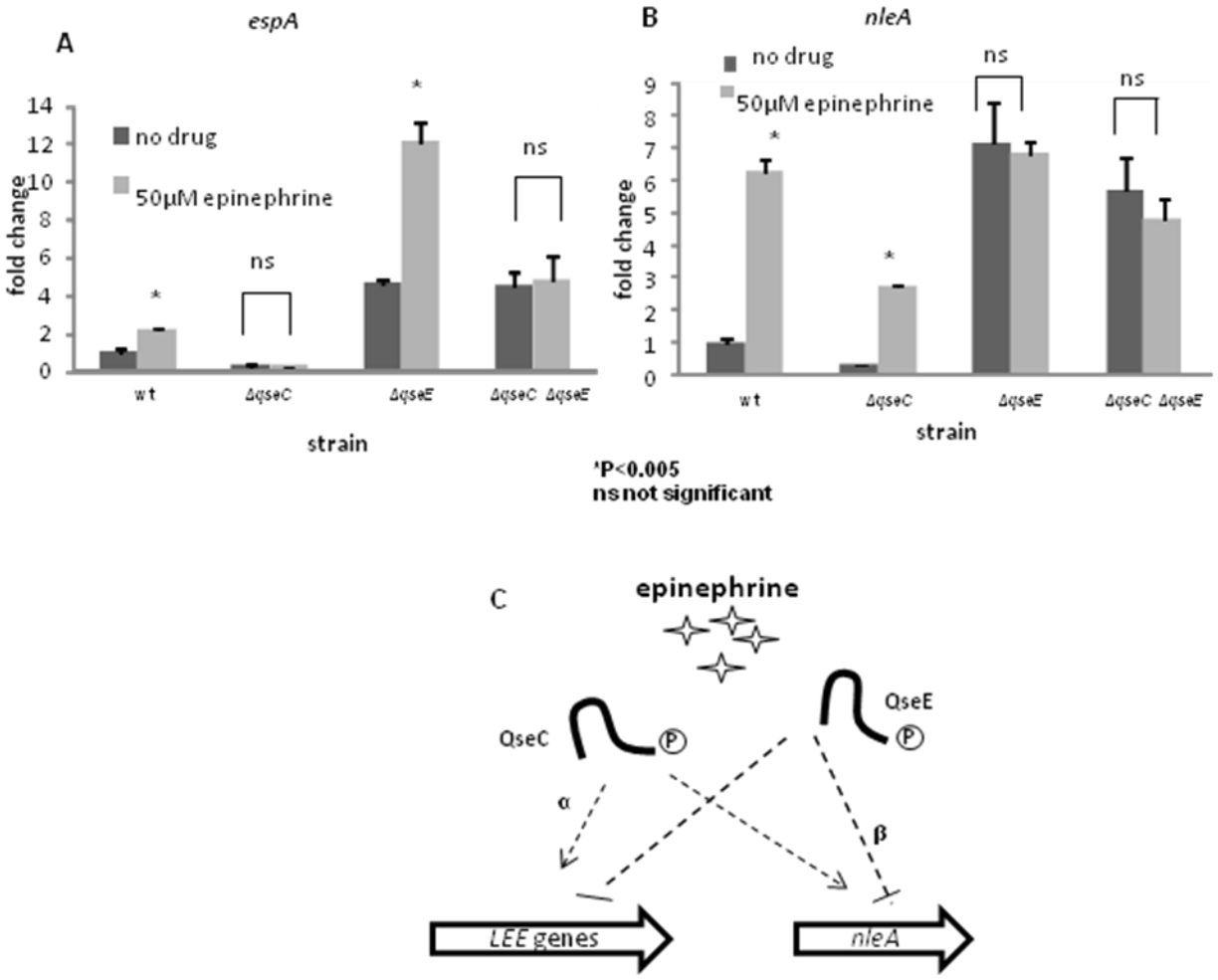


Fig.4.3. Both QseC and QseE regulate the LEE genes and *nleA.* Using qRT-PCR probes for **(A)** *tir* (*LEE5*), **(B)** *eae* (*LEE*5), **(C)** *espA* (*LEE*4) and **(D)** *nleA*, mRNA levels for all these genes were quantified and normalized to the mRNA levels of the endogenous internal control gene *rpoA.* The mRNA levels were graphed as fold changes compared to wt transcript levels. The results are from at least three independent samples.

Deletion of both kinases eliminates the epinephrine dependent regulation of virulence genes

Previous studies have shown that both QseC and QseE sense the hormone epinephrine (21, 41, 120, 205, 247). Given that both adrenergic kinases regulate the LEE genes as well as *nleA* (Fig.4.2), we next investigated the role that epinephrine plays in this regulation. We grew wt and the mutants in low glucose DMEM in the absence or presence of epinephrine (final concentration of 50µM), extracted RNA and evaluated *nleA,* and as a representative of the LEE genes, *espA* mRNA levels. In the presence of epinephrine, the mRNA levels of both genes were significantly increased in wt compared to wt with no drug (Fig.4.4), with *espA* levels increased two-fold and *nleA* levels increased six-fold. Interestingly, the epinephrine effect on transcription in the single deletions differed depending on the gene evaluated. When *espA* transcription was compared between non-treatment and treatment with epinephrine, no change was observed in *ΔqseC* while there was a three-fold increase in *espA* mRNA levels in epinephrine treated *ΔqseE* compared to non treated *ΔqseE* (Fig.4.4a). These results indicate that although both kinases are involved in *espA* gene regulation, epinephrine dependent regulation of *espA* occurs primarily via QseC. In the double mutant, no significant change was observed between non-treatment and treatment with epinephrine. When we evaluated *nleA* mRNA levels in the absence and presence of epinephrine, we observed a six-fold increase in wt (Fig.4.4b). In *ΔqseC* with epinephrine, we observed a 2.5-fold increase in *nleA* transcription compared to *ΔqseC* without epinephrine. However, there was no significant change between *ΔqseE* with and without epinephrine. These data suggests that although QseC and QseE both regulate *nleA* transcription, epinephrine dependent regulation of *nleA* occurs primarily via QseE. The double mutant *ΔqseCΔqseE* was also blind to the effects of epinephrine. Altogether, these results

support our hypothesis that QseC and QseE sense epinephrine to regulate the expression of LEE and non-LEE effectors (Fig.4.4c), and that in the absence of these two adrenergic kinases, EHEC is unable to sense this hormone and is consequently unable to differentially regulate these genes.



*P<0.005
ns not significant

Fig.4.4. Effect of epinephrine on QseC and QseE dependent regulation of LEE and non-LEE genes. Expression of **(A)***espA* (*LEE*4) and **(B)***nleA* was evaluated by q-RTPCR in wt and the mutants grown to late exponential phase in the absence and presence of epinephrine (final concentration of 50µM). The error bars indicate standard deviations of the $\Delta\Delta C_T$ values. The levels of endogenous *rpoA* mRNA were used to normalize the C_T values. **(C)** Cartoon representation showing the converse regulation of the LEE genes and *nleA* transcription by the epinephrine sensing kinases QseC and QseE. Although both kinases regulate the LEE genes and

nleA, epinephrine dependent regulation of the LEE genes is mostly via QseC (dotted arrow with α), while epinephrine dependent regulation of *nleA* is mostly via QseE (dotted line with β).

Global analysis of epinephrine dependent EHEC gene regulation by the two adrenergic kinases QseC and QseE

Since transcription of the LEE genes and *nleA* in the double kinase mutant *ΔqseCΔqseE* is epinephrine independent (Fig.4.4), we next investigated the extent of this lack of response to epinephrine. Using Affymetrix *E.coli* 2.0 microarrays we performed a global gene analysis of wt, the single and the double mutants grown in low glucose DMEM in the absence or presence of 50μM epinephrine. The microarray data indicated that there was more differential regulation when wt was treated with epinephrine than when the mutants were treated with epinephrine (Table 4.1). When wt with epinephrine was compared to wt with no treatment, 21% of the genes were up-regulated while 12% were down-regulated indicating a possible dual role for epinephrine as both an activator and a repressor of its target genes. Altered genes were observed both in the K-12 genes from strain MG1655 which contains the conserved *E.coli* backbone, and in the pathogen specific probe sets. It is interesting to note that a higher percentage of the pathogen specific genes were up-regulated than down-regulated (24% increased vs. 3% decreased). Comparison of *ΔqseCΔqseE* with epinephrine to *ΔqseCΔqseE* with no treatment indicated very few genes were differentially regulated, with 0.3% being up-regulated and 1.4% being down-regulated. This indicated to us that deletion of both *qseC a*nd *qseE* left the double mutant strain mostly unable to sense epinephrine, which correlates with the epinephrine unresponsiveness observed by qRT-PCR (Fig4.4).This relative unresponsiveness

was also observed in the single mutants. Adding epinephrine to *ΔqseC* only altered the expression of 0.4% of the total genes while addition of epinephrine to *ΔqseE* led to only 1% of the genes being differentially regulated. The fact that a total of 34% of the genes were differentially regulated when epinephrine was added to wt, while less than 2% of the genes were differentially regulated when epinephrine was added to either the single or the double mutants, indicates that deletion of QseC and QseE results in EHEC being mostly unable to sense epinephrine, and that both kinases seem to work in concert towards the proper sensing of this signal.

Table 4.1 Comparison of effect of epinephrine (epi) on wt and the mutants

		Increase	Decrease	No change	total
wt vs. *ΔqseC*	MG1655 specific	71	71	3928	4070
	Pathogen specific	78	78	5787	5943
	total	149	149	9715	10013
wt vs. *ΔqseE*	MG1655 specific	558	871	2641	4070
	Pathogen specific	724	423	4796	5943
	total	1282	1294	7437	10013
wt vs. *ΔqseCΔqseE*	MG1655 specific	268	104	3698	4070
	Pathogen specific	242	196	5505	5943
	total	510	300	9203	10013

Transcriptome comparison of the four array sets revealed that in wt, epinephrine increased the regulation of most of the LEE genes (Fig.4.5a) as well as most of the genes that code for confirmed and predicted non-LEE EHEC O157 effectors (299) (Fig.4.5b). The heat maps comparing *ΔqseC* with and without epinephrine indicated that in the presence of epinephrine,

genes encoding the non-LEE effectors were differentially regulated, while the LEE genes were unaffected. On the other hand, epinephrine increased LEE gene expression in *ΔqseE* but did not affect non-LEE effector gene expression. In the double kinase mutant *ΔqseCΔqseE,* neither set of genes responded to the addition of epinephrine. These heat maps mirrored the qRT-PCR data (Fig.4.4), which had suggested that the LEE genes were still responsive to epinephrine in *ΔqseE* but not in *ΔqseC* while non-LEE encoded effectors such as *nleA* was still responsive to epinephrine in *ΔqseC* but not in *ΔqseE.* These results also confirmed the *ΔqseEΔqseC q*RT-PCR data which had indicated that in the double kinase mutant, the transcription of both the LEE genes and *nleA* is unaffected by epinephrine.

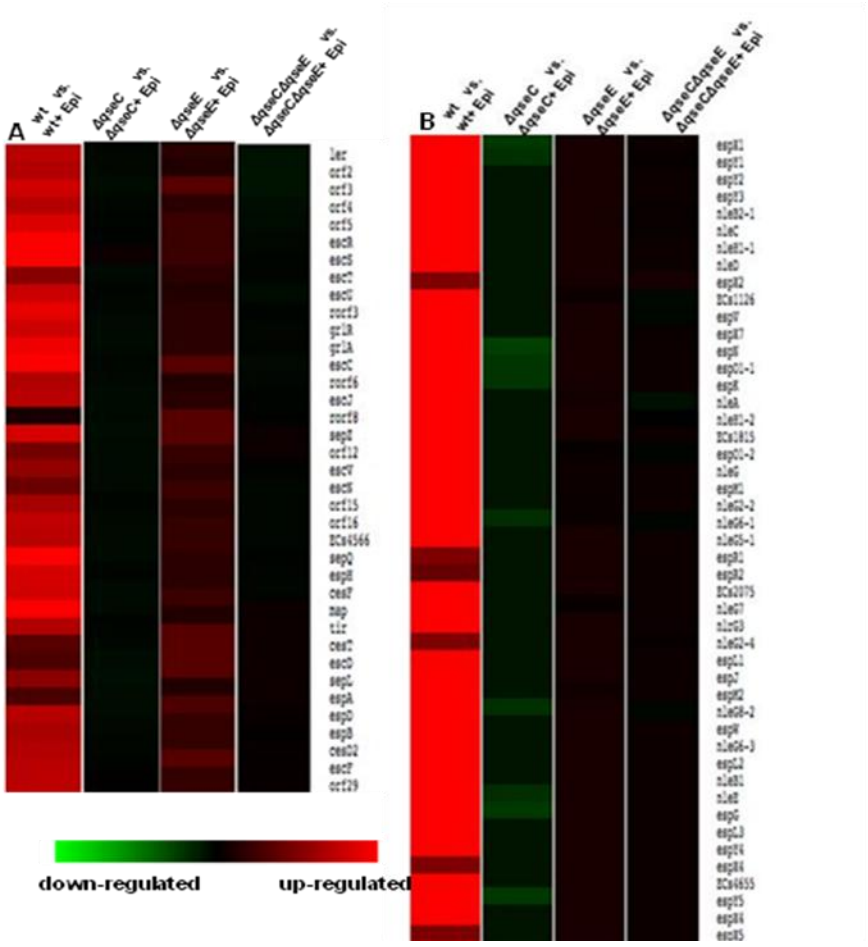


Fig.4.5.Deletion of the two adrenergic kinases QseC and QseE impairs epinephrine dependent regulation of multiple EHEC virulence factors. Heat maps from microarray analysis representing the effects of epinephrine on wt *ΔqseC, ΔqseE*and *ΔqseCΔqseE*. The strains with epinephrine were compared to the same strains with no treatment. Red indicates up-regulation, green indicates down-regulation and black indicates no change. **(A)** A heat map representing differential regulation of the LEE genes. **(B)** A heat map showing the differential expression of non-LEE encoded genes. Epi, epinephrine.

QseE regulation of the LEE and non-LEE encoded effectors occurs through RcsB

We have shown that QseC and QseE conversely regulate genes both within and outside the LEE pathogenicity island (Figs.4.3 and 4.4). We next explored the mechanism of this differential regulation. We have previously shown that QseC regulation of the LEE occurs through the KdpE RR (120)(Njoroge et al. submitted). Unlike QseC, which phosphorylates three RRs (QseB, KdpE and QseF), QseE only phosphorylates its cognate RR QseF (333). QseF is a DNA binding transcriptional regulator that binds sigma-54 dependent promoter regions (248). The transcription of *LEE4* operon (containing *espA*), as well as the *LEE5* operon (containing *tir* and *eae*) are sigma-70 dependent (164, 265). As none of these genes have a sigma-54 dependent promoter, it is unlikely that QseE-dependent regulation of these genes is through QseF. We have previously shown that QseE regulates expression of several two-component systems at the transcriptional level, including the RcsBC system (246). The response regulator of the system, RcsB, has been shown to be involved in the regulation of the LEE genes in the Sakai strain of EHEC (298). To explore whether RcsB was an intermediate in the QseE regulation of these genes, we assessed *rcsB* mRNA levels in wt, *ΔqseC, ΔqseE* and *ΔqseCΔqseE*. The transcription of *rcsB* was unaffected in *ΔqseC* but increased significantly in *ΔqseE* and *ΔqseCΔqseE* (Fig.4.6a). These results suggested that the upregulation of the *rcsB* observed in *ΔqseE* and the double mutant may be due to the fact that QseE is an inhibitor of *rcsB* transcription, which is in agreement with our previous report (246).

Next we constructed a *rcsB* non-polar mutant. RNA was then extracted from wt, the mutant and the complemented strain, and absence and rescue of *rcsB* expression in these strains was confirmed by qRT-PCR (Fig.4.6b). We then assessed the impact of RcsB regulation

on the expression of the LEE genes *tir, eae* and *espA.* Transcription of all of these genes was significantly decreased in *ΔrcsB* (2.5-fold for *tir* and *espA* and four-fold for *eae*) and expression was rescued upon complementation with *rcsB* on a plasmid (Fig.4.6c). It is worth noting that the expression of the genes assessed was much higher in the complement than in wt, probably due to the fact that the complement over-expressed *rcsB.* Because the LEE genes are activated by Ler, the master regulator of the LEE pathogenicity island, we assessed the effect of *rcsB* deletion on *ler* transcription. We observed a significant down-regulation of five-fold in *ler* transcription in the mutant. We also observed a two-fold reduction in the expression of the *nleA* gene in the *rcsB* mutant (Fig.4.6d). Altogether, these data suggest that QseE repression of the LEE and *nleA* transcription occurs indirectly via the RcsB RR.

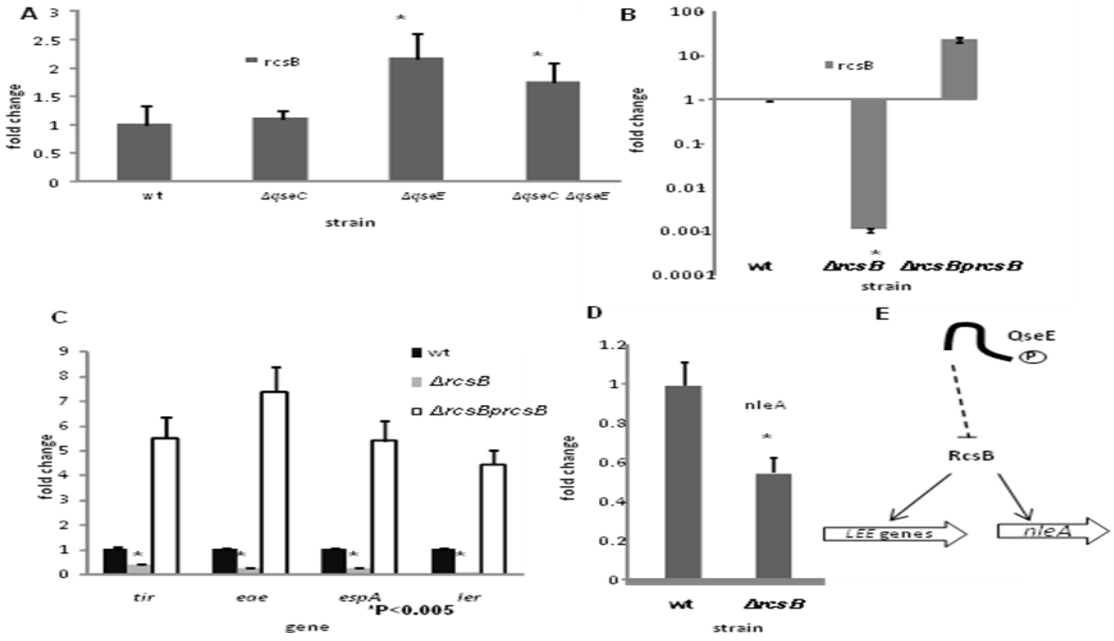


Fig.4.6. QseE regulates *nleA* and the LEE genes through its inhibition of *rcsB* transcription. (A) Transcriptional profile of the response regulator *rcsB* in wt, *ΔqseC, ΔqseE* and *ΔqseCΔqseE.* **(B)** Confirmation by qRT-PCR of the deletion and rescue in expression of *rcsB.* **(C)** Transcriptional profile of LEE gene expression for wt, *ΔrcsB* and its complement. **(D)** qRT-PCR evaluating the

transcription of *nleA* in wt and *ΔrcsB. nleA* mRNA levels were decreased in *ΔrcsB* compared to wt. Error bars indicate the standard deviations of the $\Delta\Delta C_T$ values. The mRNA levels of endogenous *rpoA* were used to normalize the C_T values. **(E)** Cartoon representation showing that the inhibition of the LEE genes and *nleA* transcription by QseE is indirect via RcsB. RcsB, whose transcription is inhibited by QseE, is a transcriptional activator of the LEE genes and *nleA.*

AE lesion formation

Since the presence of epinephrine and/or the deletion of *qseC, qseE* or both *qseC* and *qseE* together affect the expression of *nleA* as well as the LEE genes, we next used fluorescent actin staining (FAS) to investigate whether this differential regulation affected the formation of AE lesions. As most commercially available fetal bovine serum (FBS) used to supplement HeLa epithelial cell culture media contains traces of epinephrine, we used a dialyzed FBS (Gibco, Invitrogen), which has all molecules with a molecular weight less than 10,000 Da removed. HeLa epithelial cells were infected for six hours with wt or the mutant strains in the absence or presence of epinephrine to a final concentration of 50 μM. The infected cells were then fixed and stained with FITC-phalloidin (stains filamentous actin green) and propidium iodide (stains the HeLa nuclei and bacteria red). The pedestals were visualized as red bacteria cupped by bright green actin (Fig.4.7a). To ensure comparable infection by the different strains, an aliquot of the input was also serially diluted and plated to confirm similar bacterial numbers were used for infection. Infection rates were calculated as the number of HeLa cells with bacteria attached as a percentage of the total number of HeLa cells.

Incubation of HeLa cells with wt EHEC O157, in the absence of epinephrine, led to a 40% infection rate (Fig.4.7b). When the infection was carried out in the presence of epinephrine, the percentage of cells infected increased a significant two-fold. Upon *ΔqseC* incubation with these

epithelial cells in the absence of epinephrine, the percentage of infected cells decreased two-fold compared to wt. Supplementation of epinephrine did not increase infection. These results are consistent with the observation that LEE expression is decreased in *ΔqseC* (Fig.4.4a), and that addition of epinephrine to *ΔqseC* did not lead to increased LEE expression. Next, when the FAS assay was performed with *ΔqseE*, we observed that in the absence of epinephrine infection rates were two-fold higher than wt without epinephrine, and comparable to wt in the presence of epinephrine. Addition of epinephrine to the *ΔqseE* infection assay did not increase infection rates. The infection rate in *ΔqseCΔqseE* was comparable to wt but was unaffected by co-incubation with epinephrine. These results give further evidence that epinephrine dependent LEE regulation in EHEC O157 is only dependent on QseC and QseE.

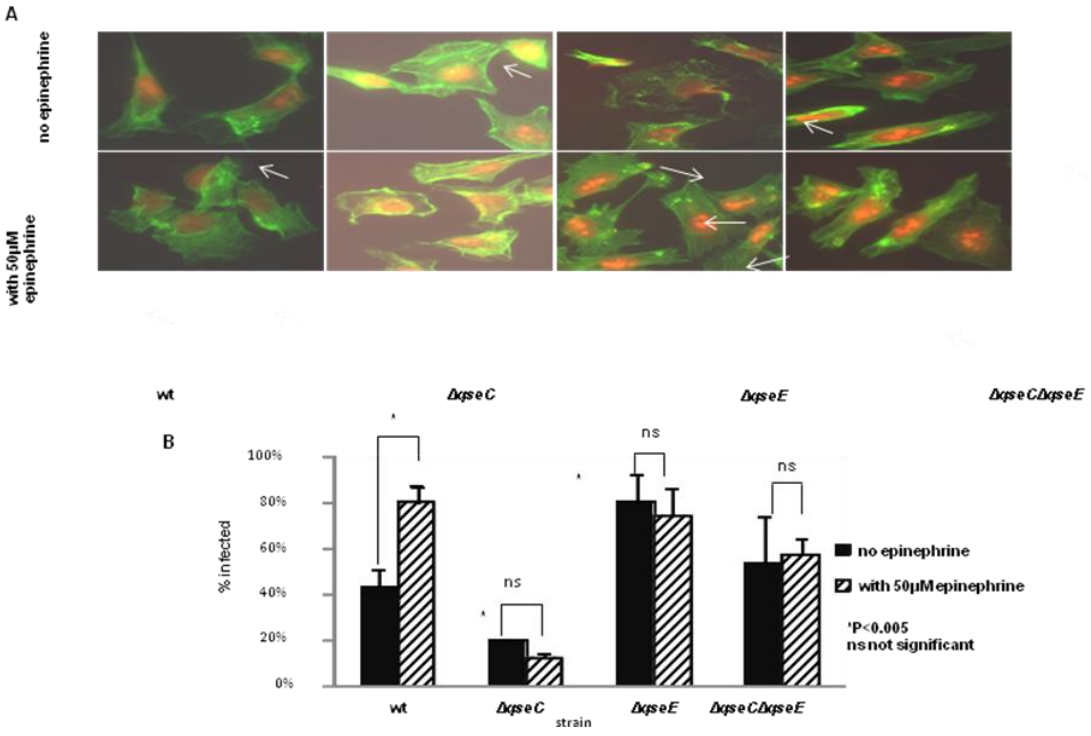


Fig.4.7. Fluroscent actin staining assays (FAS). The different strains indicated were used to infect HeLa cells for 6 hours in the absence or presence of epinephrine (final concentration

50μM). HeLa actin was stained green with FITC phalloidin while HeLa nuclei and bacteria were stained red with Propidium Iodide. Formation of pedestals was visualized as bright green (actin) cups onto which red bacterial dots bound. The experiments were performed in duplicate at least three times. For every slide at least 100 cells were evaluated. **(A)** Visualization of pedestals formed by bacteria on HeLa cells. **(B)** Graphical representation of the percentage of infected HeLa cells. Compared to wt, the infection rate of *ΔqseC* was decreased while that of *ΔqseE* was increased. The infection rate of *ΔqseCΔqseE* was comparable to wt. Treatment of the wt-HeLa infection with epinephrine doubled the infection rate while addition of epinephrine to the mutants' infection did not significantly change the infection rate.

Regulation of motility is dependent on QseC but not QseE

We have previously shown that the regulation of motility in EHEC is QseC dependent (43, 120, 285). Given that LEE gene regulation shows a converse relationship between QseC and QseE, we investigated whether this phenomenon was also observed in motility regulation. We assessed the motility of wt, *ΔqseC, ΔqseE, ΔqseCΔqseE* and their complements in 1% tryptone-agar media. As expected, motility of *ΔqseC* compared to wt was significantly diminished, with the halo diameters of the mutant reduced almost five-fold (Fig.4.8a and 4.8b). Deletion of *qseE* did not affect motility with halo diameters for *ΔqseE* being comparable to those of wt. When both *qseC* and *qseE* were deleted, the double mutant had a motility defect similar to *ΔqseC,* and this decrease in swimming could be rescued upon complementation with *qseC* and *qseE in trans.*

To confirm these motility plate results, we assessed whether the transcription of *fliC* which encodes for flagellin (Fig.4.8c), was affected by deletion of *qseC* and/or *qseE*. The strains were transformed with the *fliC-lacZ* transcription fusions and beta-galactosidase assays were performed. In both *ΔqseC* and *ΔqseCΔqseE fliC* transcription was significantly reduced compared to wt. In *ΔqseE,* transcription of *fliC* was comparable to wt (Fig.4.8c). Altogether

these results indicate that regulation of motility is QseE independent but QseC dependent. Also the double mutant data suggests that as far as motility is concerned, *qseC* is epistatic to *qseE.*

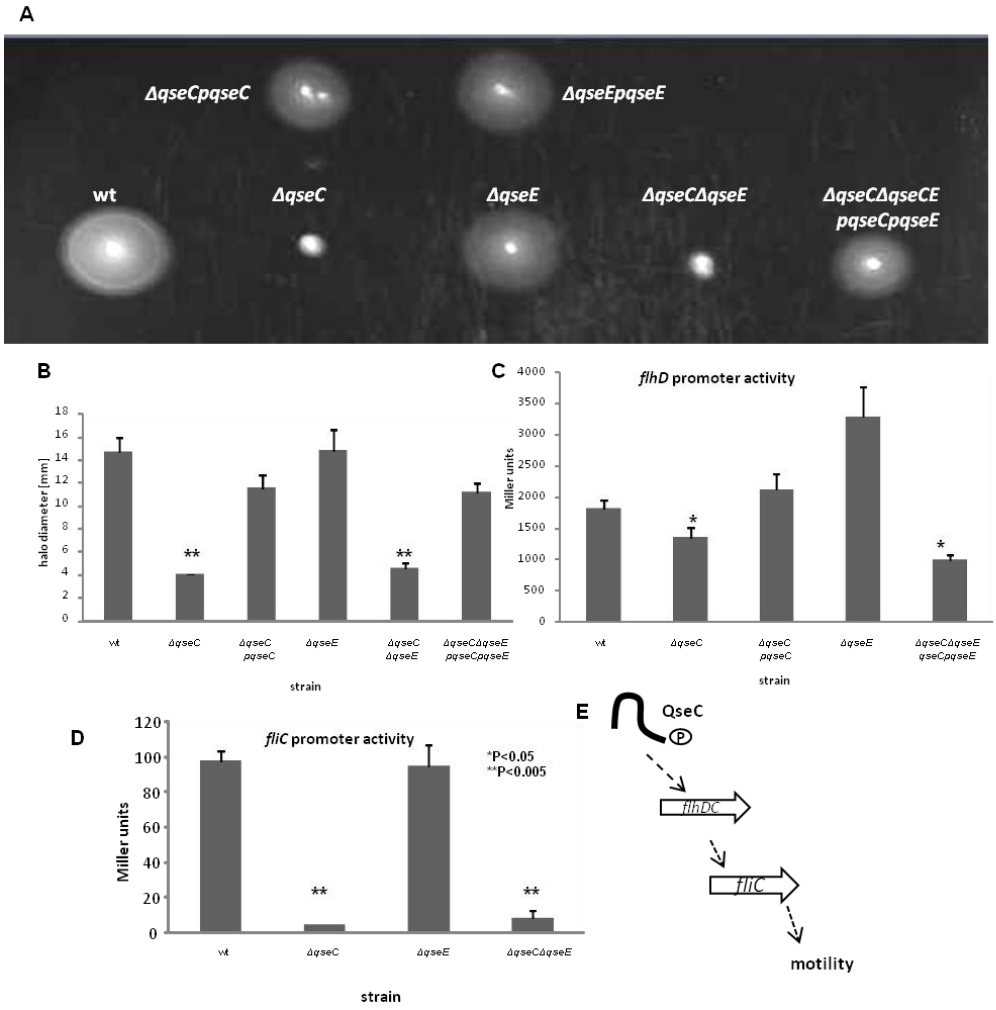


Fig.4.8.Motility regulation is QseC dependent but QseE independent. (A) Tryptone motility plates with wt, *ΔqseC, ΔqseE, ΔqseCΔqseE* and their complemented strains. **(B)** Graphical representation of the diameter of the bacterial halos. β-galactosidase assays were performed using plasmid pVS182 with *flhDC::lacZ* promoter fusion **(C)** and pVS177 with a *fliC::lacZ* promoter fusion **(D)** in wt, *ΔqseC, ΔqseCpqseC, ΔqseE* and *ΔqseCΔqseE.* **(E)** Cartoon representation indicating the QseC dependent and QseE independent activation of motility genes.

Discussion

Bacterial populations have evolved the ability to sense their surroundings through chemical signaling (274). In the 1970s, the marine bacteria *Vibrio fischeri* and *Vibrio harveyi* were shown to sense increasing concentrations of self-produced compounds (later termed autoinducers), in order to monitor their population density, and at the optimal concentration of these signals the bacteria activate expression of bioluminescence genes (62, 217, 219). Since then, a multitude of microbes have been shown to communicate within as well as outside their species (121).

Communication among bacterial species has also been reported in EHEC O157, where it has been shown that this enteric pathogen senses the AI-3, which is produced by itself as well as gut resident microbiota (85). As the infectious dose of EHEC O157 is estimated to be approximately 50 colony forming units (cfus) (142), it is unlikely that the self-produced AI3 is sufficient to promote gene regulation when this pathogen reaches the intestine. Therefore, it has been proposed that EHEC O157 senses the AI3 produced by the gut microbial flora to initiate regulation of virulence genes (283). Through the QseC AI-3 sensor, EHEC up-regulates motility, which probably allows the bacteria to swim closer to the gut epithelium where it may be exposed to the host produced epinephrine and/or norepinephrine hormones (41, 283). This exposure to these human adrenergic hormones is thought to further augment positive regulation of genes important for colonization, and formation of AE lesions.

Here we show that exposure of EHEC O157 to epinephrine increases its ability to infect HeLa cells and form pedestals. This effect is QseC and QseE dependent (Fig.4.7). QseC has been previously reported to be an activator of virulence. It has been shown to positively regulate

motility in EHEC O157, *Salmonella* and UPEC (20, 21, 43, 120, 163, 205, 285), invasion in *Salmonella* (205) and overall virulence in many other pathogens (203, 226, 243, 327). Here, we have shown that deletion of *qseC* significantly decreases formation of AE lesions on HeLa cells and that the *qseC* mutant's ability to form these lesions is unaffected by epinephrine (Fig.4.7). These data are consistent with the observation that the *qseC* mutant was unable to respond to epinephrine to activate LEE expression (Fig.4.4a). However, it is worth noting that in regards to the regulation of *nleA* transcription, the *qseC* mutant still appears to sense epinephrine (Fig.4.4b). NleA is an important virulence factor but it is not involved in AE lesion formation. This would explain why the epinephrine dependent AE lesion formation pattern (Fig.4.7) mirrored the epinephrine dependent transcription of the LEE genes (Fig.4.4a) and not the epinephrine dependent transcription of *nleA* (Fig.4.4b). A probable explanation for this may be that although both QseC and QseE regulate *nleA* transcription, QseE may play a more significant role in this gene's regulation, the result of which would be that in the *qseC* mutant, the QseE that is present still senses epinephrine, and responds to it, consequently altering *nleA* transcription. We have also shown that the other epinephrine sensor QseE inhibits pedestal formation, with *ΔqseE* forming significantly more pedestals than wt, and its infection rate is unaffected by epinephrine. Interestingly when *espA* transcription was assessed, *ΔqseE* still sensed epinephrine (Fig.4.4a). A likely reason for this observation is that in the absence of *qseE, qseC* is still present and though both kinases regulate the LEE*,* QseC is the principal epinephrine dependent regulator of *espA*. Therefore in the *qseE* mutant, the QseC that is still present senses epinephrine and alters *espA* transcription. When we tested the double *ΔqseCΔqseE* mutant in phenotypic assays with epinephrine, we observed an inability to sense this hormone (Fig.4.4a

and b, Fig.4.5, Fig.4.7a and b). Transcription of the LEE genes, and consequently AE lesion formation, were unchanged in the absence and presence of epinephrine, which indicated to us that these two kinases, QseC and QseE, are the only sensors of epinephrine in EHEC O157 involved in the regulation of the LEE. Interestingly, although the *ΔqseCΔqseE* regulatory pattern for the LEE genes is similar to QseE, the double mutant's pattern for motility regulation is similar to QseC. These data indicate that QseC and QseE have a complex interplay in the regulation of virulence in EHEC.

Bacteria have evolved complex systems to regulate their virulence with numerous points of control. The first step usually involves the sensing of an environmental signal through a membrane-bound or an intracellular sensor (235). The sensor then in turn may in a few cases directly alter transcription of target genes, or more commonly initiates a regulatory cascade that culminates in gene regulation (86, 99, 202). A multitude of sensors have been shown to be important for bacterial virulence. *Enterococcus faecalis,* a human enteric pathogen, has been reported to respond to self-produced pheromones through the kinase FsrC in order to differentially regulate virulence (215). The plant pathogen *Agrobacterium tumefaciens* uses the kinase ChvG to regulate tumorigenesis by directly or indirectly sensing extracellular acidity (173). Other examples include *cis*-2-dodecenoic acid sensing by *Burkholderia cenocepacia*'s BCAM0227 (189) and LAI-1 sensing by *Legionella pneumophilia'*s LqsS (297).

Here we show that epinephrine sensing is very complex (Fig.4.9). QseC senses AI-3, epinephrine and norepinephrine, and then through the phosphorylation of three RRs (QseB, QseF and KdpE), is able to regulate motility, Shiga toxin production and AE lesion formation (120). Adding, another layer of complexity, QseC also activate expression of the *qseEF* genes

(248). Regulation of motility depends exclusively on QseC , not on QseE (Fig.4.8). However, in concert with QseC, QseE play a role in the regulation of the LEE. QseE senses epinephrine, phosphates and sulfates, and subsequently negatively regulates expression of the LEE, and AE lesion formation (Fig.4.3, 4.4, 4.7). This regulation by QseE is indirect through inhibition of *rcsB* transcription, which is a positive regulator of the LEE and *nleA* (Fig.4.6). Tobe *et al* reported that both overexpression and deletion of *rcsB* led to increased transcription of the LEE in the Sakai strain of EHEC (298). We, however, show by qRT-PCR that in *ΔrcsB* the transcription of *ler, tir, eae* and *espA* is significantly decreased compared to wt, and this reduction could be rescued by complementation *in trans* (Fig.4.6). In agreement with Tobe *et al,* we show that overexpression of *rcsB* in the complemented strains increased LEE gene expression. It is also important to note that the strain we use in our research, an isolate from an EHEC O157:H7 hemorrhagic colitis outbreak (100), is different from the Sakai strain used by Tobe *et al* and this may explain the disparate results. Recent work by Islam *et al* and Kendall *et al* has also highlighted the occurrence of differential gene regulation among different EHEC strains (127, 148).

Here we have shown how EHEC O157 has evolved to use two histidine kinases to sense hormones produced by its host in order to fine tune the temporal and energy efficient expression of its virulence factors. This control is very complex and better understanding of the intricacies of this signaling cascade may contribute to the development of future anti-virulent therapies.

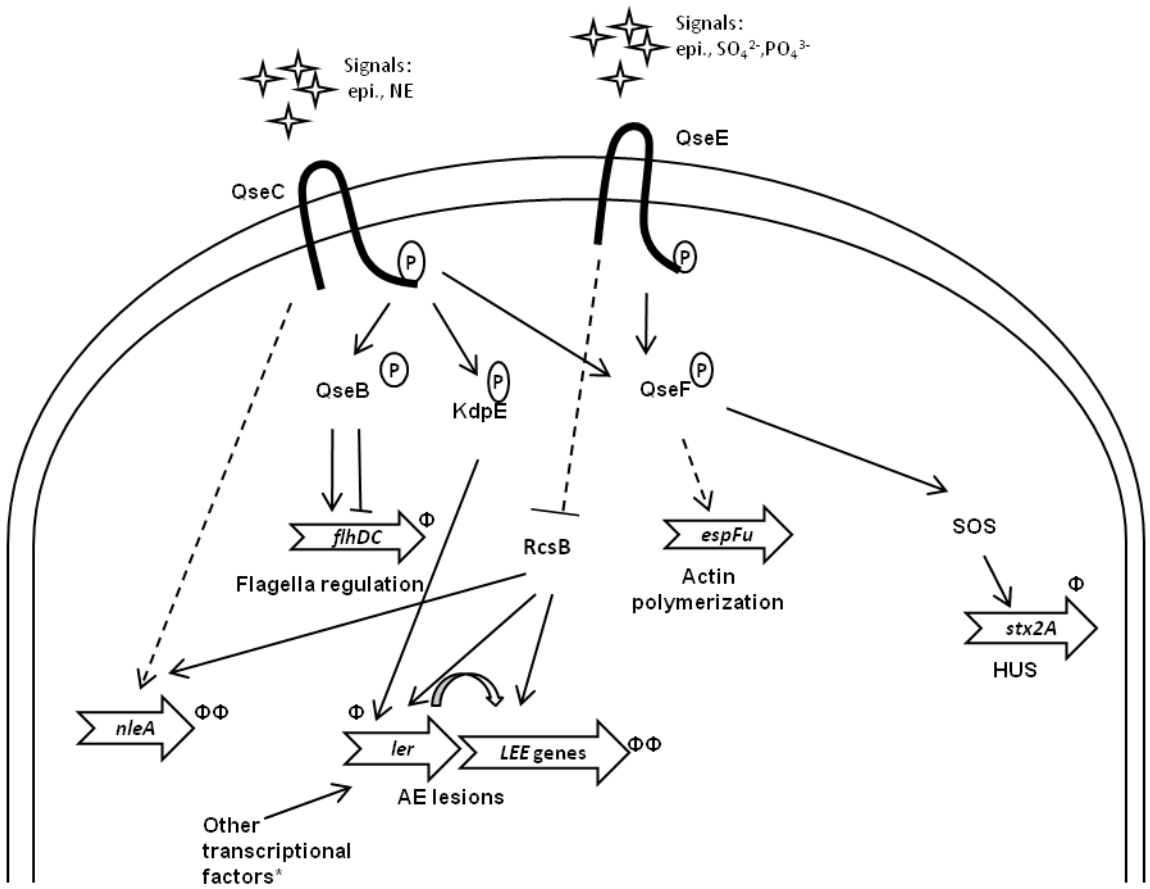


Fig.4.9. Model of the QseC and QseE regulatory cascade. Solid lines with arrows indicate positive regulation while dotted arrows indicate indirect activation. The dotted line with a bar indicates that QseE inhibits *rcsB* transcription in an as yet determined manner. Genes whose expression had been shown to be affected by epinephrine previously have Φ next to them while those whose epinephrine dependent regulation was shown in this work have ΦΦ next to them. *The *ler* promoter is highly regulated by many transcription factors including GrlA, Pch, GadE, QseA and H-NS(18, 33, 129, 140, 276). Epi, epinephrine. NE, norepinephrine. AE, attaching and effacing.

CHAPTER FIVE

When Sweets go Sour: Sugar Regulation of Bacterial Gene Expression

INTRODUCTION

One the major challenges faced by bacteria within communities is acquisition of carbon and nitrogen to synthesize primary metabolites. The mammalian GI tract harbors trillions of indigenous bacteria of approximately 1,000 different species (94) whose co-existence relies on the ability of each member to utilize one or a few limiting resources. Invading pathogens have to compete with the microbiota for these resources to establish colonization. These pathogens tend to be aggressive and greedy in search for a colonization niche, and achieve this purpose by precisely coordinating expression of an arsenal of virulence genes.

The GI pathogen EHEC causes hemorrhagic colitis and hemolytic uremic syndrome (HUS)(142). A major set of EHEC virulence factors is the type three secretion system (T3SS) whose structural components form a needle like structure through which effectors are translocated into host cells to hijack their function. The T3SS is responsible for the attachment of EHEC to the gut epithelium and the induction of extensive actin rearrangement in the host epithelial cells culminating in the formation of AE lesions (pedestal-like structures) underneath the bacteria (142). Most of the genes necessary for their formation are contained within a pathogenicity island (PI) known as the locus of enterocyte effacement (LEE)(142). The majority of the LEE genes are grouped into five major operons: *LEE1-5* and encode for the structural

components of the T3SS as well as some of the translocated effectors. The first gene of the LEE is *ler* that encodes the master regulator of the LEE genes, and is essential for the secretion of LEE and non-LEE encoded T3SS effectors, pedestal formation and overall virulence in EHEC (41, 56, 142, 193, 299). Expression of *ler* is regulated by numerous transcription factors including the response regulator (RR) KdpE (120, 193). In addition to being phosphorylated by its cognate histidine sensor kinase (HK) KdpD in response to potassium and osmotic stress, KdpE is also phosphorylated by the non-cognate HK QseC in response to the host hormones epinephrine and norepinephrine and a signaling molecule, autoinducer-3 (AI-3), produced by the GI microbiota (109, 120, 138, 283). The high level of control of *ler* expression ensures that, in response to diverse environmental signals, EHEC is able to tightly regulate the expression of the LEE and its virulence.

One important environmental signal that bacteria respond to is carbon nutrients. EHEC's ability to initiate growth and maintain colonization *in vivo* depends on whether the carbon source is glycolytic or gluconeogenic, and glucose polymers in particular have been shown to be important sources of carbon nutrition (36, 71, 136, 200). *In vitro* studies have shown that metabolites can regulate the expression of both metabolism and non-metabolism genes. The catabolite repressor/activator protein (Cra aka FruR, a member of the LacI family) is a transcription factor that uses fluctuations in sugar concentrations to activate or inhibit expression of its target genes (242). It has been shown to regulate virulence in *Salmonella enterica* and *Shigella flexneri* (98, 334). Cra's function is cAMP independent but is inhibited by the presence of micromolar concentrations of fructose-1-phosphate (F1P) or millimolar amounts of fructose-1,6-bisphosphate (FBP) (241, 263). These metabolic intermediates bind to

the inducer binding domain of Cra, decreasing its binding affinity for target promoters consequently decreasing its regulatory function.

Here we show that KdpE direct transcription regulation of *ler* is glucose dependent, and that this dependency is through Cra. We show that Cra and KdpE directly interact with each other to promote *ler* transcription and AE lesion formation. This convergence of regulation by Cra and KdpE introduces a novel mechanism of regulation that links metabolism to pathogenesis.

Results and Discussion

Carbon regulation of EHEC pathogenesis. The GI microbiota resides in the loose mucus layer, and is not in close contact with the host epithelium (310). Growth within the GI tract by a particular bacterial species is determined by the available concentration of nutrients. Consequently, for two species that compete for the same nutrients in the mucus layer and are not attached to the epithelial cells, the one that utilizes these nutrients more efficiently will eliminate the other strain (200). In the mammalian GI tract, EHEC has to compete with the γ-proteobacteria for nutrients, because they have similar preferences for carbon sources. However, commensal *E. coli* is more proficient than EHEC in the utilization of these carbon sources. EHEC uses glycolytic substrates for initial growth, but is unable to effectively compete for these carbon sources beyond the first few days, and begins to utilize gluconeogenic substrates to stay within the intestine (200). A second strategy used by EHEC to establish colonization of the GI tract is the expression of the LEE-encoded T3SS to closely attach to the host enterocytes leading to AE lesion formation (142). Activation of the expression of the LEE

relies in the sensing of the microbiota and host derived signaling molecules AI-3 and epinephrine/norepinephrine through the QseC HK (41, 120, 243, 283). Upon sensing these signals, QseC initiates a complex signaling cascade, which through the phosphorylation of the KdpE RR leads to activation of the expression of the LEE genes (120).

Bacteria share common evolutionary progenitors. EHEC diverged from its non-pathogenic relatives about 4.5 million years ago (249), obtaining virulence traits, such as the LEE, through the insertion of mobile genetic elements (4, 142). Additionally, EHEC has co-opted regulatory mechanisms such as the QseC HK, which is present in its progenitors for non-pathogenic functions, to regulate virulence (285). Given the key role that carbon sources play within the GI tract for niche competition, we investigated the role that carbon sources have on EHEC pathogenesis and their influence on the transcription of *ler*, the activator of the LEE genes. Using DMEM lacking glucose and pyruvate as our base medium we prepared assay media by adding glucose, glycerol, succinate or pyruvate (Fig.5.1A). Switching to glycolytic conditions by increasing concentrations of glucose (0.1% to 0.4%; 5.56 mM and 25 mM, respectively) or using 0.4% glycerol reduced *ler* transcription two-fold, while switching to a gluconeogenic state with 0.4% succinate increased *ler* mRNA levels four-fold compared to a glucose concentration of 0.4%. The switch from 0.1 to 0.4% glucose that alters virulence gene expression has been shown to be physiologically relevant in humans, where in cholesterol studies, the use of 0.4% vs. 0.1% glucose has been shown to increase the stimulation of cholesterol absorption in the small intestine (245). EHEC was unable to grow in 0.4% pyruvate as the sole carbon source but adding it to 0.1% glucose did not vary *ler* transcription. These data indicate that transcription of the LEE is repressed at glycolytic conditions, and activated at

gluconeogenic conditions, showing that activation of the T3SS for epithelial attachment is coordinated with the gluconeogenic shift that EHEC undergoes during intestinal colonization of mammals.

To assess whether this carbon source regulation was linked to the QseC/KdpE-dependent regulation of the LEE, we assessed whether KdpE LEE-gene regulation was affected in glycolytic or gluconeogenic conditions. Transcription of *ler* was decreased in the *kdpE* mutant compared to WT only at 0.1% glucose (gluconeogenic), and was similar to WT at 0.4% glucose (glycolytic), indicating that KdpE only activates *ler* transcription in gluconeogenic environments (Fig.5.1B). These findings were confounding, given that it has been previously reported that under conditions of high glucose availability (glycolytic), IIA^{Ntr} is dephosphorylated, and only in its dephosphorylated form binds to the KdpD HK (the cognate HK for KdpE) increasing its activity, and consequently KdpE phosphorylation, leading to higher expression of the KdpE target genes *kdpFABC* (180). Hence, through this mechanism one would predict that KdpE would activate LEE transcription under glycolytic and not gluconeogenic conditions, which is the opposite of the phenotype that we observed. We then hypothesized that KdpE might regulate the LEE in a glucose-dependent manner through interaction with another transcription factor. It has been well documented that fluctuations in glucose levels leads to different levels of cAMP within bacterial cells, and one of the prominent transcription factors involved in this regulation is CRP (aka CAP) (110). The CRP binding consensus sequence is very well defined (110), and *in silico* analysis of the *ler* regulatory region did not predict any CRP binding sites. However, these analyses identified a putative consensus sequence for Cra (Fig.5.1C), a transcription factor that senses changes in metabolite levels to differentially regulate its target genes (263). Cra is a

member of the LacI/GalR family, which activate genes encoding gluconeogenic enzymes such as FBPase, and inhibit genes encoding glycolytic enzymes such as phosphofructokinase (37, 260).

We confirmed that *ler* transcription was decreased in *Δcra* grown in 0.1% (low) glucose, and that this phenotype could be rescued by expressing Cra *in trans* (Fig.5.1D). Transcription of *ler* was similar between wt and *Δcra* in 0.4% (high) glucose (Fig.5.1A, D), suggesting that Cra-dependent activation of *ler* also only occurs in low glucose. To confirm the predicted Cra binding site around -350bp (Fig.5.1C), we performed EMSAs using a *ler* probe (-450 to -255bp) (Fig.5.1E and F). A concentration of 7nM Cra was sufficient to shift the *ler* probe while the negative control probe *kan* did not shift with up to 10μM Cra (Fig.5.1E). To confirm specificity, competition EMSAs showed that Cra binding to *ler* could be competed by an unlabeled *ler* probe with a ratio as low as a 1:1, but not by the non-specific unlabeled *kan* probe (Fig.5.1F). Using DNAse protection assays we verified the specific nucleotides in the *ler* promoter recognized by Cra (Fig.5.1I-J), and confirmed that indeed Cra binds to its *in silico* predicted binding site.

Cra binding to its targets can be displaced by μM amounts of F1P or mM amounts of FBP (241). F1P and FBP are intermediates of the glycolysis metabolic pathway (Fig.5.1G)(254). FBP is produced either through the glucose phosphorylation metabolic pathway or by the phosphorylation of F1P. To assess the role that glucose and/or its catabolites play in the binding of Cra to the *ler* promoter, EMSAs were performed with 100μM F1P, 10mM and 50mM of FBP. Fructose-6-phosphate (F6P) and glucose-6-phosphate (G6P) were used as negative controls. At a concentration of 350nM Cra completely shifted the *ler* probe (Fig.5.1H). Addition of 100μM F1P significantly reduced this shift bringing the amount of free DNA in the reaction to about

25% of the original protein free reaction in lane 1. For FBP, 50mM was sufficient to decrease binding, bringing the amount of free DNA to approximately 50%. The ability of the glucose catabolite FBP to inhibit binding of Cra to *ler in vitro* may mirror FBP's role *in vivo* as a negative inducer of the Cra-*ler* complex formation. These results support that Cra directly and specifically binds to the *ler* promoter region, and that this binding is inhibited by metabolites such as F1P and FBP that accumulate under glycolytic conditions. Increasing glucose concentrations in the media pushes the cell towards glycolytic metabolism that increases the amount of FBP in the cell. This would favor *ler* inhibition through the reduction of Cra binding. The switch to gluconeogenic metabolism using succinate decreases the amount of FBP available in the cell. This would promote Cra binding and increase *ler* transcription (Fig.5.1A).

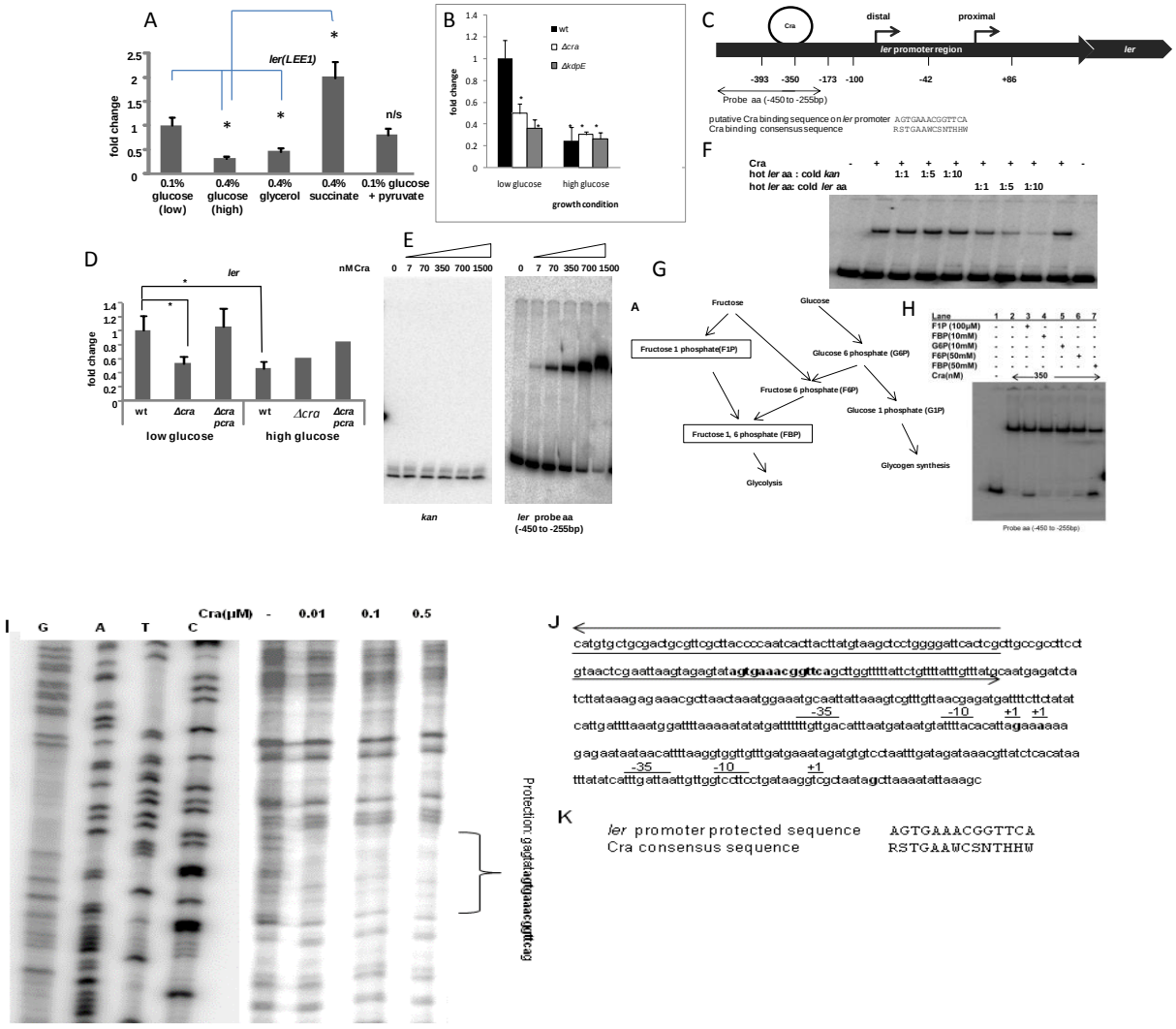


Fig.5.1. Carbon status influences EHEC pathogenesis. A. qRT-PCR of *ler* transcription in: no glucose, no pyruvate DMEM as the base media supplemented with glucose (0.1%, low), glucose (0.4%, high), glycerol (0.4%), succinate (0.4%) or low glucose + pyruvate (0.1%glucose + 0.4% pyruvate). Results were expressed as fold changes over those of low glucose DMEM. **B.** qRT-PCR analysis of of *ler* in wt, *Δcra* and *Δkdpe.* **C.** Schematic representation of the EHEC *ler* promoter. The transcriptional start sites are indicated with solid arrows. The putatitive binding site for Cra is depicted with a circle. Probe aa (450 to -255bp) was used in subsequent experiments. Underneath is the putative Cra binding sequence on the *ler* promoter and the Cra binding consensus sequence. **D.** qRT-PCR of *ler* in wt, *Δcra* and the complement in low and high glucose DMEM. *ler* transcript levels quantified as fold differences normalized to low glucose wt *ler* transcript levels. **E.** Cra EMSA using probe aa. A radiolabeled *kan* DNA probe was used as a

negative control. **F.** Competition EMSA using 70nM recombinant Cra. Increasing amounts of unlabelled *ler* or *kan* probes. **G.** Schematic representation of glucose and fructose metabolism. The catabolites known to be inducers of Cra are boxed. **H.** Inducer supplemented EMSA. Indicated concentrations of intermediates in the fructose and glucose metabolism cascade were added to 2ng (400pM) radiolabeled *ler* probe (bp-450 to -255) and 70nM Cra. G6P and F6P were used as negative controls. *P<0.05. **I.** To identify the actual nucleotides of the *ler* promoter involved in binding with KdpE and Cra, a DNase I footprint was carried out using the indicated probe and increasing amounts of Cra. The protected region is indicated within the paranthesis. **J.** DNA sequence of the *ler* promoter region showing the -35 and -10 positions of both the proximal and distal promoter. The arrow shows the position of the probe aa and the Cra binding site is indicated in bold. **K.** Alignment of the actual binding site with the consensus binding site sequence of Cra.

Cra and KdpE interplay in LEE regulation. KdpE and Cra follow a similar pattern towards sugar-dependent regulation of LEE expression, suggesting that these two transcription factors work together to integrate LEE regulation with signaling and metabolism. Through the genetic, bioinformatics and biochemical analyses depicted in Fig.5.1, we identified the Cra binding region within the *ler* promoter. Unlike Cra, which has a very well defined consensus sequence, KdpE tends to bind primarily to AT rich DNA, and does not have a very well defined consensus. Hence, to address the mechanism of KdpE-dependent *ler* regulation we performed a nested deletion analyses of the *ler* regulatory region (Fig.5.2A). This deletion analysis narrowed the region of the *ler* promoter necessary for KdpE dependent activation to between -173 and -42bp (Fig.5.2A, B). KdpE activates transcription of *ler* by directly binding to the *ler* regulatory region (Fig.5.2C), and this interaction is specific, given that in a competition EMSA (Fig.5.2E), unlabeled *ler* probe was able to compete with the labeled *ler* probe for KdpE binding but unlabeled *kan* probe (negative control) was unable to compete. Interestingly, the unphosphorylated KdpE showed higher binding affinity to the *ler* promoter than the phosphorylated KdpE (Fig.5.2F). This is in contrast to the KdpE regulation of the *kdpFABC* genes,

to which the phosphorylated KdpE has higher binding affinity (180). During glycotytic growth there is high glucose availability, IIA^{Ntr} is dephosphorylated, and binds to the KdpD HK to increase KdpE phosphorylation (180), and as a result there is increased *kdpFABC* transcription, and decreased KdpE-dependent LEE expression (Fig.5.1B), given that the phosphorylated form of KdpE has lower affinity for the *ler* promoter (Fig.5.2F). These results are in agreement with the observation that KdpE only activates *ler* transcription in gluconeogenic (low glucose) conditions (Fig.5.2B). Here, we defined that KdpE activates LEE transcription by binding within the -173 and -42bp region, while Cra binds upstream to the -393 and -255bp region (Figs.5.1, 5.2), and that under glycolytic conditions binding of both proteins to the *ler* promoter is diminished.

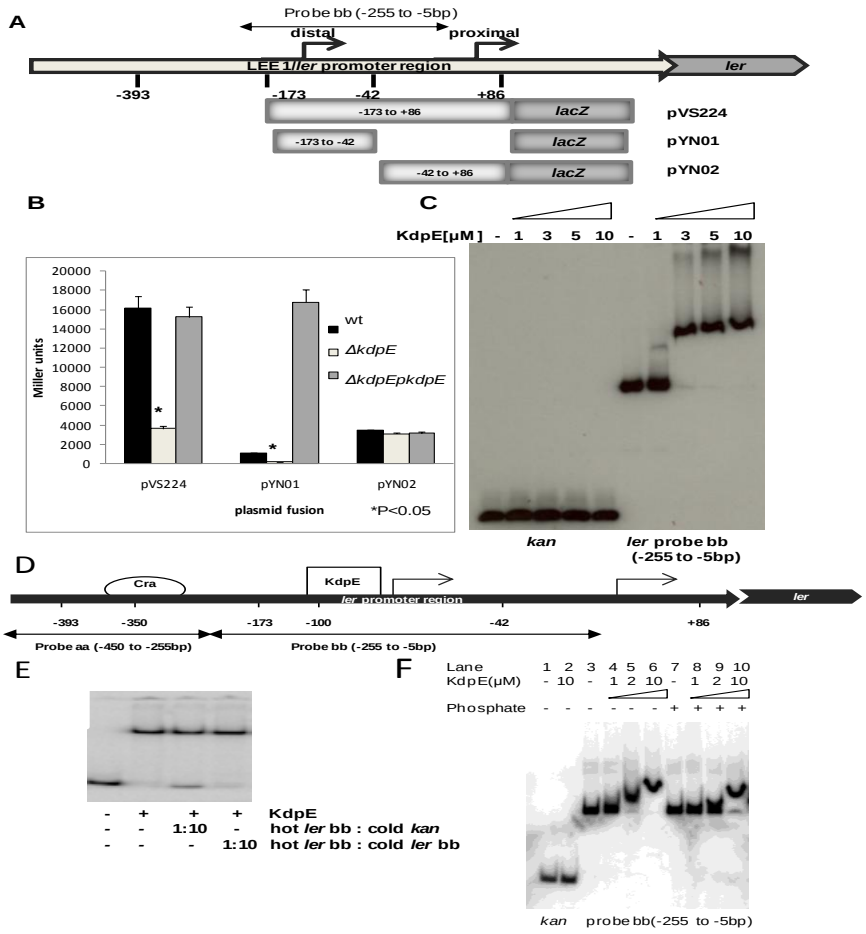


Fig.5.2 KdpE regulation of the *ler* promoter. A. Cartoon representation of plasmids used for nested deletion analysis. Fragments of the *ler* regulatory region encompass the distal (-173 to -42bp, pYN01), proximal (-42 to +86bp, pYN02) and both promoters (-173 to +86bp, pVS224). **B.** Nested deletion analysis in wt, *ΔkdpE* and the complement. The beta-galactosidase assays were performed on samples grown to OD_{600} 0.5 in low glucose DMEM (contains 1mM pyruvate and 0.1M NaCl). **C.** KdpE EMSA of the *ler* promoter region using 2ng (300pM) probe bb (-255 to -5bp). Increasing amounts of His purified recombinant KdpE was used to shift the radiolabeled *ler* DNA probe. A radiolabeled *kan* DNA probe was used as a negative control. **D.** Cartoon depicting the Cra and KdpE binding regions on *ler* and probes aa and bb used for EMSAs. **E.** Competition EMSA using 5μM recombinant KdpE and probe bb. A ratio of hot probe to cold probe of 1:10 decreased the shift due to 5μM KdpE. Unlabelled *kan* DNA probe was used as a negative control. **F**. EMSAs of KdpE and *ler* in absence and presence of acetyl phosphate. *P<0.05.

How these two transcription factors act in concert to activate *ler* expression remains undefined. Protein-protein interaction is an important mechanism for molecular processes in the cell. Different members of the LacI family have been shown to form homo-multimers, and to also interact with other proteins and metabolites as part of their regulatory mechanism (79, 272). Since both KdpE and Cra activate *ler* transcription in a glucose dependent manner, and do so by directly binding the *ler* promoter, using far western blotting we investigated the possibility that Cra and KdpE interact with each other. Pure His-tagged Cra, KdpE and, as a negative control, QseB, were run on gels, transferred to membranes then probed with whole cell lysate (wcl) of a *ΔkdpEΔcra* double mutant expressing either Flag-tagged Cra or Flag-tagged KdpE. Duplicate membranes were then washed and probed with either anti-His or anti-Flag antibody. As expected all three pure proteins were detected using Coomasie and anti His-tag antibody (Fig.5.3H,I). However, when wcl overexpressing Flag-tagged Cra was used to probe the membranes, only the lanes containing His-tagged Cra and His-tagged KdpE were detected with anti-Flag antibody, indicating that Flag-tagged Cra interacts with itself and KdpE, but not with the negative control QseB (Fig.5.3A). To further confirm this interaction, we reversed the bait-prey proteins (Fig.5.3B). The wcl overexpressing Flag-tagged KdpE interacted with His-tagged KdpE as well as with Cra but not the control QseB. As additional negative controls, we either left replicate membranes unprobed by wcl or probed with the double mutant wcl only before probing with anti-Flag (Fig.5.3J). These findings suggest that the two *ler* activating proteins Cra and KdpE interact *in vitro*.

As Cra has previously been shown to enhance CRP binding to its targets (260), we examined whether Cra could have a similar effect on KdpE binding to *ler.* Using probe bb (-255

to -5bp) that lacks the identified Cra binding site (Fig.5.3C), we conducted mixed EMSAs where the KdpE concentration was kept constant, and the Cra concentration was varied (Fig.5.3D). We also repeated this assay keeping the Cra concentration constant, and varying the concentration of KdpE (Fig.5.3E). When the concentration of KdpE was kept constant (Fig.5.3D lane 4-7), we observed an increase in the amount of DNA shifted with increasing concentrations of Cra. The maximum amount of Cra added (1.5µM, Fig.5.3D lane 3) was not sufficient to shift this probe on its own, but when supplemented with 2.5µM KdpE, it significantly altered the shifting pattern when compared to a reaction with 2.5µM KdpE only (lane 4). When this experiment was repeated keeping Cra constant and adding increasing amounts of KdpE we again observed a super shift (Fig.5.3E). These results indicate that the two proteins Cra and KdpE interact with each other to promote *ler* transcription. To further test whether the effect of Cra enhancement of KdpE binding could enhance KdpE-dependent *ler* transcription, we utilized a *ler-lacZ* fusion pVS224 (Fig.5.2A) containing only the -173 to +86bp region (which lacks the Cra binding region, Fig.5.3C) to monitor KdpE dependent *ler* transcription in the absence or presence of Cra. As predicted, transcription of this *ler-lacZ* fusion was unaffected in the *cra* mutant compared to WT (Fig.5.3F), given that Cra does not interact with this region of the *ler* promoter (Figs.5.3D and G). In agreement with our nested deletion analyses (Fig.5.2), transcription of this fusion was decreased in the *kdpE* mutant, and decreased even further in the double *kdpEcra* mutant(Fig.5.2F), suggesting that interaction between Cra and KdpE has an additive effect in the expression of *ler*. However, these proteins bind to the *ler* regulatory region at sites that are distant from one another (Figs.5.1 and 5.2), suggesting that in order for them to interact there has to be DNA bending and looping. The LEE is a horizontally acquired PI by EHEC, and has a

very low GC content (34%) compared to the GC content of the *E. coli* backbone genome (50%) (67). It has also been extensively reported that because of this low GC content feature, the regulatory region of *ler* is prone to DNA bending (335), and *ler* transcription is subject to regulation by several architectural proteins that promote DNA bending such as H-NS, Fis and IHF (193). Hence, it is feasible that through the DNA bending promoted by these architectural proteins, Cra and KdpE interact to optimally activate *ler* transcription.

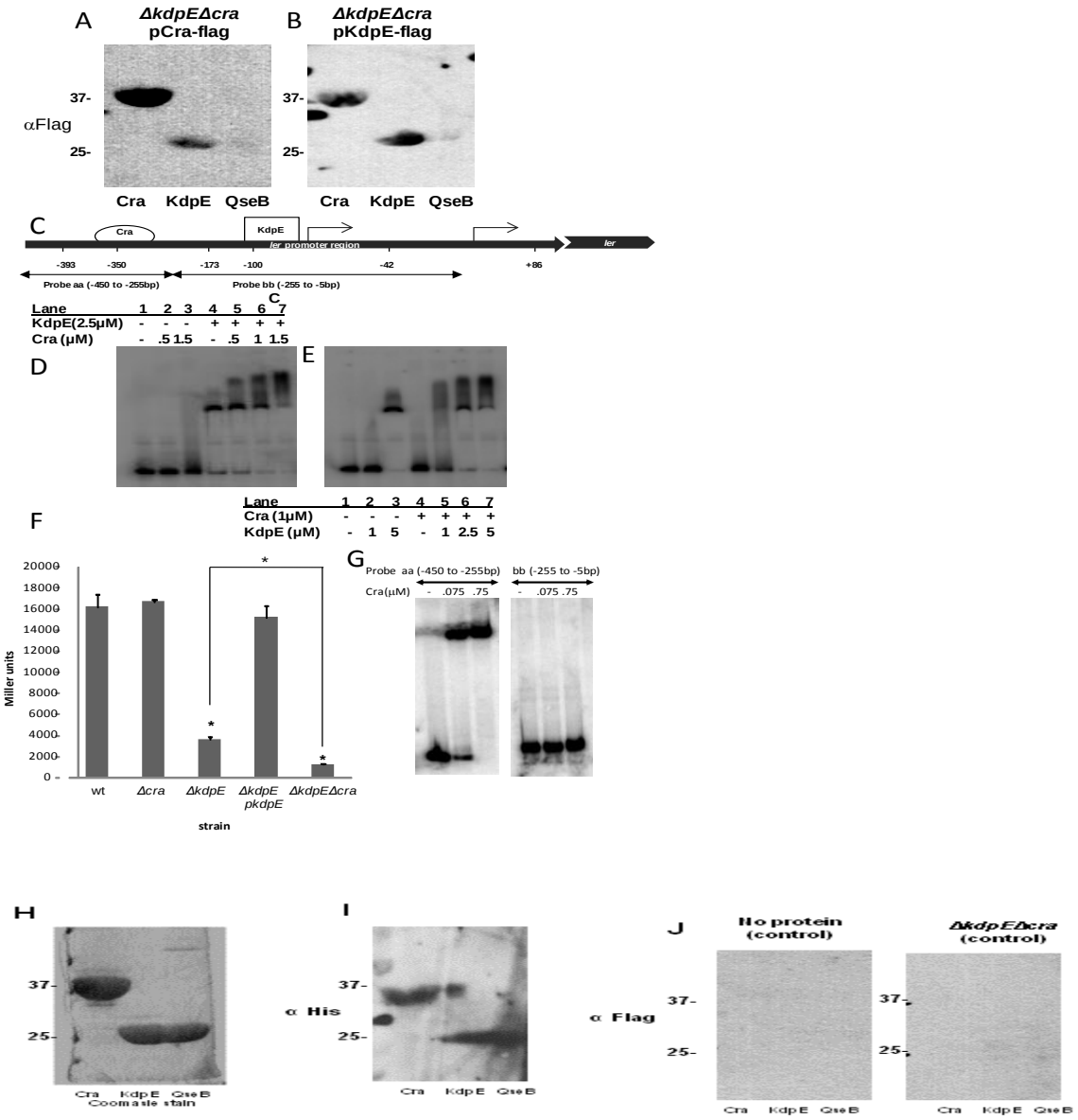


Fig.5.3. Cra and KdpE proteins interact *in vitro*. A. Far Western Blotting of the interaction between Cra and KdpE *in vitro.* Recombinant His-tagged Cra, KdpE and QseB (negative control) on a membrane were probed first with whole cell lysate (wcl) overexpressing Flag-tagged Cra or Flag-tagged KdpE, then with anti-Flag antibodies. Cra is 37kDa, KdpE and QseB are both 25kDa. Bands indicate interaction between the membrane bound His-tagged protein (bait) and the probing Flag-tagged protein (prey). Flag-Cra interacted with His-Cra and His-KdpE but not His-QseB **(A)**. Flag-KdpE interacted with His-Cra and His-KdpE but not His-QseB **(B)**. **C** Cartoon depicting the Cra and KdpE binding regions on *ler* and probes aa and bb used for EMSAs. **D and E.** Mixed protein competition EMSAs were performed using probe bb (-255 to -5bp). The EMSAs were performed with a constant concentration of KdpE and increasing concentrations of Cra **(D)** or with a constant concentration of Cra and increasing concentrations of KdpE **(E)**. **F.** β-galactosidase measurements of *ler-lacZ* fusion pVS224 (lacking the Cra binding site) in wt, Δ*cra*, Δ*kdpE* and complement, and Δ*kdpE*Δ*cra* strains. **G.** EMSAs of the *ler* probes aa and bb with Cra. **H.** Recombinant His-tagged Cra, KdpE and QseB (negative control) were purified and their size and purity confirmed using a Coomasie gel. Cra is 37kDa, Kdpe and QseB are both 25kDa. Far Western Blotting was used to visualize the interaction between Cra and KdpE *in vitro.* The three His-tagged proteins were run on a gel, transferred and blocked with 10% milk. Next the membranes were left unprobed by whole cell lysate (wcl), probed with wcl alone or wcl overexpressing Flag-tagged Cra or Flag-tagged KdpE. **I.** The membranes were probed with anti-His antibodies. **J.**The control membranes, left unprobed or probed with wcl alone did not test positive for Flag (panel 1 and 2 respectively). *P<0.05.

Cra and KdpE in AE lesion formation. Ler is the master activator of the LEE genes that encode for the T3SS that injects bacterial effectors into host cells (Fig.5.4A)(142). In low glucose, the decreased expression of the *LEE2, LEE3* and *LEE5* operons in the *cra* and *kdpE* mutants was comparable (Fig.5.4B), in agreement with the role of these two transcription factors in activating transcription of *ler* (Figs.5.1 and 5.2). However, the mRNA level of *LEE4* (measured by *espA* that encodes for the T3SS translocon, and which is itself secreted through the T3SS) was significantly decreased in *Δcra* but not in *ΔkdpE* (Fig.5.4B). It is worth noting that expression of the *LEE4* operon is also subject to high levels of post-transcriptional regulation (176), and that the RNA binding protein CsrA (involved in post transcription carbon metabolism regulation (255, 256, 261)) differentially affects expression of *LEE4* (27). Hence, a potential

explanation for the differential *LEE4* regulation between KdpE and Cra, may be that in addition to modulating *ler* transcription, these proteins also differentially affect expression of post-transcriptional regulatory systems that exclusively act on *LEE4*. Transcription of *ler* is decreased in high glucose, compared to low glucose, and this phenotype is mediated through both Cra and KdpE (Figs 5.1-5.3). Switching to high glucose reduced *espA* transcription in wt but did not affect the mRNA levels in *Δcra* and *ΔkdpE* (Fig.5.4C). Both the expression (Fig.5.4D) and the secretion (Fig.5.4E) of EspA were decreased in wt grown in high glucose. Although, *ΔkdpE* had similar levels of expression and secretion as wt in low glucose, these levels were unaffected by switching to high glucose. No EspA expression/secretion was observed in *Δcra.* Altogether these results indicate that carbon sources not only influence LEE expression transcriptionally, but also post-transcriptionally, and that KdpE and Cra act in concert in the transcriptional, but differ in the post-transcriptional regulation.

As deletion of either *cra* or *kdpE* affects expression of the LEE that affects AE lesion formation, we next investigated whether deletion of these transcription factors would directly affect pedestal formation. HeLa cells were infected with wild type or the mutant strains, and actin was stained with FITC-phalloidin (green), and HeLa nuclei and bacteria with propidium iodide (red). Pedestals were visualized as brilliant green patches underneath red bacteria. Although *ΔkdpE* formed slightly fewer pedestals than wt, *Δcra* had significantly reduced pedestal formation, and this could be complemented by introduction of a plasmid encoding *cra* (Fig.5.4F). The observation that in addition to decreased *LEE1-3*,5 expression, *Δcra* also has a severe decrease in EspA expression (Fig5.4 B-E) while *ΔkdpE* does not, may explain the disparity in pedestal formation between these two mutants. Deletion of both *kdpE* and *cra* led to an

inability to form pedestals (Fig.5.4F), further advocating an additive role for these two transcription factors in virulence regulation. Pedestal formation in the double mutant could be rescued by expressing both genes *in trans.*

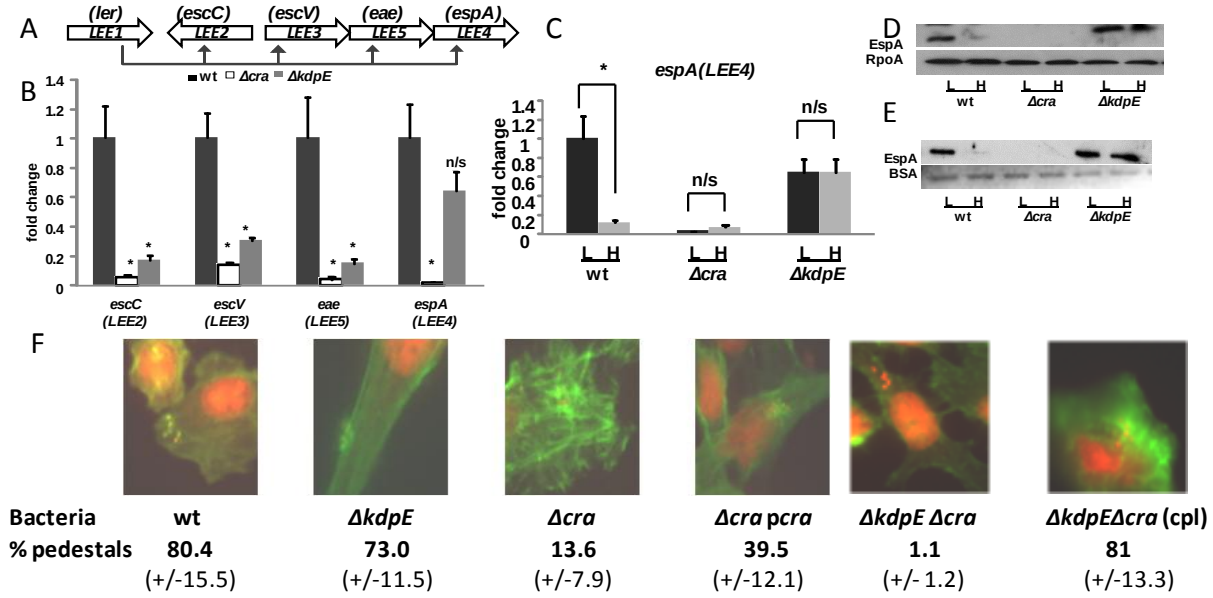


Fig.5.4. LEE and pedestal formation analyses. **A.** Schematic representation of the LEE pathogenicity island. **B.** qRT-PCR of the other LEE genes in low glucose DMEM. The mutant mRNA levels were expressed as fold changes over wt mRNA levels. **C.** qRT-PCR of *espA/LEE4* in wt, *Δcra* and *Δkdpe* in low and high glucose. For all the samples, *rpoA* mRNA levels were used as an internal control to normalize the output C_T values in order to take into account variation in bacteria numbers. Westerns of **(D)** whole cell lysates (wcl) and **(E)** secreted proteins (SP) of wt, *Δcra* and *Δkdpe* grown in low or high glucose were probed with antisera against EspA. RpoA and BSA were used as the loading controls for the wcl and SP blots respectively. L, low glucose; H, high glucose. **F.** Formation of pedestals was visualized as bright green (actin) cups onto which red (bacterial) dots were bound. These were quantified (looking at least 50 HeLa cells per slide, 3 slides each) as percentage of pedestals per attached bacteria. The standard deviation is indicated in parentheses. *P<0.05.

Conclusion

Here we show that through convergent evolution, two proteins that were originally designed to regulate essentially non-pathogenic functions, have been co-opted by a pathogen to regulate virulence factors encoded within a horizontally acquired PI. This regulation also responds to differences in metabolite concentrations and the phosphorylation state of transcription factors, which can be modulated by the availability of carbon sources. This nutrient based modulation of virulence expression is also intrinsically intertwined with inter-kingdom chemical signaling through the recognition of bacterial autoinducers and host hormones. The convergence of chemical and nutrient signaling to modulate virulence gene expression at both the transcriptional and post-transcriptional levels may be a result of the plasticity necessary for the rapid and efficient adaptation of bacteria to specific niches. In the GI tract environment, where about 1,000 different bacterial species co-exist, the exquisite integration of different cues to regulate virulence gene expression is essential for an invading pathogen to successfully establish itself within a host.

CHAPTER SIX

Regulation of Known and Putative EHEC Virulence Factors by Cra and KdpE

INTRODUCTION

In order to adapt to changes in their environment, bacteria have developed signaling mechanisms that allow the microbe to sense particular cues from their surrounding to differentially regulate appropriate genes. The signals are sensed by sensor kinases that may be membrane bound or cellular (114, 235). These signals are then transduced to response regulators (RR) that act on downstream genes, most often to regulate transcription. For example QseB, an OmpR family member, is a response regulator that is phosphorylated by its cognate kinase, QseC, and depending on its phosphorylation state, can either be an activator or inhibitor of gene expression (42, 120). The kinase QseC has also been shown to phosphorylate the non-cognate response regulators QseF and KdpE (120). KdpE and its cognate kinase KdpD form an operon that is important for K^+ transport (214, 290, 291). The membrane bound KdpD responds to K^+ limitation or salt induced high osmolarity to increase its phosphorylation state (139, 213, 314). The phosphorylated KdpD then transfers its phosphoryl group to KdpE, which by binding to the promoter region of the *kdpFABC* operon, activates the transcription of these genes, consequently adjusting intracellular K^+ levels to maintain homeostasis (291, 292, 314).

Differential gene transcription can also be controlled by transcriptional activators that bind to the promoter regions of their targets independent of phosphorylation. These regulatory

proteins respond to intercellular cues known as inducers. The catabolite activator protein (CRP) for example, is a transcriptional factor that responds to changes in intercellular cAMP levels (107, 337) to regulate its targets. Another protein, catabolite repressor protein (Cra aka FruR) has also been identified as a transcriptional factor that utilizes fluctuations in sugar concentrations to positively or negatively regulate target genes (240-242, 263). Cra has been shown to regulate virulence in *Salmonella enterica,* with the *cra* mutant being avirulent during murine infection (8, 309, 334).

EHEC uses response regulators and transcriptional regulators in general to translate signals sensed by bacteria into gene activation or inhibition. EHEC uses these regulators not only for the maintenance of bacterial homeostasis, but also to differentially regulate virulence. One important set of EHEC virulence factors is the attaching and effacing (AE) lesions. AE lesions are characterized by the attachment of bacteria to the host epithelium followed by the induction of extensive actin rearrangement within the epithelial cells culminating in the formation of pedestal-like structures underneath the bacteria (133, 158, 204, 287). Most of the genes necessary for AE lesion formation are contained within a pathogenicity island (PI) known as the locus of enterocyte effacement (LEE) (67, 190), and these genes are grouped into five operons (194). Hughes *et al.* also showed that KdpE regulates the transcription of *ler,* the first gene in the *LEE1* operon and the master regulator of the LEE PI (120). The LEE genes encode for the structural components of the T3SS as well as some effectors that are translocated through this T3SS into the host cell (130, 190). These LEE effectors along with non LEE effectors like Esp-Fu/TccP mimic mammalian signaling proteins and hijack host cell signal transduction (35, 89).

The non LEE effector EspFu has been shown to be translocated through the T3SS into epithelial cells where it contributes to the formation of AE lesions. The LEE PI and non LEE effectors are encoded within blocks of sequences (O-islands) that are unique to EHEC and are absent in the non-pathogenic *E.coli* K12 MG1655 genome. There are approximately 1,400 genes in O-islands (236) but less than 50% of them have been assigned functions. However, because O-islands are found only in pathogenic *E.coli* strains, and the genes within some of these islands that have been characterized have been found to be linked to pathogenesis has led to the hypothesis that these islands are rich sources of virulence genes.

In chapter 5, we described how KdpE is able to activate the LEE genes by directly binding to the *ler* promoter region. KdpE together with its interacting partner, Cra, are also able to regulate the LEE PI in a glucose concentration dependent manner. The aim of this chapter was to describe the transcriptional profiles of non-polar mutations of *cra* and *kdpE.* In this work, we show that Cra and KdpE share several virulence targets including the genes encoding the effectors EspFu and EspG as well as a number of genes within several O-island genes.

RESULTS

Global gene regulation by Cra and KdpE. Previous work has shown that Cra and KdpE regulate virulence in EHEC. These two transcriptional regulators directly bind to the promoter region of *ler,* activating the transcription of this gene in a metabolite and salt stress dependent manner (120) (Chapter 5). The regulation of the LEE genes by Cra and KdpE, which we showed to interact *in vitro,* culminates in significant reduction in AE lesion formation in *Δcra* and *ΔkdpEΔcra* (Njoroge e*t al,* submitted). Since Cra and KdpE regulate genes integral to EHEC

pathogenesis, we decided to elucidate the extent of these regulators' control by performing transcriptome studies using microarrays. We extracted RNA from wt, *Δcra, ΔkdpE* and *ΔkdpEΔcra* grown in low glucose Dulbeco's modified eagle medium (DMEM) to late exponential phase. Using Affymetrix *E.coli* 2.0 microarray chips, we processed and analyzed the RNA, comparing the expression profiles of the mutants to that of wt. The arrays contain over 10,000 probe sets that cover the two EHEC strains EDL933 and Sakai, the uropathogenic *E.coli* strain CFT073, the K-12 strain MG1655 as well as intergenic regions that may encode for small regulatory RNAs (sRNAs) or non-annotated small ORFs.

The microarray analysis revealed that when the *cra* mutant was compared to wt, 829 genes showed increased expression, with 43% of these being pathogen specific (Table 6.1). Additionally, a total of 515 genes had decreased expression with 49% of the genes being pathogen specific. When the *kdpE* mutant was compared to wt, 658 genes were up-regulated while 636 genes were down-regulated with the pathogen specific ratios being 34% and 60% respectively. As the deletion of both *cra* and *kdpE* results in almost complete ablation of AE lesion formation (Njoroge *et al,* submitted), we also investigated global gene expression in *ΔkdpEΔcra.* When compared to wt, *ΔkdpEΔcra* presented 997 genes as up-regulated and 305 genes down-regulated. The total number of genes differentially regulated in all three mutants was similar (1344 genes in *Δcra,* 1294 genes in *ΔkdpE* and 1302 in *ΔkdpEΔcra*). This raised the possibility that there may be more genes commonly regulated by Cra and KdpE. To test this hypothesis we looked at whether there were any commonly regulated genes in the arrays. When the *ΔkdpE* and *Δcra* arrays were compared, they had 57 genes commonly up-regulated

and 63 genes commonly down-regulated (Fig.6.1a and b). The down-regulated genes included the LEE PI and non-LEE encoded effectors (Fig.6.2), while the up-regulated ones included genes encoding sRNAs such as *micF* and *omrA.* The double mutant *ΔkdpEΔcra* had more genes in common down-regulated with *Δcra* (188) than with *ΔkdpE* (25). Also, *ΔkdpEΔcra* had significantly more genes in common up-regulated with *Δcra* (490) than with *ΔkdpE* (64). These data suggest that the double kinase mutant has a phenotype similar to *Δcra.*

Next, we investigated oppositely regulated genes in the *ΔkdpE* and *Δcra* arrays. In the single mutant arrays we identified a total of 290 genes conversely regulated (164 in the "decreased in *Δcra,* increased in *ΔkdpE*" batch, 126 in the "increased in *Δcra,* decreased in *ΔkdpE*" batch) (Fig.6.1c and Fig.6.1d). Altogether, these data indicated that there is both convergent regulation, and converse regulation of some genes by Cra and KdpE. (Array data has been deposited on the NCBI GEO database, GSE number pending).

Table 6.1. Comparison of the deletion of *cra, kdpE* or both genes on global gene expression of EHEC O157

		Increase	Decrease	No change	total
wt vs. *Δcra*	MG1655 specific	473	266	3331	4070
	Pathogen specific	356	249	5338	5943
	total	829	515	8669	10013
wt vs. *ΔkdpE*	MG1655 specific	434	253	3383	4070
	Pathogen specific	224	383	5336	5943
	total	658	636	8719	10013
wt vs. *ΔkdpECΔcra*	MG1655 specific	527	109	3434	4070
	Pathogen specific	470	196	5277	5943
	total	997	305	8711	10013

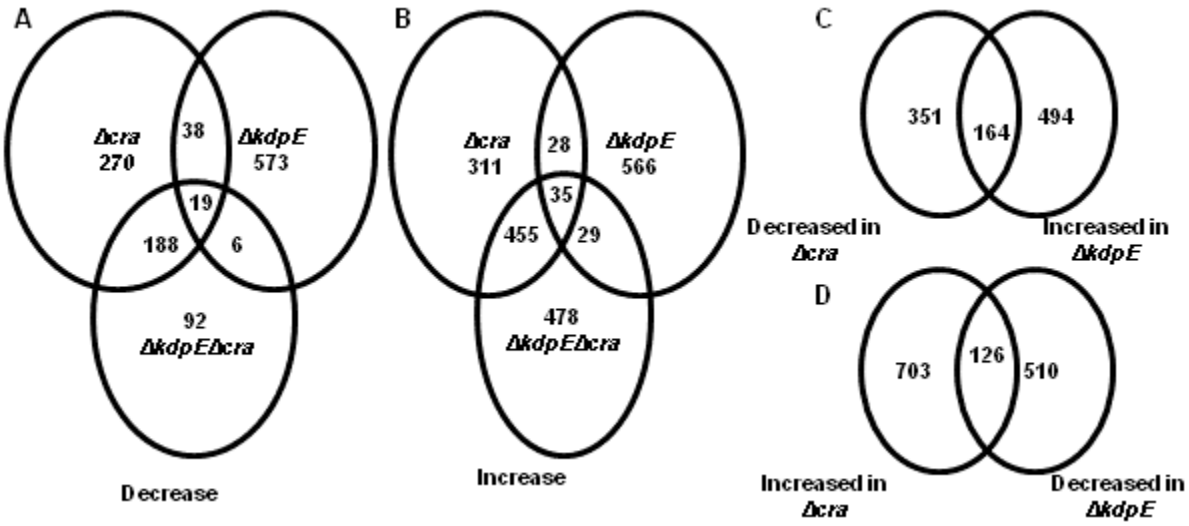


Fig.6.1. Global analysis of Cra and KdpE's effect on EHEC O157 gene transcription. Venn diagrams showing the number of overlapping **(A)** down-regulated genes and **(B)** up-regulated genes between the *cra,* the *kdpE a*nd the *kdpEcra* mutant strains compared to wt. **(C)** Venn diagram indicating genes that are decreased in *Δcra* and increased in *ΔkdpE*. **(D)** Venn diagram indicating genes that are increased in *Δcra* and decreased in *ΔkdpE*. Strains for the microarrays were grown to an OD_{600} of 1.0 in low glucose DMEM.

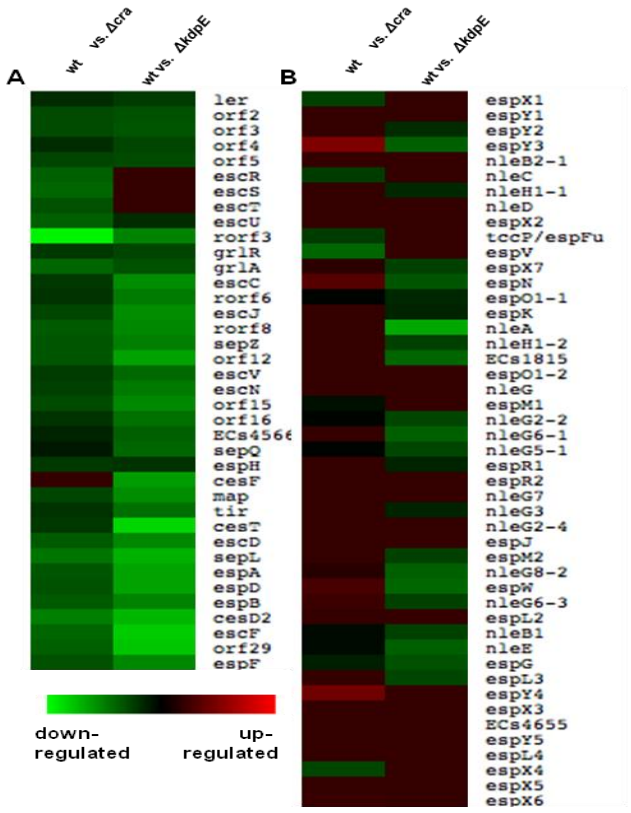


Fig.6.2. Effect of deletion of the transcriptional regulators Cra and KdpE on EHEC virulence genes. (A) A heat map representing differential regulation of the LEE genes. **(B)** A heat map showing the differential expression of non-LEE encoded genes.

Deletion of Cra and KdpE increase growth rate in a metabolite independent manner.

Due to the fact that Cra and KdpE regulate not only virulence factors (8, 334) (Njoroge *et al,* submitted) but are also involved in carbon metabolism regulation (180, 240-242), we investigated whether these regulators affected EHEC growth rates. In particular, we wanted to examine differences during growth in low glucose DMEM, conditions that have been shown to be optimum for LEE gene expression and AE lesion formation *in vitro* (33, 282). When strains

were grown in low glucose DMEM, *Δcra* and *ΔkdpE* had shorter lag times than wt and *ΔkdpEΔcra* and reached a higher density which they maintained for several hours (Fig.6.3a). A possible explanation for this is that Cra and KdpE regulate systems that delay the onset of exponential growth such that when these two genes are deleted, the mutant strains prematurely enter log phase. Interestingly, when both genes are deleted, the growth rate of the *ΔkdpEΔcra* strain is comparable to wt, suggesting that in the double mutant the systems that control exit out of lag phase nullify each other. The doubling rates of the different strains, *Δcra, ΔkdpE* and *ΔkdpEΔcra* were 37min, 42min and 41min respectively compared to wt's 48 min.

Since *ler* transcription in high glucose DMEM (0.4% glucose) is down-regulated compared to its transcription in low glucose DMEM (0.1% glucose) (Njoroge *et al,* submitted), we next examined the effect that increase in glucose had on growth. When wt was grown in high glucose, its doubling time was comparable to growth in low glucose (Fig.6.3b). However, the wt growing in high glucose had a longer log phase, which is probably due to the fact that the high glucose media provides more nutrients thus sustaining exponential growth for a longer period of time. When low/high glucose growth curve experiments were performed for the mutants (Fig.6.3c-e), a similar pattern of extended exponential growth was observed. The switch from low to high glucose increased the doubling time of *Δcra* from 37 to 46min (Fig.6.3c).

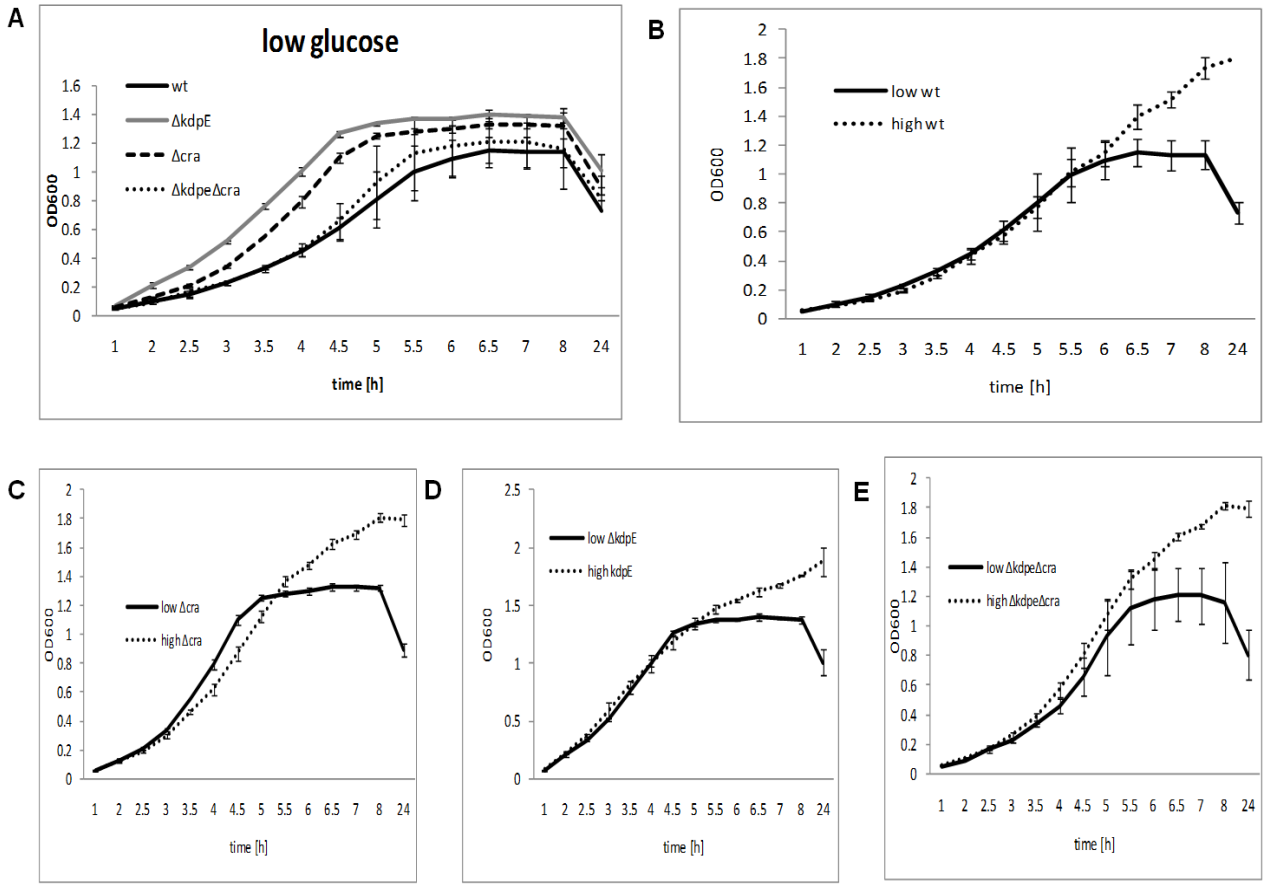


Fig.6.3. Cra and KdpE growth curve. (A) Overnight samples of wt and the mutants were diluted 1:100 in low glucose (0.1%) DMEM and their OD_{600} monitored over time. Their doubling rates were determined to be: wt, 48min; *Δcra*, 37min; *ΔkdpE*, 42min and *ΔkdpEΔcra* 41min. The strains' ability to grow in high (0.4%) vs. low glucose media was evaluated by monitoring OD_{600} for **(B)** wt, **(C)** *Δcra,* **(C)** *ΔkdpE* and **(D)** *ΔkdpEΔcra.* All strains were grown in triplicate.

***In silico* analysis of virulence factors potentially regulated by Cra.** Cra has been reported to be an activator of virulence in both *Salmonella* (8) and EHEC (Njoroge *et al,* submitted). We previously reported on Cra's ability to activate *ler, t*he master regulator of the LEE genes, and consequently the transcription and translation of genes in this PI. Since the microarray analyses suggested that numerous virulence factors were regulated by Cra, and Cra

has a very well-defined DNA-binding consensus sequence, we performed an *in silico* analysis of the EHEC genome using Virtual Footprint (VF) software version 3.0 (http://prodoric.tu-bs.de/vfp/vfp_regulon.php) (206) to search for potential Cra binding sites. This software uses the consensus binding site sequence of transcriptional regulators to identify putative targets. VF allowed us to not only narrow down the list of Cra hits, but it also allowed us to potentially identify targets that the microarray may have missed. We used Cra's consensus binding sequence **RSTGAAWCSNTHHW** (221) to scan the genomes of two EHEC strains, EDL933 and Sakai for potential targets in O-islands, regions of the EHEC genome not found in K-12 (108, 236). Since KdpE has no clear consensus binding sequence, we did not use this transcriptional factor in the bioinformatics analysis. We identified about a dozen potential targets (table 6.2) including *espG* which is located next to *ler,* and is divergently transcribed. Of the "*in silico"* predicted targets, only *ler* and e*spG* have been characterized. EspG is a secreted effector that has been reported to disrupt host cell activity (44, 106, 273, 301). Several VF hits including *Z2077* and *Z4267* were also differentially regulated in the microarrays. The fact that this software reported such few hits and that one of the positive hits was already characterized to be regulated by Cra gave us confidence in this bioinformatics analysis method.

Table 6.2. O-islands genes identified to be putative Cra targets by Virtual Footprint

name	predicted Cra binding site	O-island	function
Z0402	TGAATGGATTC	15	putative beta-barrel outer membrane protein, 55% identity to putative ATP-binding component of a transport system and adhesin protein in *Escherichia coli*, *aidA*-like
**Z0639*	TGAAGCGGTTC	29	23% identity to putative adhesion/invasion gene in *Neisseria meningitidis*
**Z0640*	TGAAGCGGTTC	29	unknown
****Z1163*	TGAATCGATC	43	36% identity to gene of unknown function in *Sinorhizobium meliloti*
****Z1602*	TGAATCGATC	48	36% identity to gene of unknown function in *Sinorhizobium meliloti*
Z2077	TGAATGGATTA	57	encoded by prophage CP-933O; *nleG7'*; secreted
Z3388	TGAATCGCTTAT	94	unknown
Z3934	TGAATGGTTTAT	108	92% identity to NinG protein [Bacteriophage 21]
Z4267	TGAAGCGTTTCA	119	32% identity to gene of unknown function in *Methanobacterium thermoautotrophicum*
***espG*	TGAACCGTTTC	148	binds p21 activated kinase, regulates endomembrane trafficking
Z5890	TGAATGGCTTA	172	74% identity to prophage P4 integrase in *Escherichia coli*
consensus	**TGAA T/G/C G/C G A/G T**		

*share an intergenic region
**shares an intergenic region and Cra binding site with *ler*
*** duplicate genes

Cra and KdpE differentially regulate the transcription of characterized and putative virulence factors. To confirm the "*in silico*" analyses we performed real-time PCR (qRT-PCR). Furthermore, since Cra has been shown to interact at least *in vitro* with another transcriptional regulator KdpE, which together with Cra directly regulates *ler* (Njoroge *et al*, submitted), we

further investigated whether KdpE also regulated the Cra targets identified bioinformatically and by the microarray. We extracted RNA from wt, *Δcra* and *ΔkdpE* strains grown in low-glucose DMEM and analyzed the changes in transcription of five genes predicted to be regulated by Cra in the "*in silico"* analysis namely *espG, Z0639, Z0640, Z3388, Z2077* and *Z4267.*

Figure 6.4a shows a cartoon representation of the potential target genes. The e*spG* gene shares 1200bp regulatory region with *ler*. Previous work has shown that Cra binds with high affinity 350bp upstream of *ler* (Njoroge *et al,* submitted), and this binding site is approximately 800bp from *espG*. This confirmed *ler* binding site, as well as the putative sites identified by the Virtual Footprint software are indicated as vertical solid arrows (Fig.6.4a). The KdpE binding site has also been identified as approximately 100bp from the *ler* translation start site and about 1100bp from *espG's* (Njoroge *et al,* submitted), and is indicated as vertical dotted arrow. When *espG*'s transcription levels were examined in the mutants, the mRNA levels were decreased 2.5 fold in *Δcra* compared to wt but in *ΔkdpE* there was only a slight increase that was not significant (Fig.6.4b). The lack of a significant effect on *espG* transcription by KdpE suggests that KdpE does not influence expression of this gene. Comparison of the mRNA levels of genes encoding the putative virulence factors in *Δcra* with wt, depicted two-fold reductions for *Z0639, Z0640* and *Z2077,* three-fold reduction for *Z4267* and a four-fold increase in *Z3388* transcription. The increase in *Z3388* mRNA levels may be explained by the fact that Cra has been shown to be both an activator and an inhibitor (241, 263). In *ΔkdpE,* the transcription of *Z0639, Z0640, Z3388* and *Z4267* was decreased 2.5 fold, three-fold, four-fold and two-fold respectively but *Z2077* mRNA levels remained unchanged (Fig.6.4b). These results indicated

that Cra and KdpE have several targets in common (*ler, Z0639, Z0640, Z3388* and *Z4267*) as well as targets whose regulation is Cra dependent but KdpE independent (*espG* and *Z2077*).

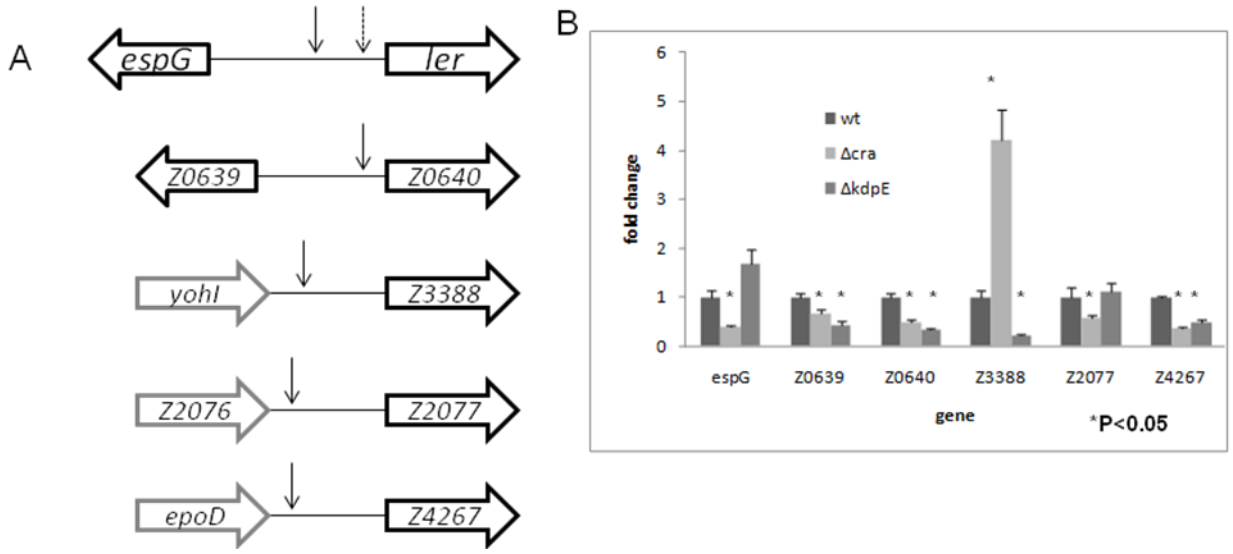


Fig.6.4. Confirmation of Cra and KdpE targets. (A) Cartoon representation of potential target genes identified using Virtual Footprint. The solid vertical arrows indicate the predicted Cra binding site while the dotted vertical arrow indicates the KdpE binding site. **(B)** qRT-PCR analysis examining the expression of the indicated genes in wt, *Δcra* and *ΔkdpE* grown to an OD_{600} of 1.0 in low glucose DMEM. The genes' transcript levels were quantified as fold differences normalized to wt gene transcription levels. The samples' *rpoA* transcript levels were used as internal controls to normalize the output C_T values. The data is from at least three independently grown replicates.

Transcriptional regulation of the putative O-island genes by Cra and KdpE is direct.

Having confirmed that several genes identified by bioinformatics and/or by the microarrays are indeed differentially targeted by Cra, we next decided to look into whether their regulation by Cra, and for some the regulation by KdpE, was due to direct binding. We designed probes encompassing the proposed binding sites of *Z0639/ Z0640, Z3388, Z2077* and *Z4267* then performed electrophoretic mobility shift assays (EMSAs). These probes were radiolabeled,

mixed with increasing amounts of either Cra or KdpE recombinant proteins, and run on a polyacrylamide gel (Fig.6.5a and b). As a negative control we used radiolabeled non-specific probe *kan.* We observed that with increasing amounts of Cra, we were able to shift all of the probes except for the negative control *kan.* When increasing concentrations of KdpE protein was incubated with the probes, all probes except the negative control *kan* and the *Z2077* probe were shifted. The fact that KdpE did not shift the *Z2077* probe correlates with the qRT-PCR data (Fig.6.5b), which showed that the mRNA levels of *Z2077* remain unchanged in *ΔkdpE.* These EMSA results provided evidence that the differential regulation of the putative virulence factors *Z0639, Z0640, Z3388* and *Z4267* by Cra and KdpE is due to direct binding. We also confirmed that although there was direct regulation of *Z2077* by Cra, there was no binding of KdpE to the promoter region of this gene which would explain the lack of regulation by this transcriptional factor. It is apparent from the qRT-PCR and the EMSAs that, although Cra and KdpE share a number of targets, not all targets regulated by Cra are also controlled by KdpE.

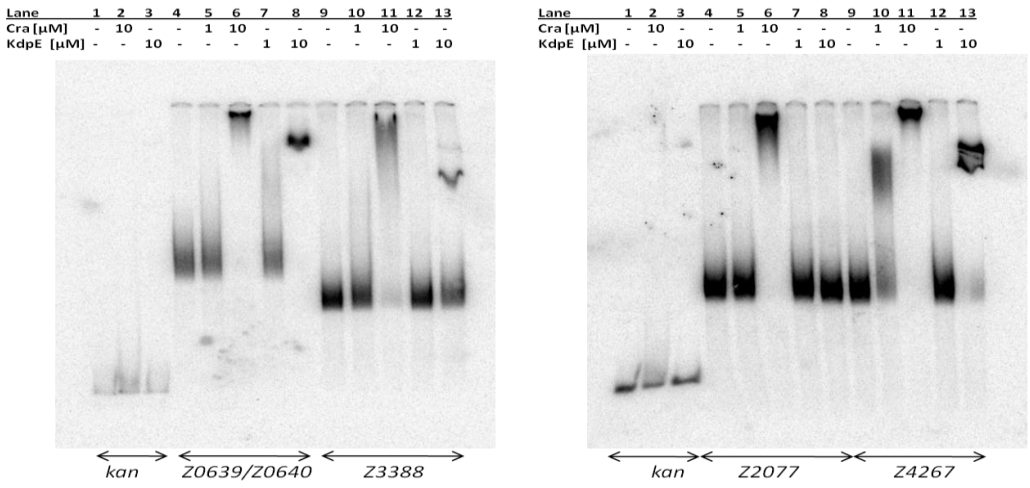


Fig.6.5. Cra and KdpE regulates O-island genes by direct interaction with their promoter regions. Increasing amounts of His purified recombinant Cra and KdpE was used to shift 2ng of

the indicated radiolabeled DNA probe. A radiolabeled *kan* DNA probe was used as a negative control.

Cra and KdpE directly regulate *espFu* expression. We have shown that the two transcription regulators Cra and KdpE, by interacting with each other, can directly regulate transcription of *ler,* the master regulator of the LEE pathogenicity island [Njoroge *et al,* submitted]. We have also shown that Cra and KdpE share O-island targets. We next wanted to investigate whether this interaction was important in other major regulatory networks, particularly those involved in virulence. From the microarrays we identified the gene *espFu* as being significantly down-regulated in the *cra, kdpE* and *kdpEcra* mutants. The gene *espFu* encodes for an effector which has been shown to be essential for pedestal formation, an important step in the progression of the disease caused by EHEC (35, 312). Using the Cra consensus binding sequence, we scanned the *espFu* promoter region and identified a putative Cra binding site (Fig.6.6a). The target *espFu* was not one of the Virtual Footprint hits and this was probably due to the stricter parameters we set for the software. We transformed an *espFu:lacZ* transcriptional fusion into the single and double mutants and performed beta-galactosidase assays. We observed a drastic reduction in *espFu* transcription in all three mutants (*Δcra, ΔkdpE* and *ΔkdpEΔcra*) (Fig.6.6b), which could be complemented by expression of these genes *in trans.* We next wanted to investigate whether this regulation was direct. Using a probe encompassing the first 500bp of the e*spFu* promoter, we performed EMSAs with Cra and KdpE. Both proteins were able to shift the labeled *espFu* probe (Fig.6.6c and Fig.6.6d) indicating that e*spFu* is indeed a direct target of both Cra and KdpE.

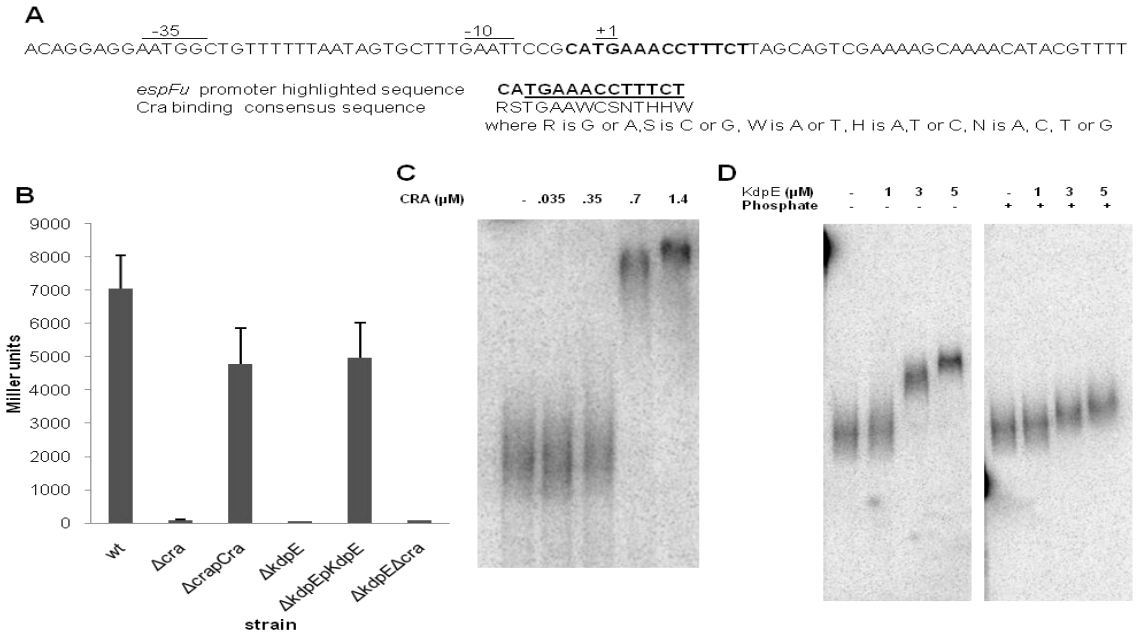


Fig.6.6. Cra and KdpE both directly regulate the *espFu* promoter region. A. DNA sequence of the *espFu* promoter region showing the -35 and -10 positions and the transcription start site. The position of the putative Cra binding site is indicated in bold. Below this is an alignment of the Cra binding site sequence from the *espFu* promoter with the consensus binding site sequence of Cra. **B.** An *espFu-lacZ* transcriptional fusion was transformed into wt, *ΔkdpE, Δcra*, their complements and *ΔkdpEΔcra*. The beta-galactosidase assay was performed at OD 0.5. In the absence of *cra* and/or *kdpE,* the transcriptional reporter's activity was significantly reduced. The activity was restored by complementing the mutant strains. **C and D.** To investigate direct binding EMSAs were performed using the *espFu* probe and increasing concentrations of recombinant Cra **(C)** or KdpE **(D)**. Both Cra and KdpE shifted the *espFu* probe. The shift due to KdpE was reduced in the presence of a phosphate donor.

DISCUSSION

Transcriptional regulators are involved in the control of gene expression by bacteria allowing these microorganisms to efficiently and effectively adapt to their colonization niche. Pathogens in particular use transcriptional factors to express their virulence factors in an energy and spatiotemporally efficient manner. Cra is a member of the LacI/GalR family, which

has been shown to positively regulate the genes encoding gluconeogenic enzymes such as fructose-1,6-diphosphatase, and negatively regulate genes encoding glycolytic enzymes such as phosphofructokinase, and the energy coupling proteins of the bacterial phosphotransferase system (PTS) (37, 38, 72, 260). Cra has also been shown to be required for virulence in *Salmonella enterica* species, and to be a facilitator of *Shigella flexneri* pathogenesis (98, 309, 334). KdpE, an OmpR/PhoB family member, positively regulates *E.coli's kdpFABC* operon in response to osmotic stress (169), and is phosphorylated by QseC to activate LEE expression. KdpE has also been recently identified as an important virulence factor in a number of pathogens. It has been shown to be important in the intracellular survival of pathogens such as *Yersinia pestis, Mycobacterium bovis* and *Photorhabdus asymbiotica* (34, 228, 313). We had previously shown that these two transcriptional factors, Cra and KdpE, directly bind to the promoter region of *ler,* the master regulator of the LEE genes and consequently activate this island leading to the formation of AE lesions. We had also presented evidence that these two transcription factors interacted. In this work, we used microarrays and Virtual Footprint to identify more targets including *espFu, espG* and several O-island genes. We confirmed that a subset of these were not only real targets of Cra and/or KdpE but also that their regulation was due to the direct interaction of these transcription factors with their promoter regions. This regulation results in the activation of EHEC virulence including the formation of the hallmark AE lesions (Fig.6.7).

One of the environmental signals that bacteria respond to is carbon nutrition. EHEC's ability to initiate growth and maintain colonization *in vivo* depends on whether the carbon

source is glycolytic or gluconeogenic (200). Glucose polymers have been shown to be important sources of carbon nutrition (36, 71, 136). *In vitro* studies have shown that metabolites can regulate the expression of both metabolism and non-metabolism related genes. In chapter five we showed that raising glucose concentrations in media from 0.1% to 0.4% inhibited not only the transcription but the translation of the LEE genes. Here we show that this difference in glucose levels also prolongs the log phase but does not significantly alter the overall growth of EHEC.

Altogether, we showed that Cra and KdpE are global regulators of gene expression, and in particular the expression of virulence genes. These regulators respond to cues that include changes in glucose levels and osmolarity. As both these transcription factors are encoded in many pathogens, understanding the Cra and KdpE regulatory cascade may provide useful information into virulence regulation in other pathogens.

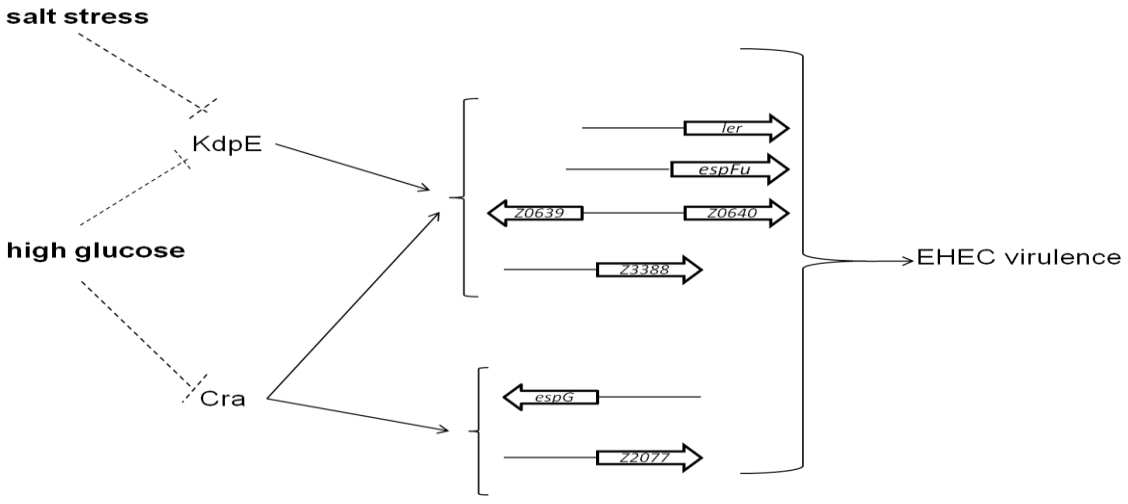


Fig.6.7. Model for Cra and KdpE regulation of EHEC known and putative virulence factors. In response to metabolites and hyperosmotic stress, Cra and KdpE positively regulate the transcription of the *ler*, *espFu, espG* and O-island and consequently EHEC virulence.

CHAPTER SEVEN

CpxA, A Bacterial Receptor for Human and Bacterial Tryptophan Derivatives

INTRODUCTION

The gastrointestinal (GI) tract is a complex environment that has a total microbial population of approximately 10^{14} cells, ten-fold more than the total number of mammalian cells in the human body (25). The intestinal microbiota plays an important role in nutrient assimilation, the development of the innate immune system, and a barrier to limit pathogen colonization (116, 117). Recently, the intestinal microbiota has also been shown to promote enteric virus replication and systemic disease (166). Given the high bacterial population density and diversity, it is plausible that these microorganisms would have a way to communicate with each other as well as with the host in order to maintain a homeostatic GI environment. However, bacterial pathogens exploit these cell-to-cell signaling systems to recognize their colonization niche and cause disease. These pathogens are therefore able to use cues in the environment for the efficient spatiotemporal expression of virulence genes. These environmental cues are normally small molecules present in the colon at concentrations that influence gene expression. The small molecules epinephrine and norepinephrine are mammalian hormones that have been shown to contribute to the pathogenesis of enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 (41, 85, 120, 243), enterotoxigenic *E.coli* (ETEC) (183), *Salmonella enterica* serovar *Typhimurium* (15, 20, 21, 205), *Vibrio*

parahaemolyticus (212) and recently in the fish and human pathogen *Edwardsiella tarda* (326). These hormones are sensed by the bacterial sensor kinases QseC and QseE to regulate important virulence factors (41, 205, 224, 243, 284). Bacteria also sense small molecules produced by other gut microbes. When these small molecules, which are known as autoinducers (AIs), reach a critical concentration threshold, they are recognized by bacteria, which then respond to them by altering their gene expression. AI-3 produced by many *E.coli* including EHEC is sensed by QseC and has also been shown to contribute to EHEC pathogenesis.

The enteric pathogen EHEC, which colonizes the human colon resulting in hemorrhagic colitis and the often fatal hemolytic uremic syndrome (HUS) (216), has several important virulence factors. These virulence determinants include chemotaxis/flagella genes (43), the production of the potent Shiga toxin that causes HUS (145, 146) and genes necessary for the attaching and effacing (AE) lesion formation on enterocytes (204, 287). AE lesion formation requires genes contained within the locus of enterocyte (LEE) pathogenicity island (PI), which encode for the structural and regulatory components of the type three secretion system (TTSS) (67, 130, 190). EHEC uses the TTSS to translocate LEE and non-LEE encoded effectors that hijack the host machinery culminating in the formation of AE lesions and contributing to overall EHEC pathogenesis.

The colon contains tryptophan derivatives, some of which have been suggested to be small molecule signals used by pathogens to regulate virulence. The protozoan *Entamoeba histolytica* has been reported to increase its virulence in response to serotonin, a mammalian neurotransmitter (191). Although a number of bacteria and viruses including EPEC,

S.typhimurium, V.cholarae, C.rodentium and rotavirus have been shown to influence 5HT signaling (70, 101, 162, 227), no study has investigated the effect that 5HT has on bacterial pathogenesis. Another tryptophan derivative, indole, has been shown to be important for the regulation of the LEE genes in EHEC (113). Bacteria produced indole has also been reported to play a role in EHEC chemotaxis, colonization, as well as in the modulation of pro- and anti-inflammatory host genes (16, 17). Although several effects of the two tryptophan derivatives on pathogenesis have been observed, their sensor(s) have not been characterized.

In this study, using genetic and biochemical phenotypic analyses, we show for the first time that the bacterial histidine kinase CpxA specifically senses the mammalian neurotransmitter serotonin and the bacteria produced small signal indole. CpxA is part of the CpxRA two-component system, which has been shown to regulate the LEE PI and curli biogenesis, both of which are significant contributors to bacterial virulence. We show that serotonin and indole inhibit CpxA's autophosphorylation consequently decreasing the expression of its targets. Altogether, these results suggest that CpxA is an important small molecule receptor crucial for cell-to-cell signaling.

RESULTS

Serotonin inhibits the LEE PI

The mammalian hormone epinephrine has been shown to differentially regulate virulence in EHEC and other pathogens (41, 243). In order to test whether serotonin, a mammalian monoamine neurotransmitter, could also influence virulence, we performed

phenotypic analyses where we grew EHEC in the absence or presence of micromolar amounts of serotonin and assessed its effect on the transcription of the LEE genes, Shiga toxin and motility genes. In the presence of 1µM serotonin, the mRNA levels of the LEE genes *ler* (*LEE1,* the LEE PI master regulator) and *espA* (*LEE4*) were decreased five-fold compared to wt with no drug (Fig.7.1a,b). However, serotonin did not significantly affect the transcription of the gene encoding Shiga toxin. Motility genes were also unaffected (data not shown). To confirm the effect that serotonin had on the LEE PI, we performed a serotonin concentration gradient Northern blot analysis. We grew wt EHEC in the absence or presence of increasing amounts of serotonin, extracted RNA and evaluated, as a representative of the LEE PI, *espA* transcripts. When we probed with an EspA cDNA probe we observed multiple bands in the absence of 5HT, with the main band detected at approximately 3kb (Fig.7.1c). With increasing concentrations of 5HT (100nM to 10µM), the main 3kb band decreased significantly. Altogether, these results provide the first evidence of a bacteria sensing serotonin, and suggests a role for this monoamine neurotransmitter as an inhibitor of virulence.

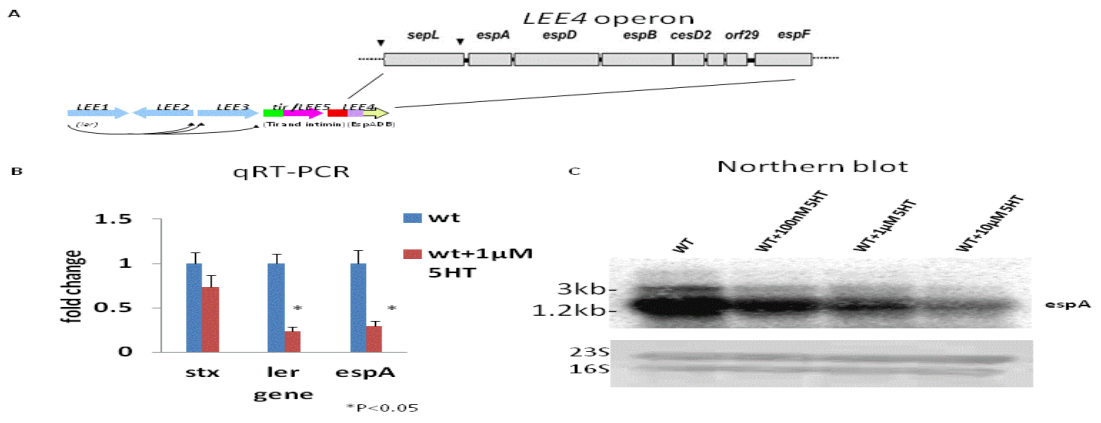


Fig.7.1. Effect of serotonin on EHEC virulence. (A) Cartoon representation of the LEE pathogenicity island with acloseup of the *LEE4* operon. **(B)**qRT-PCR evaluating the transcription

of *stx, ler(LEE1)* and e*spA(LEE4)* in wt grown in the absence or presence of 1µM 5HT. Error bars indicate the standard deviations of the $\Delta\Delta C_T$ values. The mRNA levels of endogenous *rpoA* were used to normalize the C_T values. **(C)** Northern blot showing the effect of increasing concentrations of 5HT on *espA* transcripts. The main (3kb) band decreased with increasing concentrations of 5HT. 23S and 16S rRNA were used as loading controls.

Global analysis of serotonin dependent EHEC gene regulation

Since 5HT inhibited the transcription of the LEE PI, we next investigated the extent of gene regulation in response to this mammalian hormone. Using Affymetrix *E.coli* 2.0 microarrays we performed a global analysis of wt grown in low glucose DMEM to OD_{600} 1.0 in the absence or presence of 10µM serotonin creatinine sulfate complex. This form of serotonin is the form commonly used in neuronal studies and was used in all initial studies before being replaced with the simpler compound serotonin hydrochloride. However, both forms of 5HT inhibit the LEE genes in the same manner (data not shown). We also compared the global gene regulation of serotonin to that of epinephrine (Chapter 4). The microarray analysis indicated that there were twice as many down-regulated genes (747) than up-regulated genes (342) when wt with serotonin was compared to wt with no treatment (Table 7.1). When this regulation was compared to that of epinephrine (Table 4.2), we noticed that not only did serotonin have fewer genes differentially regulated (2150 genes were up and 1196 genes were down-regulated with epinephrine), the pattern of regulation was also different. Use of epinephrine resulted in more genes up-regulated than down-regulated, while serotonin had the opposite effect with more genes down-regulated than up-regulated.

Table 7.1. Effect of serotonin on global gene expression of EHEC O157

		Increase	Decrease	No change	total
wt no signal vs. wt with 10μM serotonin creatinine sulfate complex	MG1655 specific	183	270	3617	4070
	Pathogen specific	134	353	5456	5943
	Intergenic	25	124	1148	1297
	total	342	747	10221	11310

Next, we investigated genes that were commonly regulated between 5HT and epinephrine. When growth in serotonin and epinephrine were compared, the two hormones had 4 genes commonly up-regulated and 13 genes commonly down-regulated (Fig.7.2a and b). The down-regulated genes included the *purDEK* and *asnAB* genes while the up-regulated ones included *srlA* and *hokA.* We also looked at conversely regulated genes in the 5HT and epinephrine arrays. We identified a total of 19 genes conversely regulated (11 in the "decreased in 5HT, increased in epinephrine" batch, 8 in the "increased in 5HT decreased in epinephrine" batch) (Fig.7.2c and Fig.7.2d). The conversely regulated genes included the *csgF* and sRNAs such as *micF* and *omrAB.* Altogether, these data indicated that there is very little co-regulation of EHEC genes by these two hormones.

We next decided to investigate the effect of the hormone on the general expression of sRNAs that are known to participate in post transcriptional regulation (PTM), as well as uncharacterized intergenic regions (IGs). Transcriptome comparison revealed that when wt was grown with 5HT, 149 IGs were differentially regulated (25 up-regulated, 124 IGs down-regulated) (Table 7.1). In comparison only 52 IGs were differentially regulated when wt was grown with epinephrine (47 up-regulated, 5 IGs down-regulated). This high number of

differentially regulated IGs suggests a role for 5HT as a regulator of non-coding RNAs. Furthermore, the fact that almost five times as many IGs were down-regulated as up-regulated, suggests that this 5HT dependent regulation of non-coding RNAs is mostly inhibitory. A heat map comparing sRNA expression in wt with and without 5HT indicated that this monoamine neurotransmitter down-regulated many of the characterized and putative sRNAs (Fig.7.2e). Altogether, these data indicate that 5HT is a mammalian hormone which is sensed by EHEC leading to the inhibition of global gene regulation. (Array data has been deposited in the NCBI GEO database, GSE number pending).

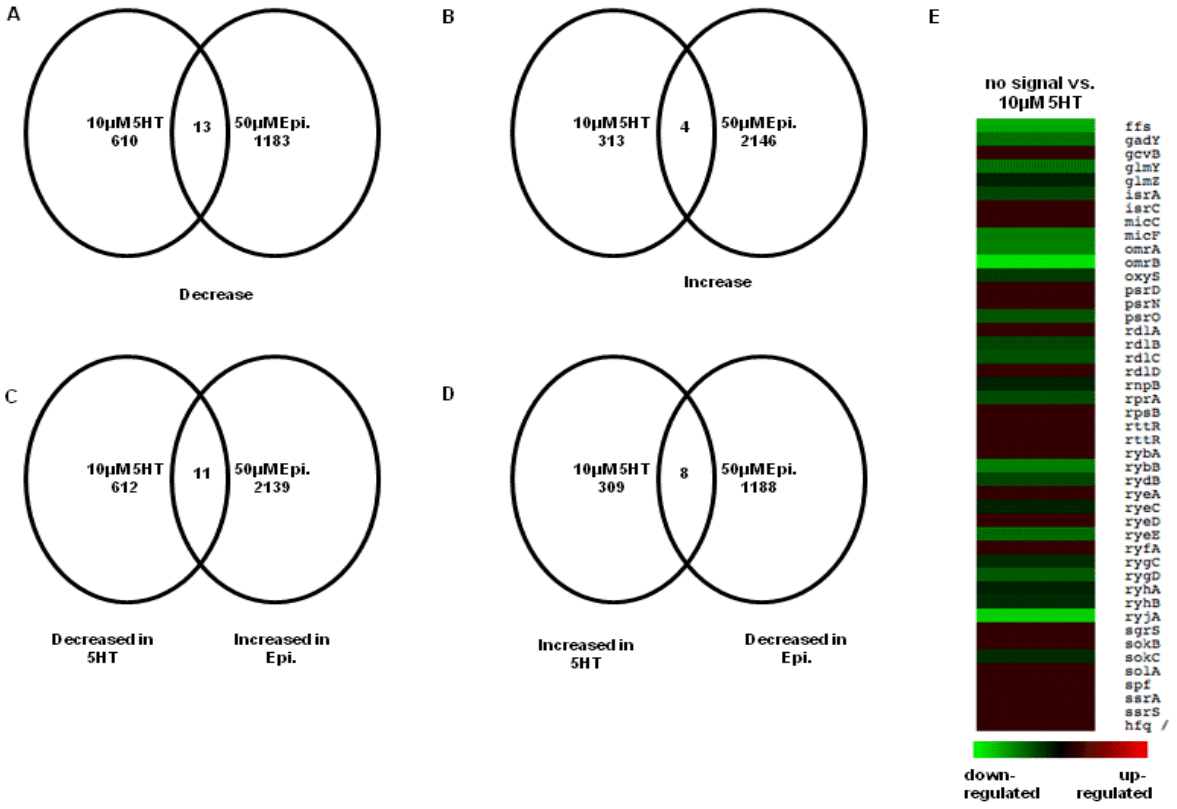


Fig.7.2. Global analysis of serotonin and epinephrine's effect on EHEC O157 gene transcription. Venn diagrams showing the number of overlapping **(A)** down-regulated genes and **(B)** up-regulated genes between the addition of serotonin to wt and the addition of

epinephrine to wt (all compared to wt with no signal). **(C)** Venn diagram indicating genes that are decreased with serotonin and increased with epinephrine. **(D)** Venn diagram indicating genes that are increased with serotonin and decreased with epinephrine. **(E)** A heat map showing the differential expression of sRNAs and the sRNA chaperone in wt with serotonin compared to wt with no signal. Strains for the microarrays were grown to an OD_{600} of 1.0 in low glucose DMEM. 5HT, serotonin; epi, epinephrine

The serotonin antagonist, ritanserin, rescues serotonin dependent inhibition of EHEC genes

Using qRT-PCR, Northern blot and microarrays we have shown that serotonin is an inhibitor of the expression of many EHEC genes including the LEE genes and sRNAs. To further confirm the regulatory specificity of 5HT, we investigated whether commercial 5HT antagonists could reverse the effect the hormone had on its targets. 5HT antagonists inhibit the action of 5HT receptors which can be G protein-coupled receptors or ligand-gated ion channels (118). 5HT receptors are responsible for the modulation of the release of many neurotransmitters and hormones including epinephrine, dopamine, cortisol and acetylcholine consequently affecting numerous neurological and biological processes such as appetite, nausea, aggression and anxiety. To perform the inhibition rescue studies we used the antagonist ritanserin, which has been shown to act on most of the 5HT receptors found in the GI tract (7), such as 5HT receptors 2A, 2B, 2C and 7. We grew wt EHEC to OD_{600} 1.0 in the absence of signal, in the presence of 1µM ritanserin, 1µM 5HT or both of these molecules, extracted RNA and investigated the effect on the transcription of several genes. Ritanserin did not affect the transcription of genes encoding for the sensor kinase CpxA which was highly down-regulated in the 5HT microarray, neither did it significantly affect the transcription of *ler* (Fig.7.3). 1µM 5HT decreased *cpxA* and *ler* transcription two-fold and 2.5-fold respectively. Co-culturing wt with both 5HT and

ritanserin led to the rescue of gene transcription to wt levels for *cpxA* and *ler.* The results of this antagonist study confirm the specificity of 5HT regulation of EHEC genes.

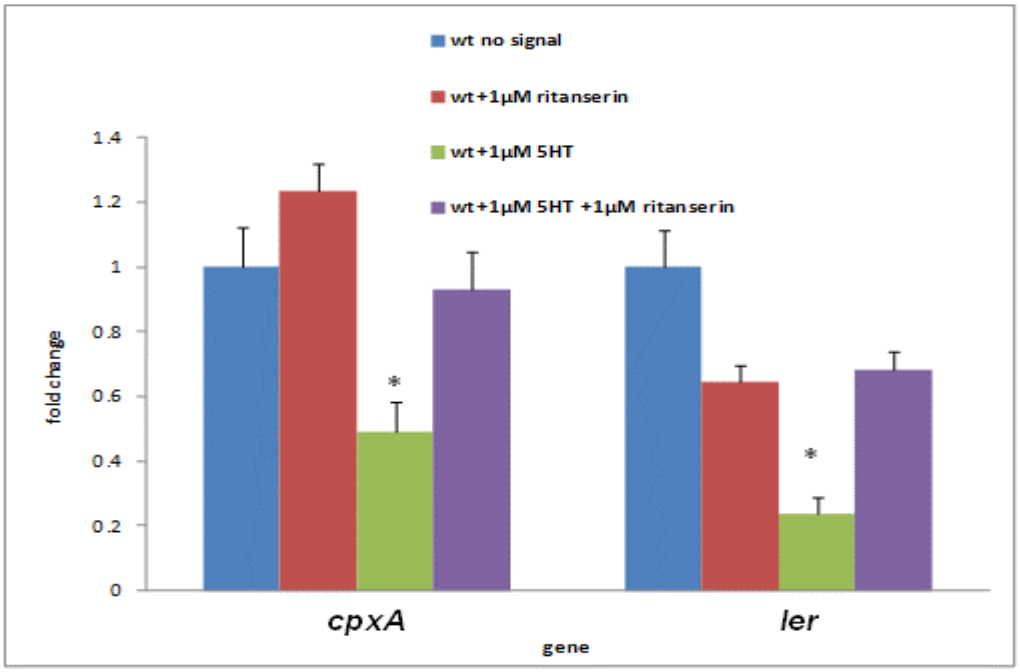


Fig.7.3. Effect of ritanserin on serotonin induced transcriptional inhibition. qRT-PCR evaluating the transcription of *cpxA, stx* and *ler(LEE1)* in wt grown in the absence or presence of 1µM 5HT and/or 1µM ritanserin . The mRNA levels were expressed as fold changes over wt mRNA levels. For all the samples, *rpoA* mRNA levels were used as an internal control to normalize the output C_T values in order to take into account variation in bacteria numbers. *P<0.05 according to student t test.

Cumulative sensing of indole and serotonin

We have shown that serotonin inhibits transcription of the LEE genes. Another tryptophan derivative, indole, is produced exclusively by bacteria. There is evidence, albeit contradictory, suggesting that indole has an effect on EHEC virulence and host response to *E.coli* colonization (16, 113). The concentration of indole present in the human colon is not

known. However, commensal and pathogenic strains of *E.coli* have been shown to produce approximately 500µM indole in cultures (16, 57, 113) and indole in human feces has been detected at between 250 and 1000µM (143, 338). We therefore decided to use 500µM indole to test whether this tryptophan derivative could also regulate the LEE PI as 5HT did. Furthermore, because both signals are found together in the gut, we also grew wt EHEC in the presence of both signals. In the presence of indole, the transcription of both *espA* and e*ae* (*LEE5*) was decreased two-fold, which was comparable to the effect of 5HT on these two genes (Fig.7.4a). When we assessed the transcription of these two LEE genes in the presence of both signals, we observed an even greater decrease in mRNA levels (10-fold compared to no signal and five-fold compared to single signal). This additive inhibition of transcription due to the combined signals suggests a similar and additive role *in vivo* where small molecules are not found on their own but as part of a signaling mixture.

Because the microarrays had intimated a role for 5HT in some small RNAs (sRNA) regulation, we also investigated the effect of this monoamine on sRNA expression. Global analysis of the effect of 5HT on sRNAs had indicated that many of these non-coding RNAs are down-regulated in the presence of 5HT. We therefore selected a number of these sRNAs to test for transcriptional regulation by 5HT. We assessed the expression of *glmY* which promotes *glmS* translation (308), *micF* which inhibits *ompF* translation (307), *omrB* which inhibits *csgD* translation (115), and *ryjA* which was one of the most highly down-regulated sRNAs in our microarray but has no assigned function . The transcription of all these sRNAs was significantly decreased in the presence of combined signals (Fig.7.4b). In the presence of just indole, only

the transcription of *glmY* was significantly decreased while in the presence of just 5HT, none of the sRNAs were significantly affected. These data suggest that the combination of the signals serotonin and indole can inhibit these sRNAs, further reiterating our hypothesis that performing studies using signal mixtures that mimick the *in vivo* environment could yield relevant information.

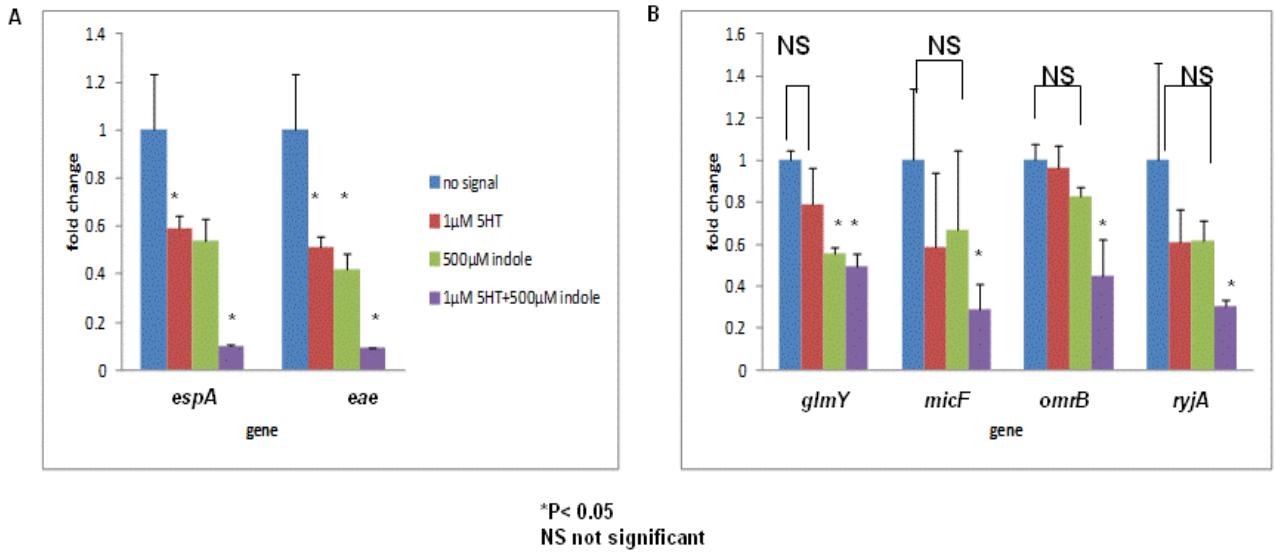


Fig.7.4. Additive effect of serotonin and indole. (A) qRT-PCR evaluating the transcription of e*spA(LEE4)* and *ler(LEE1)* in wt grown in the absence or presence of 1µM 5HT and/or 500µM indole . **(B)** Evaluation of effect of 1µM 5HT and/or 500µM indole on wt mRNA levels of sRNAs by qRT-PCR. The mRNA levels were expressed as fold changes over wt mRNA levels. For all the samples, *rpoA* mRNA levels were used as an internal control to normalize the output C_T values in order to take into account variation in bacteria numbers. *P<0.05 according to student t test.

Indole and serotonin sensing occurs through the sensor kinase CpxA

Previous studies have linked signal sensing by bacterial pathogens to sensor kinases. QseC and QseE, for example, have been shown to sense the hormone epinephrine (41, 205,

247). Small molecules that are sensed by bacteria for differential gene regulation also tend to differentially regulate expression of their sensor kinase (41). Using the same concept, we searched the 5HT transcriptome for sensor kinases that were differentially regulated by this hormone. Among these kinases was CpxA. CpxA is part of the CpxAR two component system which has been suggested to be involved in indole dependent activation of genes encoding for drug exporters (112) and has been shown to be involved in the regulation of the LEE PI (184). Therefore, we hypothesized that CpxA was the sensor of either 5HT or indole or both of these tryptophan derivatives. To test this hypothesis we constructed a non-polar deletion of *cpxA,* and evaluated the ability of this mutant to express LEE genes in the absence or presence of 5HT. We first assessed at the transcription of *ler,* the master regulator of the LEE PI. We grew wt and *ΔcpxA* in low glucose DMEM with either no 5HT or with two concentrations of this signal: 10µM, which was the concentration used in the microarray and 1µM, which is the concentration of 5HT in the colon's lumen. In the absence of 5HT, *ΔcpxA* had a 2.5-fold decrease in *ler* transcription indicating that CpxA is an activator of the LEE genes (Fig.7.5a). Although 1µM and 10µM 5HT decreased the transcription of *ler* in wt 2.5-fold, *ΔcpxA* was not significantly affected by either one of the 5HT concentrations. These results indicated that CpxA was involved in the 5HT dependent regulation of *ler*.

Next we investigated whether CpxA was also involved in the indole dependent regulation of the LEE PI. First, we wanted to ensure that any effect we saw was not due to self produced indole. An *E.coli* liquid culture can contain up to 600µM indole that may interfere with our analysis of indole dependent gene regulation and our investigation of gene regulation

due to combined indole/5HT signaling (16, 113). Therefore, to ensure there was no endogenous indole in our assays we deleted the gene *tnaA* which encodes for tryptophanase, the enzyme that catalyzes L-tryptophan conversion to indole (55, 322). These deletions were perfomed both in a wt and a *ΔcpxA* background. We also constructed an arabinose inducible tnaA expression plasmid for complementation. Using the indole spot reagent (p Dimethylaminocinnamaldehyde, DMACA) we tested the ability of the resultant strains to produce indole. We grew the different strains in LB (which has been shown to induce maximum indole production) in the absence or presence of arabinose. EHEC wt and *ΔcpxA* were both positive for indole (blue) while EHEC *ΔtnaA* and *ΔtnaAΔcpxA* were negative for indole (pink) (Fig.7.5b). *C.rodentium,* which naturally does not encode for the TnaA enzyme, was also indole negative, but we were able to make it indole positive by expressing EHEC's *tnaA in trans.*

Using wt, *ΔcpxA* and the newly constructed strains *ΔtnaA* and *ΔtnaAΔcpxA* we investigated the effect of the two tryptophan derivatives on the transcription of the LEE PI. We grew the different strains to an OD_{600} 1.0 in low glucose DMEM in the absence of signal, in the presence of 5HT, indole or both, extracted RNA and assessed *espA* (*LEE4*) and *eae*(*LEE5*) mRNA levels. Both 5HT and indole decreased the transcription of *eae* in wt and combining the two further decreased transcription (Fig.7.5c). Both *ΔcpxA* and *ΔtnaAΔcpxA* with no signal had decreased *eae* mRNA levels compared to wt with no signal and these mRNA levels were unaffected by the addition of either one or both of the signals. The *tnaA* mutant in the absence of signal had *eae* mRNA levels comparable to wt with no signal. However the phenotype of the *tnaA* mutant was very interesting with addition of 5HT increasing *eae* mRNA levels three-fold,

addition of indole decreasing transcription 100-fold and combining both signals leading to mRNA levels similar to the mutant with no signal. As had been previously observed, both 5HT and indole decreased the transcription of *espA* in wt and combining the two further decreased transcription (Fig.7.5d). The transcription pattern for *espA* in the mutants was similar to that observed with *eae* transcription. However, the results for the effect of indole on *espA* mRNA levels in *ΔtnaA* are different from what was reported by Hirakawa *et al* (113). They reported that the addition of indole to *ΔtnaA* increased *espA* expression which is the converse of what we observed. One possible explanation for this disparity may be due to the fact that we assessed *espA* transcription while they assessed EspA protein expression; given that the *LEE4* operon is highly post-transcriptionally regulated (176), this may be the reason for the difference. Another possible reason for the different results is the fact that they performed their experiments in LB, which although it induces more endogenous indole production, is not the optimal media to investige LEE gene expression.

Altogether, these results support our hypothesis that CpxA is the sensor kinase of both 5HT and indole, and that this kinase uses these signals to regulate the expression of the LEE PI. Consequently, in the absence of CpxA, EHEC is unable to sense these two small signals and is consequently unable to differentially regulate its virulence factor. Interestingly, growing *ΔcpxA* and *ΔtnaAΔcpxA* with the combined signals led to a slight increase in transcription of *espA* compared to the mutants with no signals. This may be due to the *LEE4* post-transcriptional regulation interfering with the effects of the combined signals or may indicate the presence of another (minor) sensor for these signals.

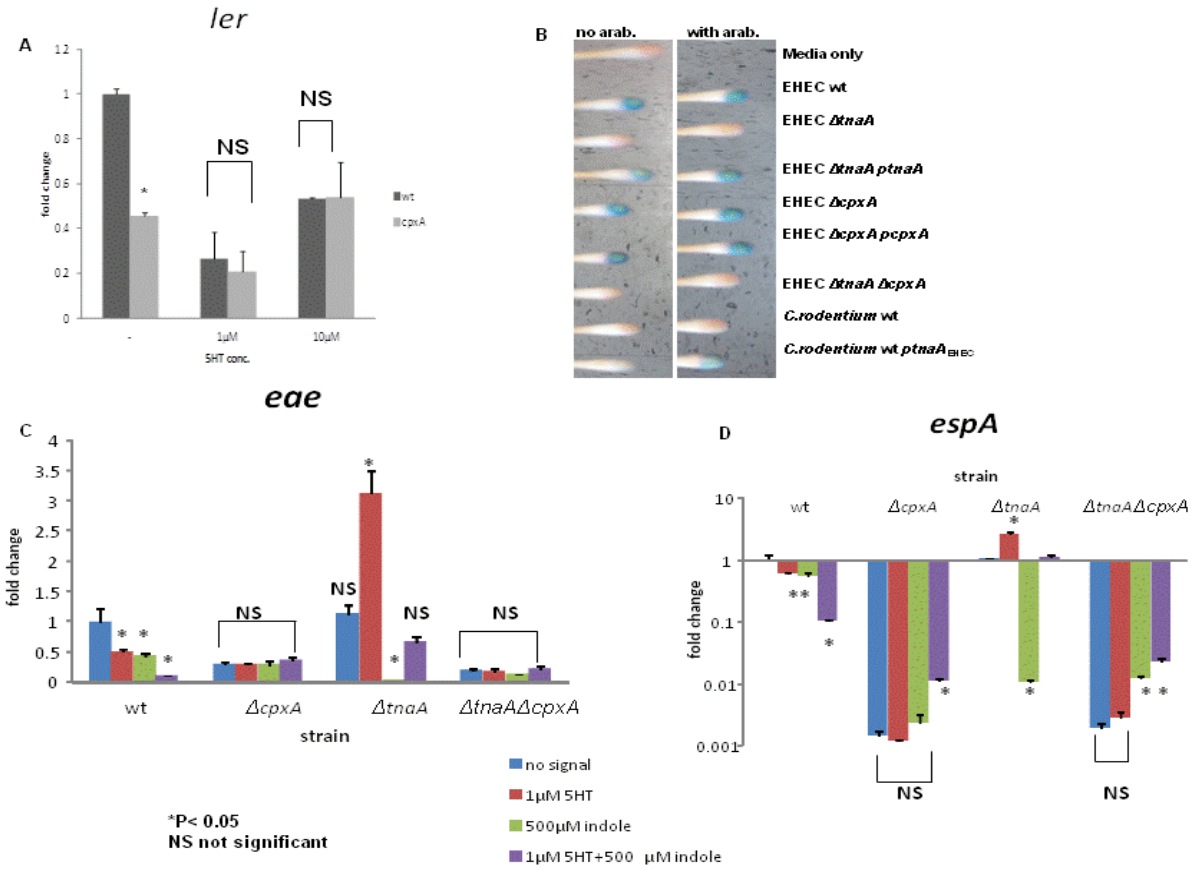


Fig.7.5. CpxA is the bacterial sensor of serotonin and indole. (A) qRT-PCR evaluating the transcription of *ler* in wt or *ΔcpxA* grown in the absence or presence of 1μM or 10μM 5HT. **(B)** An Indole spot test was used to test the ability of different strains to produce indole. Pink or yellow, negative; Blue or green, positive. qRT-PCR was used to evaluate the effect of 1μM 5HT and/or 500μM indole on the transcription of **(C)** *eae* or **(D)** *espA* in wt or the mutants. The mRNA levels were expressed as fold changes over wt mRNA levels. For all the samples, *rpoA* mRNA levels were used as an internal control to normalize the output C_T values in order to take into account variation in bacteria numbers. *P<0.05 according to student t test.

CpxA and TnaA regulation of AE lesions

We have shown that CpxA is an activator of the LEE genes and that TnaA is necessary and sufficient for the production of indole, a small molecule that has been reported to reduce

EHEC attachment to HeLa cells (17). Given that the LEE genes are important for AE lesion formation and attachment is among the first steps towards the formation of these lesions, we hypothesized that CpxA and TnaA may be involved in this phenomenon. As the formation of the EspA translocon is integral to the translocation of effectors that are responsible for the formation of AE lesions, we decided to first investigate whether the strains' protein levels mirrored their transcriptional profile. Strains were grown in low glucose DMEM to an OD_{600} 1.0. The supernatant, which contained secreted proteins, was then concentrated and probed with anti-EspA polyclonal antibodies. Samples were spiked with equal amounts of BSA (loading control) before concentration. The *ΔtnaA* strain secreted the same amount of EspA as wt (Fig.7.6a). *ΔcpxA* was unable to secrete EspA and this could be partially rescued by expressing *cpxA in trans.* The double mutant *ΔtnaAΔcpxA* was also unable to secrete any detectable EspA. These results mirrored the transcriptional studies.

Next, we assessed the ability of these strains to form pedestals. HeLa cells were infected for six hours with wild type or the mutant strains. Actin was stained with FITC-phalloidin, HeLa nuclei and bacteria with propidium iodide, and pedestals were visualized as brilliant green patches underneath red bacteria. *ΔcpxA* had significantly reduced pedestal formation compared to wt (6% vs. 55%) and this could be complemented by introduction of a plasmid encoding *cpxA* (Fig.7.6b). Although *ΔtnaA* formed comparable numbers of pedestals to wt (48%), overexpressing *tnaA in trans* in *ΔtnaA* resulted in an interesting phenotype. *ΔtnaAptnaA* not only had significantly decreased pedestals (16%), there was also a stacking pattern to the

attaching bacteria reminiscent of biofilm formation. The double mutant *ΔtnaAΔcpxA* had decreased AE lesion formation (23%).

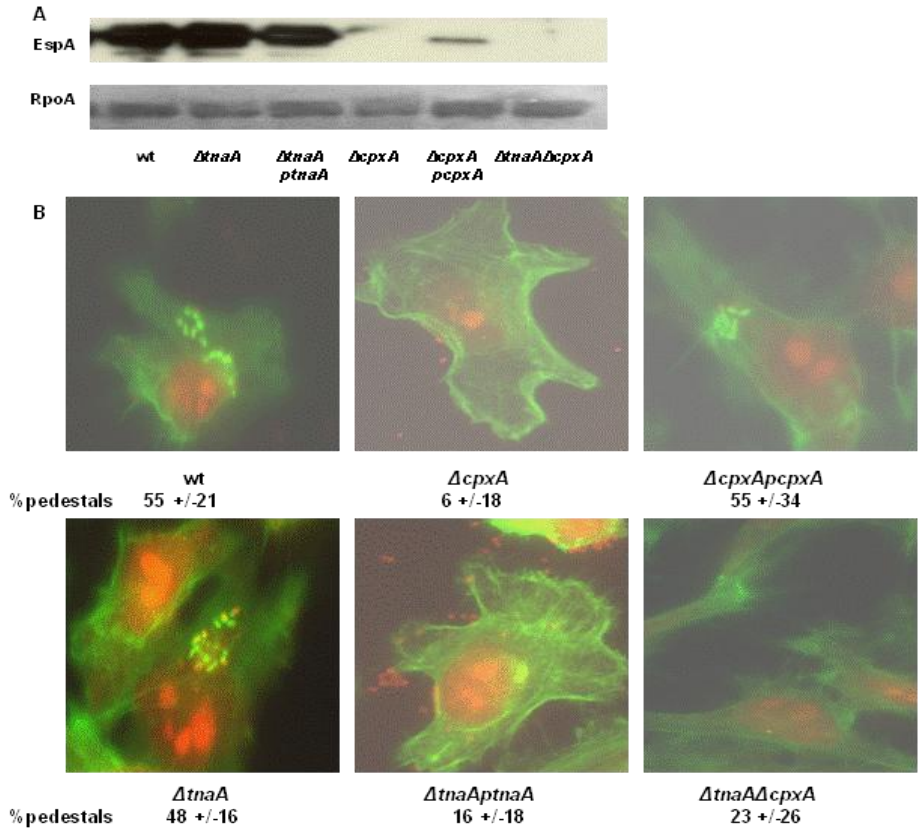


Fig.7.6. CpxA and TnaA regulation of AE lesion formation (A) Western of secreted proteins of wt, *ΔcpxA, ΔtnaA, ΔtnaAΔcpxA* and complements grown in low glucose DMEM were probed with antisera against EspA. BSA was used as the loading control. **(B)** Fluorescent actin staining assays (FAS).The different strains indicated were used to infect HeLa cells for 6 hours in the absence or presence of epinephrine (final concentration 50µM). HeLa actin was stained green with FITC phalloidin while HeLa nuclei and bacteria were stained red with Propidium Iodide. Formation of pedestals was visualized as bright green (actin) cups onto which red bacterial dots bound. The experiments were performed in duplicate at least three times. For every slide at least 100 cells were evaluated. The numbers underneath each panel indicate the percentage of infected HeLa cells.

CpxA auto-phosphorylation is decreased in response to indole and serotonin

Our genetic analyses suggested that CpxA is the bacterial sensor for 5HT and indole. *ΔcpxA* is unable to activate the expression of LEE *g*enes in response to 5HT and/or indole. 5HT inhibits the transcription of *cpxA,* and this could be a further mechanism of inhibition of the LEE genes. However, we hypothesized that in addition to down-regulating the LEE PI by inhibiting their activator CpxA, 5HT may also decrease CpxA activity by inhibiting the ability of this sensor kinase to autophosphorylate. The epinephrine sensor QseC has been shown to respond to this hormone by increasing its autophosphorylation state leading to an increase in virulence (41, 243) (Chapter 4). To test whether CpxA's autophosphorylation could also be affected by the tryptophan derivatives, we performed *in vitro auto*phosphorylation assays. We reconstituted the normally membrane bound sensor kinase CpxA into liposomes. In the liposomes, CpxA adopts an inside-out orientation with the periplasmic signal recognition domain inside the liposome vacuole (Fig.7.7c)(138). CpxA loaded liposomes were treated with a final concentration of 1μM, 60μM 5HT or 500μM indole. Addition of either 5HT or indole to the liposomes resulted in a decrease in autophosphorylation levels over time (Fig.7.7a, b, d and e). This confirmed that 5HT not only decreased the transcription of *cpxA* but also that it and indole were able to decrease CpxA autophosphorylation proving that CpxA was indeed the sensor for both tryptophan derivatives.

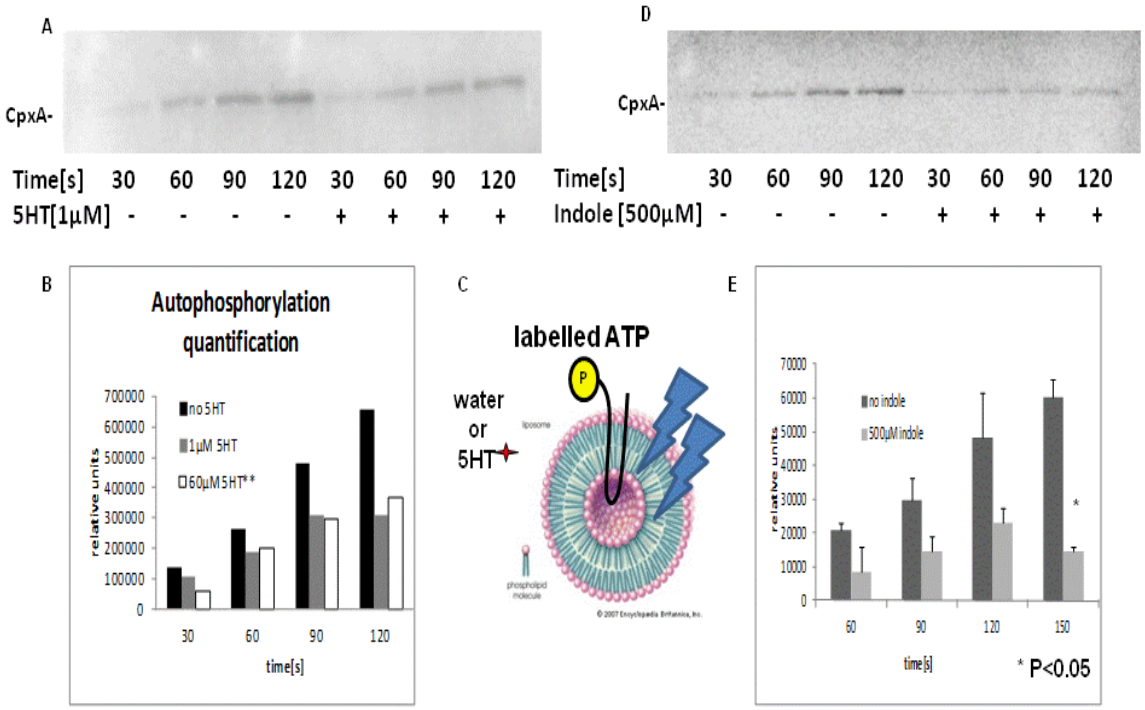


Fig.7.7. CpxA decreases its autophosphorylation in response to 5HT and indole. CpxA's autophosphorylation in liposomes is decreased in the presence of 5HT **(A and B)** and 500µM indole **(D and E).** The decrease in phosphorylation was visualized on SDS gels **(A and D)** and the bands' intensity graphed as relative units **(B and E). (C)** Cartoon representation of the inside-out orientation of CpxA in the liposome. Lightning graphics indicate the freeze/thaw process required to get the signals into the liposome.

DISCUSSION

Cell-to-cell signaling allows bacteria to sense and respond to their environment in a manner that culminates in the most spatiotemporal efficient expression of their genes. The first evidence of multicellular-like behavior was reported in *Vibrio fischeri* and *V.harveyi* where cell-to-cell signaling is used to regulate bioluminescence (218, 219). Since then many small molecules have been identified that are produced by bacteria and are sensed by these microbes to synchronize gene expression (82, 122). Pathogens in particular take advantage of

these small molecules to coordinate the expression of their virulence factors (120, 205, 225). Bacteria produced indole has been shown to inhibit biofilm formation, motility and attachment to epithelial cells in EHEC, and has been implicated in the regulation of the TTSS (16, 17, 113). Serotonin is another tryptophan derivative produced by animals, fungi and plants but not by bacteria, and it has a profound effect on gut physiology (153). Although the level of this mammalian neurotransmitter has been shown to be affected by bacterial pathogens, little was known about whether the microbes themselves were affected by serotonin. Here we show that the bacterial histidine kinase CpxA senses both indole and serotonin. Upon sensing these signals, the autophosphorylation of CpxA is inhibited leading to the down-regulation of CpxA's targets, which include the LEE PI, an important regulator of EHEC virulence (Fig.7.8).

Interestingly, the presence of both signals, at physiological concentrations found in the colon lumen, further inhibits EHEC virulence genes. An explanation for the inhibition of EHEC virulence by these two tryptophan derivatives has to do with spatiotemporally efficient expression of the genes that regulate this microbe's virulence. The majority of the serotonin producing enterochromaffin cells are located in the ileum and there is a decreasing gradient of this neurotransmitter as it enters the colon with the lowest gut concentration being found at the distal colon (196, 270). This is the colonization niche of EHEC therefore, it is likely that this pathogen senses the relatively high concentrations of serotonin in other parts of the gut as a cue that it hasn't reached its destination. Through CpxA, EHEC senses these serotonin concentrations to inhibit the LEE PI, and consequently AE lesions and overall virulence. Once it reaches the colon, EHEC still needs to get from the colon's lumen to through the mucosa to

reach the epithelium where it would form AE lesions. In the lumen EHEC senses, through CpxA, the relatively high levels of indole produced by the dense microflora population, as well as serotonin which is still present albeit at lower concentrations in the lumen consequently maintaining the inhibition of the LEE PI in this GI compartment, where LEE expression is an undesirable expenditure of energy. As EHEC swims closer to the colon epithelium where there is no gut flora to produce indole, and there is barely any serotonin, the inhibition on CpxA autophosphorylation is lifted, and the pathogen is able to attach and eventually form the characteristic AE lesions. Bansal *et al* suggested that SdiA, a LuxR homologue, mediated indole sensing (170). However their *sdiA* mutant was still responsive to indole in all phenotypic tests they performed indicating that although SdiA may play a role in biofilm formation and motility which are both regulated by indole, it is probably not the sensor/mediator of this small molecule. Here we show that the *cpxA* mutant is unable to respond to the addition of either indole, serotonin or both. The serotonin receptor antagonist, ritanserin, is able to rescue serotonin-dependent LEE gene inhibition, which substantiates the specifity of this neurotransmitter. Furthermore, we show that both these tryptophan derivatives inhibit the autophosphorylation of CpxA thus confirming CpxA as their sensor.

CpxRA two component system has been shown to be important for the regulation of virulence genes in many bacterial pathogens including EPEC (315), *Shigella* (201), UPEC(124) and *Legionella pneumophila* (84). Numerous two component systems including QseBC, PhoPQ and GacSA have been linked to virulence (99). The histidine kinases of these systems have been targeted for antivirulent therapy (63, 99, 243). LED209 and walkmycin C target epinephrine

dependent QseC and WalK autophosphorylation respectively. Identifying agonists of serotonin and indole that inhibit CpxA autophosphorylation and the consequent virulence gene activation may lead to the identification of more lead targets for antivirulent therapy. Since this histidine kinase is found in many pathogens both enteric and non-enteric, these antimicrobials can be effectively used for multi-pathogen treatment.

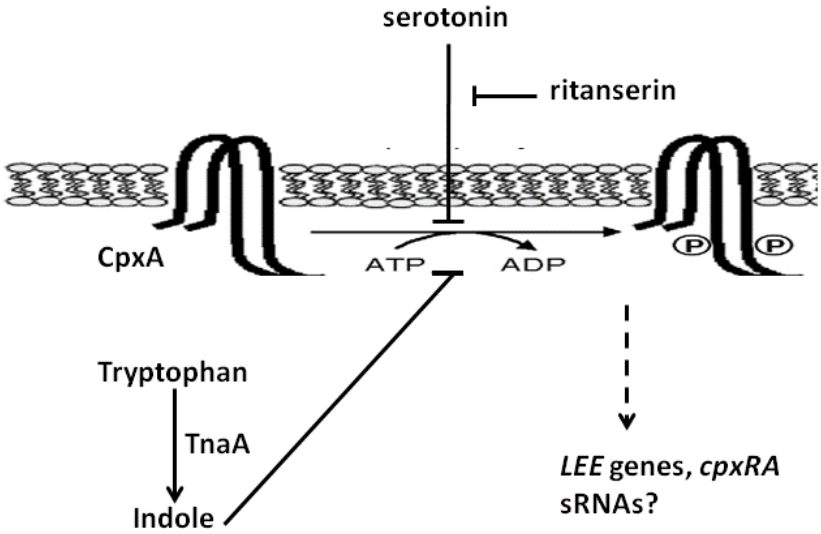


Fig.7.8. Summary of serotonin and indole signaling in EHEC. (modified from Gotoh *et al* (99))

CHAPTER EIGHT

DISCUSSION AND FUTURE DIRECTIONS

The underlying concept behind this dissertation was to describe in-depth the studies that were performed to identify and characterize elements of the mammalian host environment that influence the virulence of pathogens. In particular, we investigated the ability of small molecules to differentially regulate the pathogenesis of EHEC, a human pathogen that is responsible for major outbreaks of hemorrhagic colitis and the hemolytic uremic syndrome. Similar to many other disease causing bacteria, the enteric pathogen EHEC requires very complex regulation to ensure energy efficient spatiotemporal expression of colonization factors and virulence genes. The main virulence factors in EHEC are the ability to form attaching and effacing (AE) lesions on epithelial cells, Shiga toxin production and motility. AE lesions require the expression of genes contained within the locus of enterocyte effacement (LEE) pathogenicity island (PI). The LEE PI encodes for the structural and regulatory components of the type three secretion system (T3SS) as well as some of the effectors that it translocates. Through the T3SS, LEE encoded and non-LEE encoded effectors are translocated into host cells where they hijack the host cellular machinery culminating in the characteristic AE lesions as well as general virulence.

Before this work, it was known that EHEC responded to the mammalian adrenergic hormone epinephrine by increasing the expression of all three virulence traits (41, 243, 284). It

was also shown that two bacterial kinases, QseC and QseE, responded to epinephrine by increasing their autophosphorylation (41, 247). Although previous work had linked the epinephrine dependent increase in QseC autophosphorylation to epinephrine dependent virulence gene up-regulation, the same had not been done for QseE. Furthermore, the fact that *in vivo* work in infant rabbits had shown that a *qseC* mutant was attenuated for virulence while a *qseE* mutant had increased virulence, suggested a more complex mechanism of regulation that needed to be mapped. We theorized that first, QseC and QseE are the only bacterial sensors of epinephrine, and secondly that these sensor kinases regulate virulence genes in a converse manner. Therefore to test these theories, we set out to map the QseC-QseE-epinephrine signaling cascade.

In chapter four we were able to confirm that QseC is an activator of the LEE genes and the non-LEE effector *nleA,* and showed for the first time that QseE is an inhibitor of these genes. Using a q*seCqseE* double mutant we were also able to show that these two kinases are the only sensors of epinephrine in EHEC. We showed that even though both these kinases regulated the LEE genes and *nleA,* epinephrine dependent regulation of the LEE PI is mostly dependent on QseC while epinephrine dependent regulation of *nleA* is mostly dependent on QseE. We were also able to show for the first time that epinephrine increases the ability of EHEC to form AE lesions on HeLa cells. Previous work had suggested that QseC's regulation of the LEE PI was through the phosphorylation of one of its response regulators (RR), KdpE, and the subsequent activation of *ler*, the master regulator of the LEE genes, by this RR (120). We confirmed this in chapter five where we showed that KdpE bound to the promoter region of the

ler gene, and consequently activated the transcription of not only *ler* but also the other LEE genes. We also showed that the regulation of the LEE PI and *nleA* by QseE is not through its cognate RR QseF but through the QseE's ability to inhibit the transcription of *rcsB,* a RR that activates the LEE genes as well as *nleA.*

Although we were able to map the intricate regulatory cascade involving epinephrine and the two kinases QseC and QseE, we did not fully describe the role of RcsB in this cascade. The RcsBC two component system has been shown to be important in the virulence of other pathogens such as *S.enterica* and *Erwinia amylovora* (87, 323). RcsB has also recently been shown to regulate the type six secretion system of *S.typhi* (325). Hence, it would be interesting to investigate the extent of RcsB dependent regulation in EHEC. For example, we showed that QseE decreased the transcription of *rcsB* but it is also possible that QseE may affect the phosphorylation state of this RR. Therefore to test this hypothesis we propose that *in vitro* phosphorylation/dephosphorylation studies be performed between QseE and RcsB with QseE's cognate RR QseF and RcsB's cognate kinase RcsC as controls. It would also be interesting to test whether the activation of the LEE genes and *nleA* by RcsB is through the direct binding of this DNA binding RR to the promoter region of these genes. Other targets of QseE such as the non-LEE effector *espFu* could also be tested for direct regulation by RcsB. Some preliminary EMSA results indicate that RcsB is able to bind to the promoter regions of *ler* (Fig.8.1). Transcriptome analyses of an *rcsB* mutant could also identify other virulence targets regulated by this RR. Another aspect of RcsB regulation that has not been explored is whether RcsB's effect on the translation of its targets follows the transcription pattern described in chapter four. RcsB has

been shown to regulate the sRNA *rprA,* which is important for the translation of the sigma factor RpoS (185, 186). It would therefore be interesting to investigate the effect that RcsB has on the protein levels of the *LEE4's* EspA and NleA.

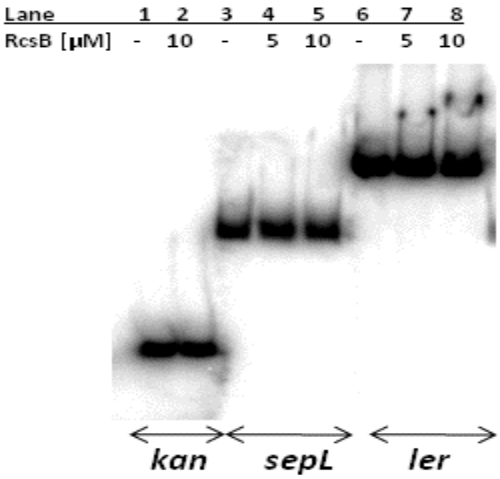


Fig.8.1. RcsB directly interacts with the *ler* promoter region. Increasing amounts of His purified recombinant RcsB was used to shift 2ng of the indicated radiolabeled DNA probe. A radiolabeled *kan* DNA probe was used as a negative control. RcsB can bind to the *ler* but not the *sepL* promoter.

In chapter five and six we characterized carbon source dependent regulation of virulence factors. Before this work, it was known that colonization by EHEC *in vivo* was favored by gluconeogenic substrates, and inhibited by glycolytic ones (36, 71, 136, 200). However, it was not known if these substrates also differentially regulated virulence factors. In order to test the hypothesis that this was the case, we assessed the effect of both gluconeogenic and glycolytic substrates on the transcription of *ler,* the master regulator of the LEE PI. We showed that *ler* transcription was activated by gluconeogenic substrates like succinate as well low

glucose, and inhibited by glycolytic ones such as high glucose congruent with the colonization data. We then focused on the interesting pattern of regulation between low and high glucose and showed that two transcription factors (TF), KdpE and Cra, were responsible for this regulation. This was the first report of Cra, a metabolite dependent TF, being an activator of *ler* and consequently the LEE PI. We went on to show that deletion of *cra* led to significantly decreased AE lesion formation. We also showed that both KdpE and Cra bound to the promoter region of *ler* in order to activate transcription of this gene. Furthermore, we showed that this regulation was metabolite (fructose 1 phosphate and fructose 1,6 bisphosphate) dependent for Cra. Using EMSAs and Far Western blots, we were able to show that these two TF interacted *in vitro.* A double deletion of *cra* and *kdpE* resulted in almost complete loss AE lesion formation. A microarray analysis identified additional targets of Cra and KdpE. However, we also used "*in silico*" analyses to pinpoint other targets that might have been missed by the microarray, an approach that proved better at providing information on global gene regulation, but lacked the sensitivity to identify all targets. Using a bioinformatics program, Virtual Footprint to find other targets of Cra, we identified known and putative virulence factors that were predicted to be regulated by Cra, including some that were missed in the microarray analyses. We showed that these predicted targets were indeed regulated by Cra, and that some of them were also regulated by KdpE. This regulation was shown to be due to direct binding by these two TFs.

These data provided evidence that Cra and KdpE interact with each other in order to regulate one or more of the targets they have in common. To further confirm this, we propose that *in vitro* transcription assays be performed. Pull down assays should also be performed to

test the two proteins' interaction *in vivo.* The Virtual Footprint, qRT-PCRs and EMSAs provided several targets including O-island genes that have not been characterized. Therefore a possible future direction to pursue would be to characterize these putative virulence factors. Preliminary BLAST analysis and literature review (Table 6.2) showed that some of these targets have homology to known virulence factors of other pathogens while others have been predicted to be secreted effectors.

In chapter seven, we characterized the signaling cascade of the tryptophan derivative serotonin. Ninety percent of the serotonin in the human body is found in the gut, which makes it a plausible signal that an enteric bacteria like EHEC may recognize. Before this work, this mammalian hormone had been shown to increase the virulence of the amoeba *E. histolytica* (191). It had also been suggested that since another tryptophan derivative, indole, had been linked to EHEC virulence regulation, other derivatives and indole based compounds such as serotonin, melatonin and indole-3-acetic acid could also influence EHEC pathogenesis (113). These reports led us to hypothesize that serotonin could regulate one or more of the main EHEC virulence factors. Preliminary analysis showed that although serotonin did not affect the regulation of Shiga toxin or motility, it did inhibit the transcription of the LEE PI. We also confirmed that indole regulates the LEE genes. However, unlike what was previously alluded to by Hirakawa *et al.*, we found that indole did not increase the activation of the LEE gene *espA* but actually decreased it (113). Furthermore, we showed that combining the two signals had an additive effect on the inhibition of the LEE PI. Through genetic and biochemical analyses we identified the bacterial kinase CpxA as the sensor of both indole and serotonin. We showed that

the mechanism of serotonin regulation involved the inhibition of *cpxA* transcription, as well as the inhibition of CpxA autophosphorylation. Since *tnaA* is required for the production of indole in bacteria (279, 322), we decided to delete it to prevent endogenous indole production distorting the data. *C. rodentium,* which is used as a model for EHEC disease in mouse infections, lacks the *tnaA* gene. Therefore, performing *in vitro* assays with *tnaA* deleted in EHEC would allow for better comparison with future *in vivo* assays performed with *C. rodentium*. Double deletion of both *cpxA* and *tnaA* led to a LEE PI transcription phenotype similar to the single *cpxA* mutant, and an indole production phenotype similar to the single t*naA* mutant. We also showed that AE lesion formation was decreased in the *ΔcpxA* and *ΔtnaAΔcpxA* mutants. Although we were able to characterize some major aspects of the serotonin regulation, a lot of work remains to be done in order to satisfactorily map the serotonin regulatory cascade. In chapter 7, we investigated at the effect of *cpxA* and *tnaA* on LEE gene translation as well as on AE lesion but we did not assess the effect of serotonin and/or indole on these two phenomena. Preliminary EspA secreted protein assays showed a protein levels' pattern that was different from the signal dependent transcription data we had (Fig.8.2). This disparity suggests that there is signal-dependent post-transcriptional modification (PTM). The mechanism for this PTM, which may involve sRNAs, needs to be further characterized.

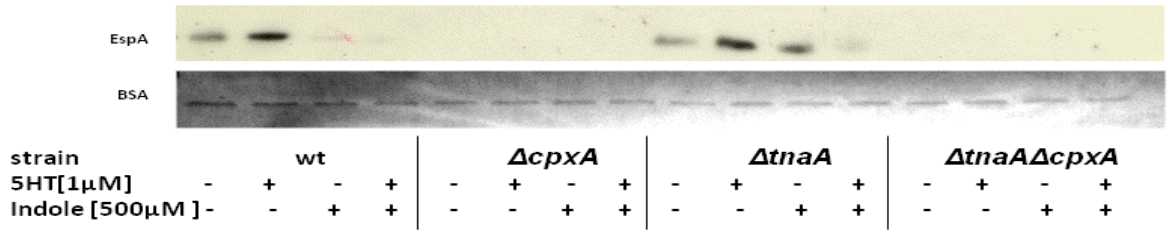


Fig.8.2. Effect of serotonin and indole on EspA protein levels. Secreted proteins' Western for wt, *ΔcpxA, ΔtnaA* and *ΔtnaAΔcpxA* in the absence or presence of 1μM 5HT and/or 500μM indole. Samples were probed with antisera against EspA. BSA was used as the loading control.

Another important set of experiments that need to be done are the *in vivo* (mouse) studies. We plan to use C57BL/6J mice, either wt strains or *tph1* knockout (KO) strains. The gene *tph1* encodes for the enzyme tryptophan hydroxylase-1 (TPH1) which is involved in the rate limiting step of serotonin synthesis from tryptophan (46, 266). As a result, the *tph1* KO strain have no detectable serotonin in the gut, particularly in the colon (266). We will perform single and competition infections on wt and *tph1* KO mice using either wt and/or *cpxA* deletion strains of *C. rodentium.* We will look at survival, bacterial shedding, histopathology and bacterial loads in different organs. In order to ensure that *C. rodentium* senses serotonin in the similar manner to EHEC, we constructed a *C. rodentium ΔcpxA* and tested its ability to regulate its LEE PI on exposure to this signal (Fig.8.3). The preliminary data shows that *C. rodentium* senses serotonin in a CpxA dependent manner. *C. rodentium* wt and *ΔcpxA* will be used in the future *in vivo* work.

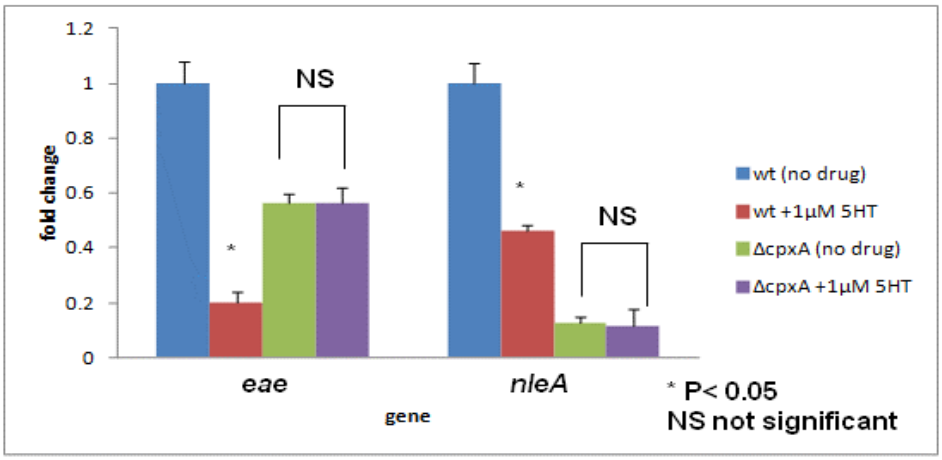


Fig.8.3 CpxA is the sensor of serotonin in *C.rodentium*. qRT-PCR evaluating the transcription of *eae a*nd *nleA* in *C.rodentium* wt and *ΔcpxA* grown in the absence and presence of 1µM serotonin. Error bars indicate the standard deviations of the $\Delta\Delta C_T$ values. The mRNA levels of endogenous *rpoA* were used to normalize the C_T values. *P<0.05 according to student t test.

In addition to the *in vivo* work, we need to map the RR-target component of the serotonin-signaling cascade. CpxA is part of the CpxAR two-component system; CpxA is the kinase and CpxR is the RR. We theorize that the serotonin-signaling cascade, which involves CpxA, also utilizes CpxR in its regulation of downstream targets. CpxR has been shown to be involved in the regulation of the LEE genes in enteropathogenic *E. coli* (EPEC) (184). As a first step towards characterizing CpxR regulation of EHEC, we have constructed a *cpxR* mutant. We propose to perform microarrays on this mutant, as well as the *cpxA* mutant in order to study the global gene regulation by these genes. This will also provide a source of potential new targets, which can be confirmed by qRT-PCR and EMSAs. Using the Virtual Footprint software we were able to obtain preliminary data that identified several potential targets of CpxR

including *cpxR* itself as well as two O-island genes *Z0639* and *Z0640;* we plan confirm these targets using qRT-PCRs and EMSAs.

The biochemical assays we performed involving the influence of serotonin and indole on the autophosphorylation of CpxA suggested that these small molecules interact with the kinase. Liposome studies involving QseC and radiolabeled epinephrine confirmed that the epinephrine dependent increase in QseC autophosphorylation is due to the direct interaction of the protein and this hormone (41). Therefore, as an additional future direction, we plan to identify the binding site of serotonin and/or indole using radiolabeled versions of these small molecules as well as antibodies to them. We will also assess the potential of tryptophan, the precursor of both serotonin and indole, to act as a signal for EHEC. Studies using *Caenorhabditis elegans* have shown that tryptophan spiking of the EPEC lawn is required in order to observe EPEC-dependent killing of *C.elegans* (12, 13). Therefore, it is possible that this amino acid has the ability to influence EHEC pathogenesis as well. Finally, it would be interesting to test whether other bacterial pathogens sense tryptophan and its derivatives. Preliminary data showed that serotonin did not significantly affect *S. enterica's* ability to invade macrophages or HeLa cells (data not shown). However both serotonin and indole affected the transcription of UPEC's *csgE* gene, which is involved in curli and biofilm production (Fig. 8.4), mechanisms that are both coincidentally regulated by CpxR (137).

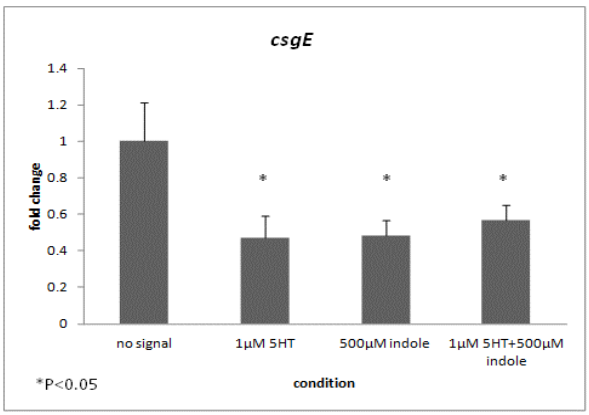


Fig.8.4. Inhibitory effect of serotonin and indole on UPEC curli . (A) qRT-PCR evaluating the transcription of *csgE* in wt UPEC grown in the absence or presence of 1µM 5HT and/or 500µM indole. The mRNA levels were expressed as fold changes over wt mRNA levels. For all the samples, *rpoA* mRNA levels were used as an internal control to normalize the output C_T values in order to take into account variation in bacteria numbers. *P<0.05 according to student t test.

In conclusion, in this thesis we have described small molecules in the host environment that EHEC and other bacterial pathogens use as signals to either augment their virulence or diminish it (Fig.8.5). We showed that bacterial histidine kinases are important sensors of mammalian hormones and bacterial produced autoinducers (AIs). They use these signals to differentially regulate virulence factors. QseC senses epinephrine, norepinephrine and AI-3 to increase its activation of the LEE PI via KdpE, the motility genes via QseB and Shiga toxin via QseF. QseE on the other hand senses epinephrine, sulfates and phosphates to inhibit the LEE genes and non-LEE effectors via RcsB. CpxA senses indole and serotonin to inhibit the LEE PI and consequently EHEC virulence. We also showed that pathogens have co-opted non-pathogenic regulatory systems to regulate virulence. EHEC uses two transcription factors Cra and KdpE to activate virulence genes. Furthermore, we showed that carbon nutrition plays a

role in virulence regulation. Carbon substrates influence EHEC virulence differently depending on whether they are gluconeogenic or glycolytic. Understanding how mammalian hormones, bacteria AIs and the host environment in general affects the virulence of EHEC and other bacterial pathogens may aid in the design of novel antivirulent therapies and better pathogen prevention and control technologies.

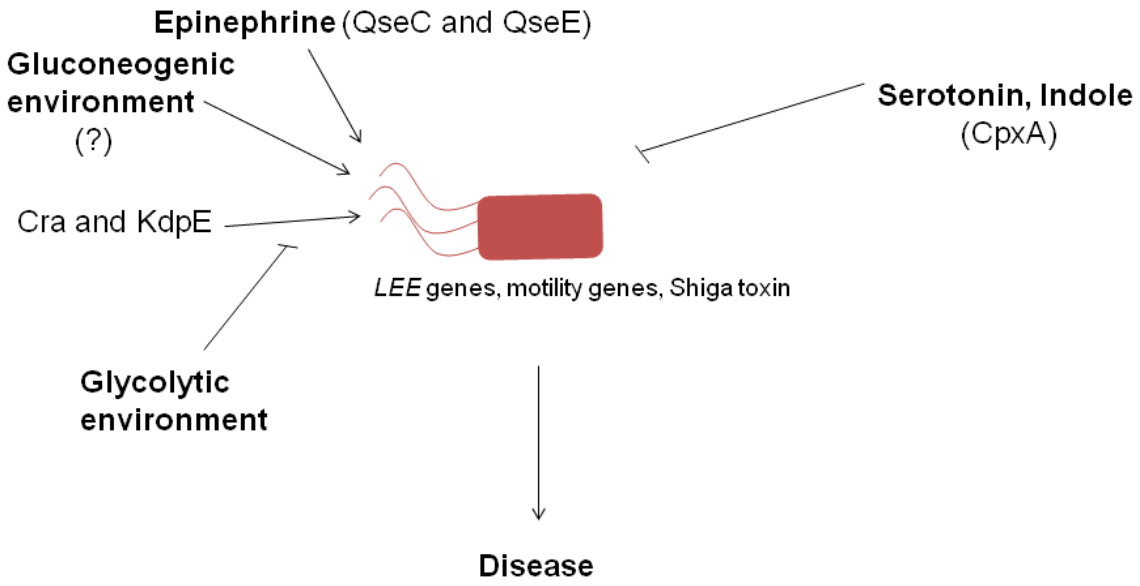


Fig.8.5. Summary of the effect of small molecules in the host environment on EHEC virulence.

ACKNOWLEDGEMENTS

First, I would like to thank my mentor, Dr. Vanessa Sperandio, for giving me the opportunity to train in her lab. Her guidance, support and endless patience made me appreciate research and science in general. Because of her, I feel confident and well prepared for a successful career in science. I would also like to thank my co-workers from the Sperandio lab (past and present): Regan Russell, David Rasko, Nicola Reading, David Hughes, Ben Habdas, Dasha Terekhova, Cristiano Moreira, Christopher Parker, Melissa Kendall, Meredith Curtis, Alline Pacheco, Y Nguyen, Charley Gruber and Reed Pifer. I appreciate you answering all my incessant questions, putting up with my Mountain Dew- Sour Patch induced frenzies, and for all the "interesting" groundbreaking lab conversations.

I would like to thank the Division of Basic Sciences and the Molecular Microbiology Program, and especially my committee members (past and present): Dr. Kim Orth, Dr. Wade Winkler, Dr. Dave Hendrixson, Dr. Ron Taussig and Dr. Michael Shiloh. I would also like to thank Dr. Nancy Street for her career advice and mentoring.

I thank my family for always believing in me even when they thought I was nuts to want to do this. Thanks to all my friends here in Dallas and scattered all over the world; you allowed me to live vicariously through you when I had to spend countless hours in the lab. Finally, a special thank you to my sweetie Mark Barfield; you made sacrifices so I could fulfill my dream.

Thank you all for your encouragement, support, hugs and advice. I could not have done it without you!

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