Characterization of complex regulatory circuits in enterohemorrhagic *Escherichia coli* O157:H7

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List of abbreviations

AE lesions: attaching and effacing lesions

CFU: Colony forming units

DMEM: Dulbecco's modified Eagle's medium

DPI: days post infection

EA: ethanolamine

EHEC: Enterohemorrhagic *E. coli*

EMSA: electrophoretic mobility shift assay

FAS: Fluorescein acting staining

GI: gastrointestinal

H: hours

HUS: hemolytic uremic syndrome

LB: Luria broth

LEE: locus of enterocyte effacement

Log: logarithmic

MBP: maltose binding protein

PE: phosphatidylethanolamine

T3SS: type III secretion system

Tir: translocated intimin receptor

WT: wild type

Abstract

Pathogens have complex networks of overlapping and integrated signaling pathways that provide highly adapted responses to different external stimuli. Pathogens utilize external stimuli to sense the host environment and regulate the expression of virulence traits according to location. For gastrointestinal pathogens, this equates to pathogens sensing the host gastrointestinal tract and colonizing. Previous studies have demonstrated that the signaling molecule ethanolamine (EA) plays an extensive role in the regulation of virulence traits in EHEC. We hypothesized that EA is involved in the regulation of virulence traits through multiple signaling cascades. We determined that the EA-utilization operon encoded regulatory protein EutR directly binds to the promoter regions of *ler*, the master regulator of the locus of enterocyte effacement (LEE), to regulate transcription. We further explored signaling cascades regulating the LEE by investigating the function of previously uncharacterized open-reading frame, *etrB*. We determined that expression of EtrB is directly regulated by QseA, and that EtrB functions as a direct regulator of the LEE and influences the expression of other virulence traits, including non-LEE encoded effectors and fimbrial locus 11. Previous studies demonstrated that EA promotes the expression of fimbrial genes in EHEC. Data from our lab suggests that the fimbrial loci Erf1 and Erf2, while not important for mediating early attachment events, are necessary for the expression of virulence traits important for later stages of infection, including AE lesion formation and Shiga toxin expression. We determined that this phenotype of fimbriae influencing AE lesion formation is not conferred to all fimbrial loci as a deletion in fimbrial locus 3

has no effect on AE lesion formation to HeLa cells. Furthermore, we determined that expression of Erf1 or Erf2 as surface structures is not necessary to modulate LEE expression and AE lesion formation. Overall, this work has demonstrated how pathogens, such as EHEC, utilize widely conserved transcriptional regulators to coordinate virulence gene expression in response to the host environment. Many pathogens encode the EA-utilization operon, the ETT2, and fimbriae. Therefore, our studies may describe a general mechanism used by pathogens to coordinate the expression of virulence traits.

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Chapter One: Introduction to enterohemorrhagic *Escherichia coli* O157:H7

Part of this chapter has been adapted from "Commensal 'trail of breadcrumbs' provide pathogens with a map to the intestinal landscape"

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Escherichia coli

E. coli is a gram-negative, rod-shaped, facultative anaerobic bacterium that colonizes the gastrointestinal (GI) tract of mammals. It is a very diverse group that consists of both commensal and pathogenic *E. coli*. Commensal *E. coli* exist as part of the normal human flora and are in a mutually beneficial relationship; the human host provides *E. coli* with a continuous food source and colonization niche while *E. coli* functions to break down food products, producing useful nutrient vitamins and protecting the host from colonization by pathogens [1-3]. Commensal *E. coli* is one of the most abundant facultative anaerobes and colonizes the mucosal layer of the GI tract. However, some *E. coli* have acquired various virulence attributes that confer the ability to cause disease in humans. These *E. coli* strains can be categorized into pathotypes, which all contain similar virulence factors to cause similar diseases. The eight well-characterized *E. coli* pathotypes are uropathogenic, meningitis-associated, enteropathogenic, enterohemorrhagic, enterotoxigenic, enteroaggregative, enteroinvasive, and diffusely adherent *E. coli* [4]. The clinical symptoms manifest as urinary tract infections and sepsis/meningitis respectively for the first two pathotypes, and diarrhea for the last six pathotypes. The various pathotypes of *E. coli* tend to be clonal groups with shared serotypes that have similar O antigen (lipopolysacharride) and H antigen (flagellar) [5]. One similarity among all *E. coli* pathotypes is that they must colonize a specific niche to multiply and cause disease [4]. Niche specificity is achieved through differential expression of virulence traits at specific locations. By restricting the expression of virulence

factors to anatomically relevant sites, pathogenic *E. coli* gain a fitness advantage during colonization.

Enterohemorrhagic *Escherichia coli* O157:H7

Enterohemorrhagic *E. coli* O157:H7 (EHEC) is a major cause of severe foodborne disease and mortality worldwide. In the United States EHEC causes annual outbreaks resulting in 25-45% of infected people requiring hospitalization [6]. Cattle are the primary reservoir for EHEC and are asymptomatically colonized [7-9], which makes the identification of EHEC-infected cattle difficult. Most cattle are colonized by EHEC at low levels, but some cattle, named super-shedders, excrete much higher numbers of bacteria compared to other cattle [10]. Super-shedder cattle are estimated to consist of less than 10% of the total population of cattle, but release up to 99% of EHEC found in the environment [10]. EHEC survives in bovine feces for weeks leading to the contamination of food products that come in contact with feces from infected cattle [11]. The first outbreak in the United States was associated with the consumption of undercooked hamburgers at a fast-food chain restaurant [12]. Incidences of EHEC infection are currently often associated with the consumption of contaminated foods such as raw or undercooked meats, unpasteurized milk, fruits, and vegetables [7, 12].

EHEC infection is characterized by bloody diarrhea and hemorrhagic colitis. A major complication that arises in up to 20% of EHEC-infected patients is the development of hemolytic uremic syndrome (HUS) [13, 14]. HUS may present as renal failure, thrombocytopenia, and hemolytic uremia and has a fatality rate of 3-

5%. Additional complications that develop in survivors from HUS include neurological disorders and mild chronic renal disease, which affect about 25% and 50% of survivors respectively [14]. Renal dialysis is required for 50% of patients who develop HUS [15], thereby increasing the hospital stay for patients and the financial burden associated with EHEC infections. These complications are caused by Shiga toxin release during EHEC infection. Shiga toxin is encoded on a lambdoid prophage [16] and is highly expressed during an EHEC SOS response [17], which is discussed in more detail in the following section. The use of antibiotics and antimotility agents to treat EHEC infection induces an SOS response in EHEC, leading to increased production and release of Shiga toxin [18, 19]. Thus, the use of antibiotic and antimotility agents is contraindicated in order to decrease the risk of patients developing HUS [18, 20]. Consequently, the combined EHEC economic costs exceed 400 million dollars annually [21]. No treatment options are currently available for EHEC infections besides supportive care [22].

The National Institutes of Health classifies EHEC as a Category B pathogen, the second highest priority agent. This classification is due to the morbidity and mortality associated with EHEC infection, and because of the ease of dissemination. The infectious dose for EHEC is very low with ingestion of 50-100 colony-forming units (CFUs) sufficient to cause disease [23, 24]. Together the low infectious dose, the severe clinical manifestations of disease, and the lack of treatment options make EHEC a significant public health concern.

Virulence factors

The locus of enterocyte effacement (LEE) and Shiga toxin are two of the defining virulence traits of EHEC and are crucial for classification of the EHEC pathotype [4]. EHEC also possesses other, less well-defined virulence traits, such as flagella, fimbriae, and a second cryptic T3SS. Virulence traits are regulated by transcription factors.

Transcription Factors

E. coli gene expression is regulated by a network of overlapping and integrated signaling pathways. Transcription factors have an important role in this network by modulating gene expression in response to environmental and cellular stimuli. Transcription factors can be categorized into families of related proteins based on conserved motifs contained in the protein sequences [25-28].

The AraC/XylS family of transcription factors is one of the most common positive regulators [29, 30]. AraC/XylS regulators contain a helix-turn-helix DNA binding domain and typically activate transcription, except in rare cases were they act to repress transcription [31, 32]. These regulators usually respond to a ligand and directly bind target DNA to promote expression [33-35]. Some AraC/XylS regulators promote gene expression by antagonizing H-NS repression [36-38]. EutR of the EA utilization operon is an AraC/XylS-type regulator.

The LysR-type transcriptional regulator family of proteins is one of the most abundant types of bacterial DNA-binding proteins [39-41]. These regulators have a conserved DNA binding helix-turn-helix domain [42] and they can activate or

repress transcription through direct or indirect interactions [30]. LysR regulators typically repress their own expression by binding to their promoter region [30]. LysR-type regulators are regarded as global transcriptional regulators that activate or repress target genes [42-45]. QseA is a LysR-type protein in EHEC that acts as a global regulator [46].

Transcriptional regulators in the NarL-family directly bind DNA to regulate transcription [47]. These regulators contain a helix-turn-helix motif that binds DNA to activate or repress expression [47]. Proteins in NarL-family can function independently or as part of a two-component system [48, 49]. The regulatory protein EtrB, previously known as YgeK, is a NarL-like protein.

A common feature of the AraC/XylS, LysR, and NarL families is the DNA binding domain. The helix-turn-helix portion of the transcription factor directly binds to DNA to modulate gene expression [50]. These transcription factors can promote gene expression by relieving existing repression (Figure 1.1A) [51, 52], binding upstream of the promoter to help recruit RNA polymerase (Figure 1.1B) [52-54], or by binding adjacent to the promoter and interacting with RNA polymerase (Figure 1.1C) [54, 55]. A transcription factor can activate gene expression through multiple mechanisms, such as by relieving existing repression of one gene and helping recruite RNA polymerase to another separate gene (Figure 1.1) [52, 54, 55]. Transcription factors can repress gene expression by inhibiting binding of RNA polymerase (Figure 1.1D) [56]. The mechanism of EutR and EtrB regulation of target genes has not been demonstrated. A previous study suggests

that QseA inhibits its own transcription by inhibiting binding of RNA polymerase to -35 promoter element [46].

LEE

The main pathogenicity island in EHEC is the LEE [57]. Pathogenicity islands are horizontally acquired genomic islands that play a pivotal role in pathogen virulence [58]. The LEE is composed of five operons, *LEE1-LEE5* encoding most of the genes involved in attaching and effacing (AE) lesion formation (Figure 1.2). The first gene in *LEE1, ler*, encodes the LEE-encoded regulator that activates transcription of all five LEE operons [59, 60]. The LEE also encodes a type three secretion system (T3SS), secreted effectors, the adhesin intimin, and the translocated intimin receptor, Tir [61, 62]. The T3SS injects LEE- and non-LEE-encoded effectors directly into the host cell to mediate colonization, AE lesion formation, and further alterations to host epithelial cells including tight junction disruption and mitochondrial damage [63-70]. EHEC infection is characterized by the formation of AE lesions on the intestinal epithelium. AE lesions are intimate, receptor-mediated bacterial attachment to epithelial cells causing effacement of the microvilli and host actin reorganization, resulting in the presentation of EHEC on an actin-rich pedestal-like structure [71, 72].

The translocation of effector proteins into host cells is mediated by the LEE-encoded T3SS. The T3SS is composed of products from 20 genes that make up the multicomponent organelle, which form the basal body (protein rings spanning both the inner and outer bacterial membrane), a needle-like structure extending from the

bacterial cell to the host cell, and the translocation pore on the host cell [73]. The first step in formation of the T3SS is *sec*-dependent export of the membrane-bound components [74]. These components form the basic structure of the basal body otherwise known as the needle base. Following formation of the needle base, cytosolic components are added. This machinery is then used to secrete components to from the needle structure and translocation pore. Exportation or secretion of proteins through the T3SS is thought to be dependent on cytoplasmic ATPases which energize the T3SS [75]. A unique feature of EHEC T3SS is the formation of a filamentous extension from the needle component to the translocation pore composed of EspA filament [76-79]. EspA also plays an important role in transient adhesion between EHEC and the host cell, allowing effector protein translocation and the formation of intimate Tir-mediate attachment [77, 78, 80].

Intimin is an outer membrane adhesion molecule responsible for adhering to the host epithelium. Recent evidence suggests that intimin binds the host-encoded receptors β_1 integrin [81] and nucleolin [82, 83]; however, the binding affinity for these non-cognate receptors is unknown. The primary binding component of intimin is the LEE-encoded Tir (translocated intimin receptor). Both intimin and Tir form multimers and bind leading to a signaling cascade that drives actin reorganization and AE lesion formation [84-86]. A non-LEE encoded effector, EspFu, has Nck-activity allowing it to recruit and bind neuronal Wiskott-Aldrich syndrome protein (N-WASP) to activate actin polymerization in a Nck-independent manner [87]. The result of this signaling cascade is the accumulation of actin at the site of bacterial attachment, forming pedestal-like structures underneath adherent EHEC.

EHEC encodes many effector proteins located outside the LEE, termed non-LEE-encoded effectors. A number of these effectors, including NleA, NleB, NleE and NleF, are important for EHEC survival and colonization [69, 88, 89]. NleA, also termed EspI, is a secreted protein [90] that colocalizes with the Golgi apparatus and affects protein trafficking and secretion [70, 91]. NleA is regulated by Ler and H-NS [90, 92, 93], and by a variety of other signals including quorum-sensing [94] and DNA-damage [95]. Many studies utilize NleA as a measurement of non-LEE-encoded effector protein expression because NleA has a known role in EHEC pathogenesis, and influences disease in the AE lesion forming *Citrobacter rodentium*, a commonly used mouse model for EHEC infection [70].

Expression of the LEE is regulated by a complex network of regulator proteins encoded both within the LEE and outside of the LEE (Figure 1.3). The first gene encoded in *LEE1* is *ler* (LEE-encoded regulator), which acts as a global regulator of LEE expression by promoting expression of *LEE2-5* [59, 96]. Ler also regulates the expression of genes encoded outside of the LEE, such as *nleA* and *lpf* [90, 96, 97]. Expression of the LEE is silenced by H-NS (histone-like nucleoid-structuring protein) repression, and Ler counteracts H-NS repression by displacing H-NS to activate expression of the LEE [59, 98, 99]. The LEE also encodes GrlA (global regulator of LEE activator) and GrlR (global regulator of LEE repressor) [100]. GrlA functions to activate expression of the LEE by increasing expression of *ler* and *grlRA*, while GrlR negatively regulates LEE expression by repressing GrlA activity post-translationally through direct interaction [101-103]. Regulation of GrlR

is at the post-translation level through protease degradation, acting to increase *ler* expression by relieving repression of GrlA [104].

Many regulators encoded outside of the LEE regulate expression of the LEE through regulation of *ler*. Ler is positively regulated by IHF [105], QseA [106, 107], QseEF [108], EutR [109, 110], PchA and PchB [111], NsrR [112], QseC and KdpE/Cra [94, 113, 114], Hfq [115], RpoS/DsrA [116], ppGpp [117], RgdR [118], EtrB [119], and GrvA [120]. Expression of *ler* is negatively regulated by GadX [112], FusKR [121], SdiA [122, 123], EivF [124], EtrA [124], and Hha [125]. The LEE operons can also be regulated independent of Ler. GadE represses expression of LEE4/5 [112] and YhiEF represses expression of LEE2/4 [126]. The complex regulation of the LEE by many regulator proteins highlights the importance of this pathogenicity island in EHEC virulence.

Shiga toxin

Shiga toxin is a major virulence factor in EHEC causing renal damage and hemolytic uremic syndrome (HUS) in patients as well as contributing to the development of hemorrhagic colitis [13, 127]. Shiga toxin was first identified in *Shigella dysenteriae* over 100 years ago by Kiyoshi Shiga and was originally called Verotoxin due to its cytotoxicity against Vero cells [128]. In 1983, Shiga toxin was reported in EHEC and provided a link between Shiga toxin and HUS development in EHEC-infected patients [129, 130]. Shiga toxin is an AB_5 toxin, consisting of a catalytic A subunit bound to a pentamer of B subunits [131, 132]. The B subunits are responsible for binding to target cells via the globotriaosylceramide (Gb3) receptor

and the A subunit is responsible for toxin activity [131, 133-135]. Gb3 is highly expressed by epithelial cells, particularly kidney epithelial cells, which is why the kidneys are highly susceptible to intoxication [134, 135]. Gbs3 is also expressed in the brain and Shiga toxin can cause neurological complications such as cognitive impairments, seizures, and coma [136, 137]. Shiga toxin accesses the kidneys through systemic circulation after absorption by the epithelium, which is aided by the GI damage due to EHEC infection [138, 139]. After binding to Gb3, Shiga toxin is endocytosed and inhibits protein synthesis through the N-glycosidase activity of the catalytic subunit A by cleaving an adenosine residue from the 28S ribosomal RNA [140, 141]. Inhibition of protein synthesis causes apoptosis in target cells.

EHEC is associated with two main groups of Shiga toxin, Stx1 and Stx2. While Stx1 and Stx2 are similar in structure and enzymatic activity, they only share 55% homology by the amino acid level [142, 143]. Stx1 is almost identical to Shiga toxin produced by *Shigella*, and Stx2 is more commonly associated with increased disease severity in humans including increased incidence of hemorrhagic colitis and HUS [142, 144]. Different clinically isolated strains of EHEC have different combinations of Stx1 and Stx2. Previous studies suggest that EHEC strains encoding only Stx2 are more likely to cause severe disease than isolates containing only Stx1 or both Stx1 and Stx2 [145]. The EHEC strain EDL933 isolated in 1982 from undercooked fast food hamburgers, encodes both *stx1* and *stx2* [12, 146-148] and the EHEC strain 86-24, isolated in 1986 from a patient, encodes only *stx2* [149]. EHEC strain 86-24 is more virulent than EDL933 and this is hypothesized to be because of the presence of *stx2.* Strains that express *stx2* are more commonly associated with patient

progression to HUS development and have increased incidences of bloody diarrhea [144, 150-152]. The genome of 86-24 has not been annotated, therefore the genome similarity between 86-24 and EDL933 is unknown. However, the sequences of 86-24 and EDL933 are highly similar and EDL933 is often used as a reference genome for studies using 86-24.

Shiga toxin is encoded on a chromosomally inserted lambda-bacteriophage [153, 154]. Production of Shiga toxin is activated by the phage lytic cycle, which is induced by the bacterial SOS response and released upon bacterial cell lysis [155, 156]. DNA damage triggers the SOS response, leading to production and activation of RecA which in turn activates expression of phage genes, including Shiga toxin [156]. The use of antibiotics to treat EHEC infection induces the phage lytic cycle by activation of the bacterial SOS response, leading to increased Shiga toxin production and increased risk of HUS development [13, 18, 157-160].

Fimbriae

Fimbriae are appendages that protrude from bacterial cells and help bacteria avoid displacement by the continuous flow of the luminal contents by adherence to epithelial cells [161]. Fimbriae mediate early EHEC attachment events before AE lesion formation [162]. The EHEC genome contains 16 fimbrial loci [163], suggesting fimbriae are an important virulence determinant in EHEC. However, very little is known about the contribution of fimbriae to EHEC colonization and disease, partly due to difficulties in understanding environmental conditions that promote EHEC fimbriae expression *in vitro* [162, 164, 165]. Recent data from the Kendall lab

demonstrate that ethanolamine (EA), an abundant molecule in the GI tract [166], activates expression of 15 of the 16 EHEC fimbriae [166]. This provides a useful tool to assess the function of these fimbriae in EHEC colonization and pathogenesis in the lab.

Most EHEC fimbriae are assembled by the chaperone/usher mechanism, but EHEC also encodes type IV fimbriae and curli [163, 164]. Fimbriae assembled by chaperone/usher pathway at minimum encode a chaperone to assist with assembly, fimbrial subunits to form the major structure of the fimbriae, and an usher to facilitate assembly at the outer membrane. Many fimbrial operons also encode additional fimbrial subunits, transcriptional regulators, or additional chaperones.

The most highly studied EHEC fimbriae are long polar fimbriae 1 (Lpf1, locus 12) and Lpf2 (locus 13), which are highly similar to Lpf fimbriae in *Salmonella enterica* Serovar Typhimurium [167]. Both Lpf1 and Lpf2 are not encoded in the commensal *E. coli* K12 strain [168, 169]. Expression of *lpf1* is upregulated in late exponential growth and the *lpf1* locus is repressed by H-NS [168, 170-173]. H-NS repression of *lpf1* is relieved by direct interaction of Ler with the *lpf1* promoter region [171, 172]. Lpf1 facilitates EHEC adhesion to epithelial cells, as an EHEC *lpf1* mutant strain has decreased adherence and an altered adherence pattern to epithelial cells [168, 174]. Lpf1 binds to fibronectin, laminin, and collagen IV in the extracellular matrix [175]. Expression of Lpf1 in a non-fimbriated *E. coli* K12 strain increases *E. coli* adherence to epithelial cells and results in the formation of short, peritrichous fimbrial structures on the surface of *E. coli* [168]. An *lpf1* mutant

exhibits decreased colonization as measured by reduced fecal shedding in lambs compared to WT EHEC [170].

Lpf2 is expressed during late exponential growth and is decreased during iron depletion [170]. The ferric-uptake-regulator (Fur) directly binds to the *lpf2* promoter region suggesting that Fur represses expression of *lpf2* in response to iron availability [176]. It is unknown what Lpf2 binds to on epithelial cells. When Lpf2 is expressed in a non-fimbriated *E. coli* strain, adherence to epithelial cells is decreased, however an *lpf2* deletion strain displayed decreased adherence to Caco-2 epithelial cells at early time points [174]. The natural AE-lesion forming, murine pathogen *Citrobacter rodentium* contains the Lpf2 locus. An *lpf2* deletion strain in *C. rodentium* has no defect in mouse colonization or AE lesion formation [177].

The contribution of Lpf1 and Lpf2 has been studied *in vivo* using double mutants through different infection models. The *lpf1/lpf2* double mutant displayed a colonization defect in crossbred lambs and infant rabbits [170, 178]. A colonization defect for the *lpf1/lpf2* double mutant was also observed during early colonization in germ-free pigs and in sheep infections [179].

The *E. coli* common pilus (Ecp, locus 15), previously referred at as Mat (meningitis-associated fimbriae), is encoded in both pathogenic and nonpathogenic *E. coli* strains [180]. The *ecp* locus encodes EcpR, a LuxR-like regulator that directly regulates expression of *ecp* [181]. Ecp is also regulated by integration host factor (IHF), which is essential for relieving H-NS repression of *ecp* [181]. Deletion of *ecp* from EHEC results in decreased adherence to epithelial cell lines [180, 182].

The hemorrhagic *E. coli* pilus (Hcp, locus 16) is a type IV pilus that forms bundles and binds to the extracellular matrix proteins laminin and fibronectin [183, 184]. Expression of *hcp* is induced in Minca minimal medium and expression of *hcp* contributes to EHEC adhesion as *hcp* mutants had decreased adherence to epithelial cells [183]. Hcp is likely expressed during infection in humans as the sera from patients who presented with HUS recognized the major pilin subunit HcpA [183].

Curli (locus 7) is an adhesin that is important for EHEC adherence and persistence to food products, such as leaves or spinach, and in the environment [185-189]. Expression of curli is promoted by numerous environmental signals including nutrient limitations, pH, and temperature, supporting a role for curli in environmental persistence [190-192]. Expression of curli is also influenced by the expression of other fimbriae, as a *lpf1/lpf2* double mutant causes an increase in curli expression resulting in increased epithelial cell adherence [178]. Curli forms thin, aggregative fimbriae on the surface of cells and binds the extracellular matrix proteins fibronectin and laminin, and other host proteins such as MHC Class 1 molecules and plasminogen [185, 193, 194]. Mutants in curli production alone do not influence EHEC adhesion [182], however other studies in *E. coli* and *Salmonella enterica* have demonstrated that curli plays a role in epithelial cell invasion and biofilm formation [195-197]. The relevance of these curli studies for EHEC pathogenesis is unclear as EHEC does not form biofilms.

EHEC fimbrial locus 8 encodes the F9 fimbriae, originally identified in an EHEC transposon mutagenesis screen for being defective in colonization of very young calves [198]. F9 fimbriae expression in EHEC decreases adherence to bovine

epithelial cells, but expression of F9 fimbriae in *E. coli* K12 increases adherence [199]. In a previous study, colonization of weaned calves with the F9 fimbriae mutant strain resulted in reduced colonization as measured by bacterial shedding [199]. Interestingly, the F9 mutant was still able to colonize calves at the terminal rectum indicating that F9 fimbriae is not involved in tissue tropism in calves but may contribute to colonization at other sites.

The *E. coli* laminin-binding fimbriae (Elf, locus 5) is expressed on the surface of EHEC during epithelial cell adhesion and forms fine, flexible fibers [200]. Elf binds to the extracellular matrix protein laminin but not fibronectin or collagen IV and is maximally expressed in Minca minimal medium and in the presence of host cells [200]. A mutant in *elf* had decreased adherence to epithelial cells and bovine explants [200] .

Of the remaining fimbrial loci, very little is known. The EHEC fimbrial locus 14 encodes type 1 fimbriae that appears to be non-functional in EHEC because of deletions within the promoter region [201, 202]. EHEC fimbrial locus 2 is increased in expression under acid stress conditions, and expression of *loc2* promotes EHEC adhesion during acid stress [203]. A study to identify conditions that promote fimbrial gene expression utilized promoter fusions of 15 EHEC fimbrial loci (locus 14, the type 1 fimbriae, was not included) and assessed expression under varying growth phases, media, temperatures, and aeration [164]. The study only determined the expression of one previously uncharacterized fimbriae, locus 9, which was expressed significantly higher in stationary phase [164]. It is important to note that this study determined that 11 of the 15 fimbrial fusions were not expressed under

the study conditions, highlighting how little is known about the contribution of fimbriae to EHEC pathogenesis [164]. Three of the aforementioned fimbrial loci (*elf*, *lpf1,* and *lpf2*) contain premature stop codons in the *elf* usher, *lpf1* usher, and *lpf2* chaperone genes [164]. These three loci all produce function fimbriae [168, 174, 200] suggesting that components from other fimbrial loci, such as the usher and chaperone, can complement [204, 205].

Overall despite many studies, the role of EHEC fimbriae and their contribution to pathogenesis is largely unknown (summarized in Table 1.1). Additionally, many EHEC fimbriae have not been studied due to difficulties in promoting their expression *in vitro*. Additional studies are needed to elucidate the function of these fimbriae in regards to EHEC colonization and pathogenesis.

E. coli T3SS-2

EHEC encodes a second cryptic T3SS termed the *E. coli* T3SS-2 (ETT2), which shares homology to the *Salmonella* T3SS-1 [206, 207]. The ETT2 is present in a majority of *E. coli* strains but has undergone mutational attrition to make the ETT2-encoded T3SS nonfunctional in a majority of these strains [206]. In EHEC, the ETT2 contains many multiple frameshift mutations in secretion apparatus genes that are necessary to form a function secretion system, by analogy to the *Salmonella* T3SS-1 [206, 208]. However, despite being unable to encode a functional T3SS, some of the genes encoded in the ETT2 are predicted to be functional, such as the five predicted or characterized transcription factors. A previous genetic study demonstrated that two ETT2-encoded regulators, EtrA and EivF, repress LEE expression [209],

suggesting that cross talk can occur between the LEE and ETT2-encoded regulators. Despite this study, the regulation of the ETT2 and the function of the ETT2, including the function of two uncharacterized, putative ETT2-encoded transcription factors YqeI and Ygek (also called EtrB), are unknown.

Signals

The expression of EHEC virulence traits is tightly regulated and is controlled by multiple environmental stimuli. Signals that stimulate the expression of EHEC virulence traits include both bacterial- and host-produced molecules, such as bacterial-derived autoinducers and host-derived hormones involved in quorum sensing, and the bacterial- and host-produced compound ethanolamine (EA).

Quorum sensing

Quorum sensing is a mechanism bacteria utilize to communicate [210]. Bacteria produce hormone-like compounds called autoinducers that are then sensed resulting in differential expression of target genes [211]. EHEC produces the signaling molecule autoinducer-3, which is involved in EHEC quorum sensing [211, 212]. In addition, EHEC quorum sensing involves not only bacterially derived signals, but also host-derived signals, such as the host hormone epinephrine [213]. Cross talk with quorum sensing autoinducer-3 and epinephrine activates virulence gene expression, including the LEE, non-LEE encoded effectors, and many O-islands, through the LysR-like transcriptional regulatory QseA [46, 106, 214-216]. The sensing of these bacterial and host-derived cues plays a critical role in EHEC

pathogenesis and is an example of how EHEC utilizes interkingdom signaling to sense and respond to the host environment.

Ethanolamine

EA (Figure 1.4A) is a component of phosphatidylethanolamine (PE), an abundant lipid in eukaryotic and bacterial cell membranes [217]. As a component of PE and other modified lipid molecules, EA is an important signaling molecule and influences immunomodulation, cell division, nutritional intake, and energy balance [218-221]. The exfoliation of enterocytes as well as the turnover of bacterial cells releases an abundant and replenished supply of EA in the GI tract. EHEC and other members of the Enterobacteriaceae carry the ethanolamine utilization operon (*eut*), which contains 17 genes that encode for the transport and breakdown of EA [222, 223]. Although EA can serve as a carbon and/or nitrogen source for bacteria, the resident microbiota do not readily metabolize EA [224]. Thus, intestinal pathogens, including EHEC, utilize EA to sidestep nutritional competition and enhance growth during infection [221, 224-227].

Significantly, bacterial pathogens respond to EA as a signaling molecule to activate virulence gene expression [109, 110, 166, 228]. In EHEC, EA activates the expression of genes critical for colonization of the GI tract, including fimbrial adhesins and the LEE, as well as genes encoding Shiga toxin (Figure 1.4B) [109, 166, 228]. As mentioned above, EHEC encodes 16 distinct fimbrial loci [163, 229], and these fimbriae may be important for initial adherence to enterocytes, that precedes intimate, LEE-dependent adherence [230], but the contribution of many fimbrial loci

to EHEC pathogenesis has been elusive due to the difficulties of expressing fimbrial genes in vitro [164]. Thus, the finding that the biologically relevant molecule EA promotes expression of EHEC fimbriae suggests that these fimbriae play a role in the ability of EHEC to establish infection. Additionally, EA activates expression of global regulators in EHEC [109], suggesting that EA plays a central role in integrating multiple cues to optimize timing of virulence gene expression.

Crosstalk between signaling pathways

Despite the abundance of research identifying transcription factors and how they regulate virulence traits, very little research has focused on the crosstalk between these regulatory proteins. In other words, there are very few studies investigating the integration of the complex network of the overlapping signaling pathways that modulate EHEC virulence traits.

One of the few known examples of regulatory crosstalk in EHEC is the interaction of SdiA, a transcription factor that responds to acyl-homoserine lactones produced by the commensal microbiota to repress expression of the LEE [122, 123, 231]. SdiA activates expression of *gad* acid-resistance genes, including the LEE-repressor *gad*X [123]. GadX is activated by nitric oxide [112] and acidic pH [232]. Crosstalk between SdiA and GadX represses LEE expression in response to these signals while activating the EHEC acid-response, which is hypothesized to aid in EHEC passage through the stomach.

As mentioned above, crosstalk between the host hormone epinephrine and bacterial derived, quorum sensing molecules is integrated in EHEC to regulate the

expression of genes involved in virulence such as the LEE and the flagella regulon [233, 234]. This crosstalk is an example of an interkingdom signaling pathway that EHEC exploits to regulate the expression of virulence traits.

An example of a positive feedback mechanism to regulate EHEC virulence. is through EHEC sensing butyrate, a short chain fatty acid abundant in the colon [235]. In response to butyrate, the leucine-responsive regulatory (Lrp) protein initiates a signaling cascade that promotes expression of *pchA* [236], which encodes a direct activator (PchA) of the LEE [237, 238]. Recent work by Takao *et al*. revealed that butyrate-dependent activation of the LEE is even more complex and also includes the transcription factor LeuO [239]. Lrp directly promotes expression of *leuO*, which in turn also binds the *ler* promoter to activate expression of the LEE and microcolony formation. Interestingly, LeuO activation of the LEE genes required PchA and both PchA and Ler activated *leuO* expression. This positive feedback mechanism is hypothesized to function in prolonging expression of the LEE.

Overall, much of the literature concerning EHEC virulence describes how a transcription factor regulates virulence gene expression in response to a signal. Studies concerning how EHEC assimilates environmental information from multiple signaling cues to regulate traits important for pathogenesis are lacking.

Project rationale

EHEC responds to a wide variety of signals, including EA, to colonize the host and cause disease [46, 109, 110, 166, 215]. We hypothesize that signaling cascades involving EA and quorum sensing to regulate EHEC virulence are linked. We

investigated the mechanism of EutR as a virulence regulator, the involvement of QseA-regulated proteins in EHEC virulence, and how fimbriae regulate virulence traits in EHEC. Understanding the intersection of complex signaling cascades to regulate virulence traits and how environmental cues contribute to virulence can provide important information in regards to bacterial pathogenesis. Furthering our understanding of signaling cascades involved in pathogenesis is critical for the development of novel therapeutics to treat EHEC infection because of the limited treatment options available. Many bacterial pathogens encode the EA-utilization operon and fimbriae. Therefore, our studies may describe a general mechanism used by pathogens to coordinate the expression of virulence traits.

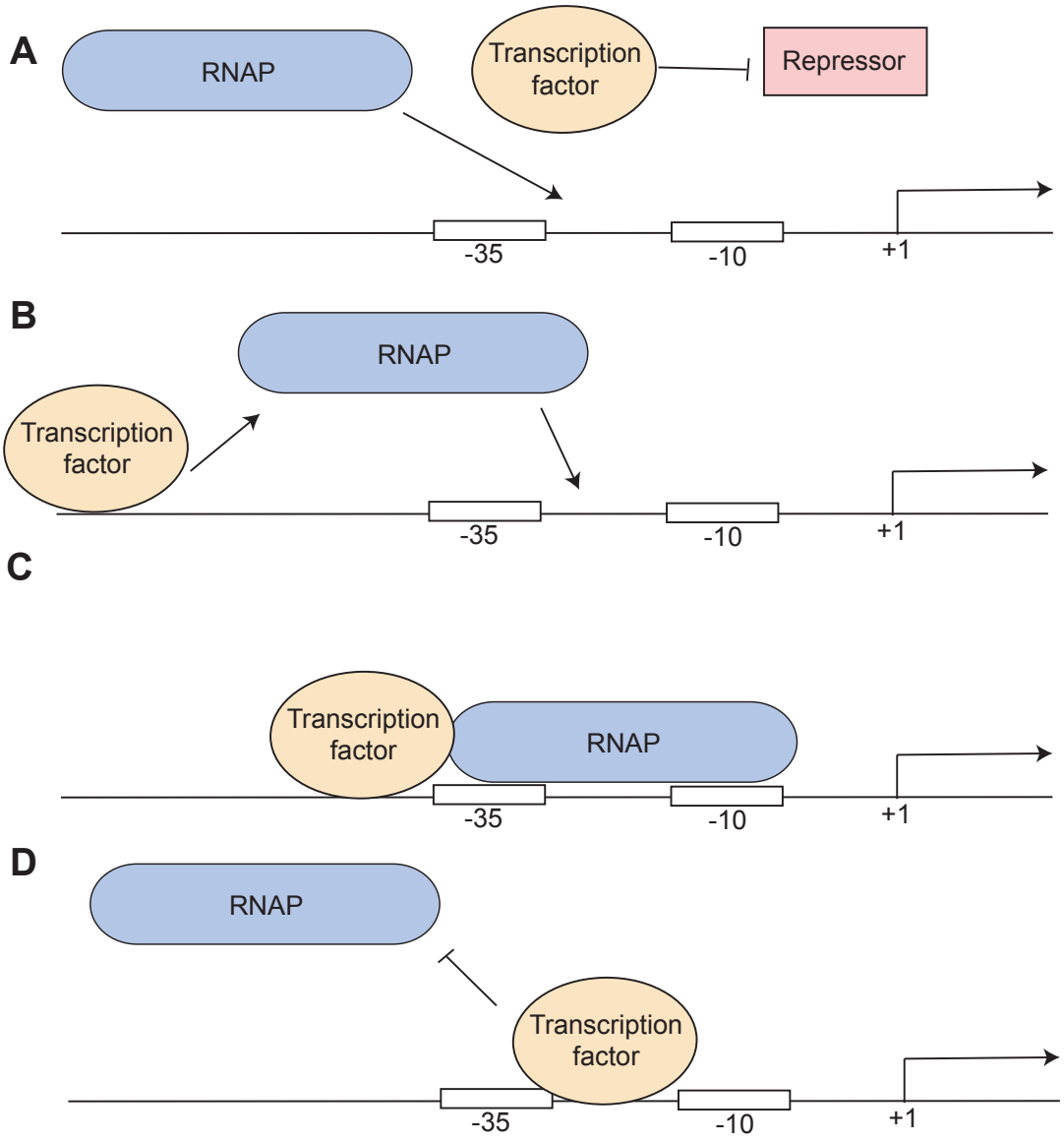

Figure 1.1 Transcription factors activate or repress the expression of target genes. Transcription factors can activate gene expression by (A) relieving existing repression to allow RNA polymerase (RNAP) to bind to the promoter region, (B) binding upstream of the promoter to help recruit RNAP, or (C) binding adjacent to the -35 promoter region to interact with RNAP. (D) Transcription factors can inhibit RNAP binding to repress gene expression.

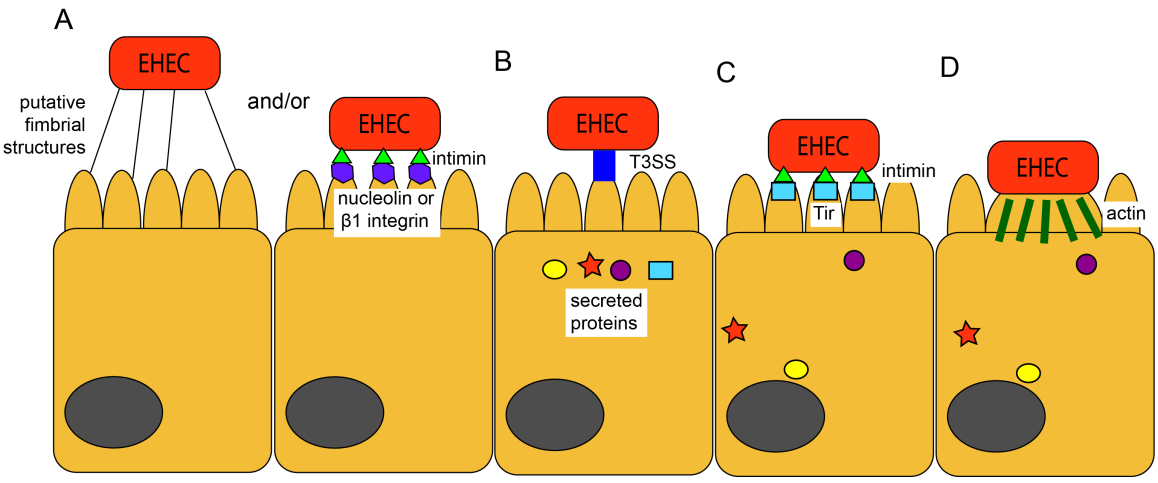

Figure 1.2 EHEC disease progression to attaching and effacing (AE) lesion formation. (A) EHEC may form initial, transient contact with epithelial cells through fimbriae-mediated adhesion to unknown host targets and/or intimin adherence to nucleolin and β_1 integrin. (B) A type III secretion system (T3SS) translocates effector proteins including the T3SS-encoded Tir, the translocated intimin receptor into the host cytoplasm. (C) Intimin on the bacterial surface (shown in green triangles) and Tir (shown in light blue squares) bind to form a tight attachment of EHEC to the host cell. (D) Effector proteins cause host actin reorganization (shown in green rectangles) to display EHEC on an actin rich, pedestal-like structure.

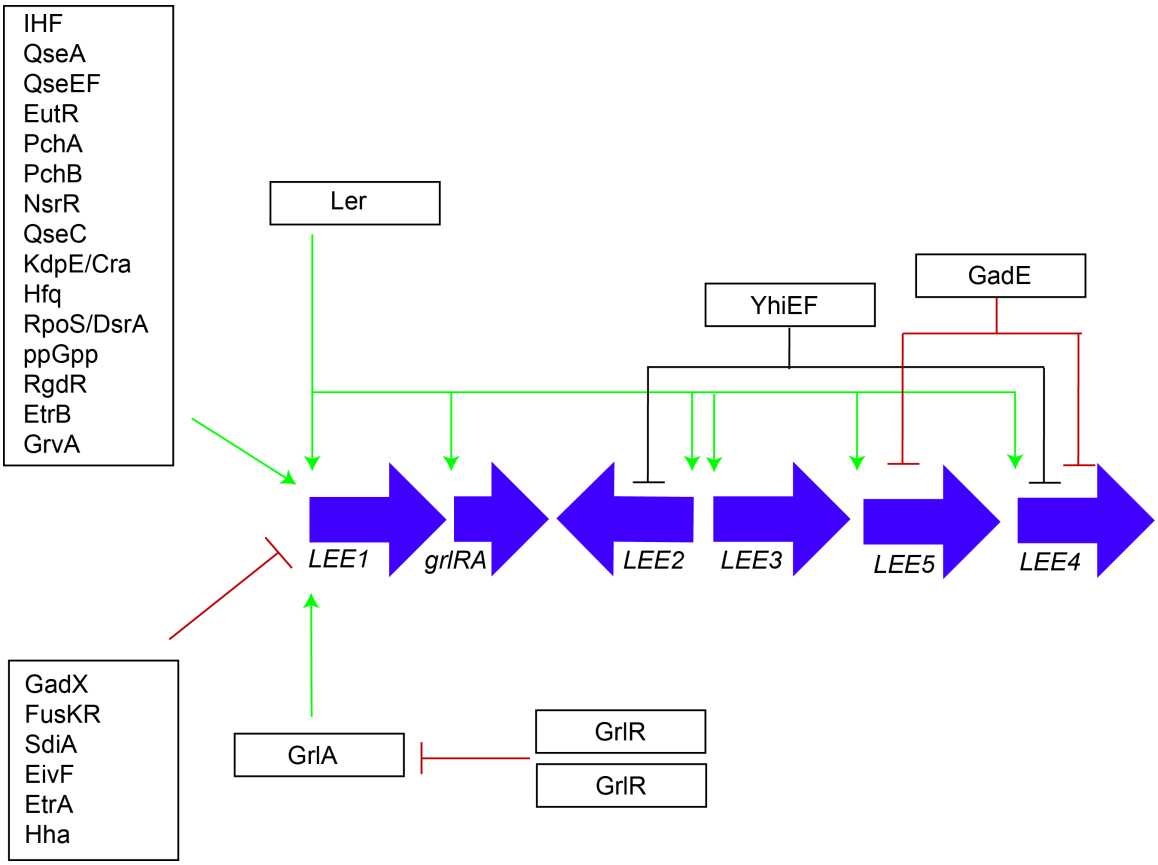

Figure 1.3 Regulation of the Locus of Enterocyte Effacement (LEE). A schematic representation of known regulators of the LEE. Positive regulation of the LEE is shown by green arrows. Negative regulation of the LEE is shown in red and black blunt arrows. Direct regulation of the LEE has only been demonstrated for some of the regulatory proteins listed.

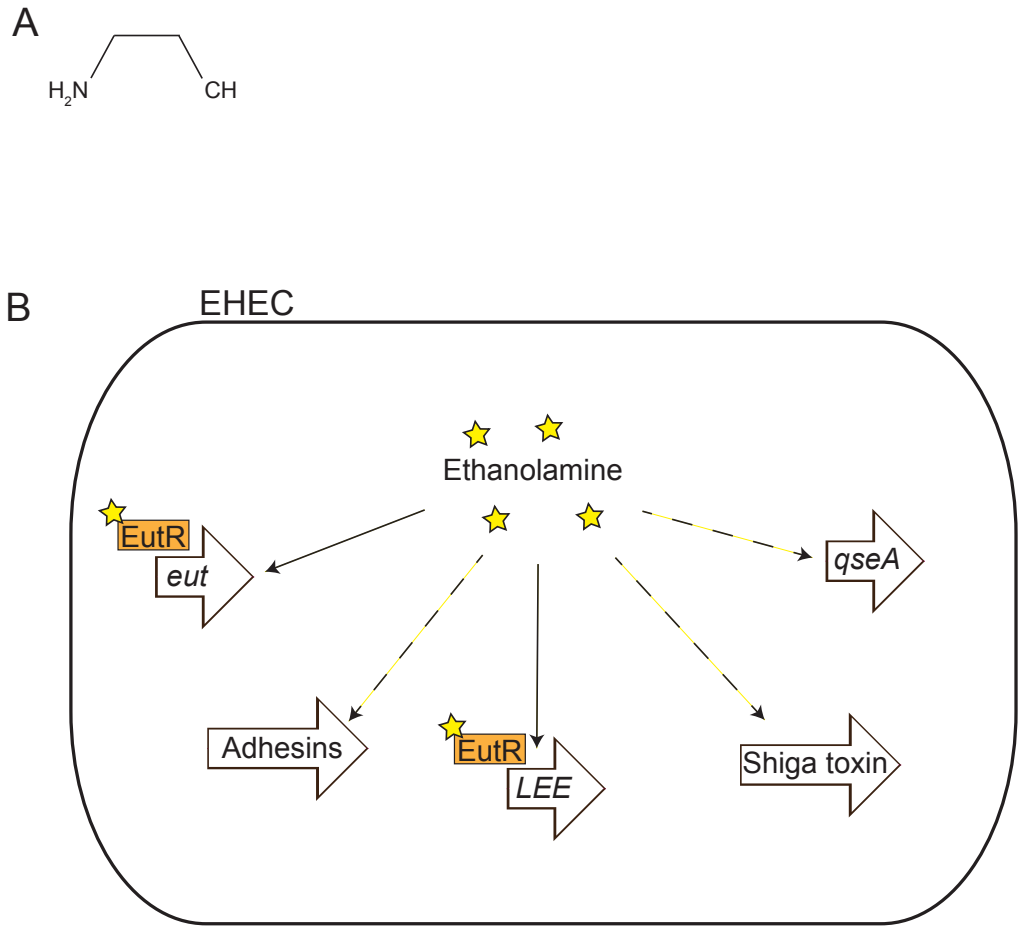

Figure 1.4 Ethanolamine (EA) signaling in EHEC. (A) The structure of EA. (B) EHEC senses EA and activates the expression of genes located in the *EA utilization operon* (*eut*), responsible for utilization of EA as a metabolite. In addition, EA activates the expression of multiple virulence traits including fimbrial adhesions, the LEE, Shiga toxin, and the regulatory protein QseA. The EA sensor, EutR, directly regulates the expression of the indicated genes.

Table 1.1 Previous studies on the contribution of fimbriae to EHEC pathogenesis

Locus	Forms a fimbrial structure	*In vitro* adherence	*In vivo* adherence	References
loc1	-	NT	NT	
loc2	Yes	Increase	NT	[165, 240]
loc3	Yes	NT	NT	[165]
loc4	-	NT	NT	
loc5/elf	Yes	Increase	NT	[200]
loc6	-	NT	NT	
loc7/curli	Yes	Increase/no phenotype	NT	[185, 194]
loc8	Yes	Increase/no phenotype	No phenotype	[164, 198, 199]
loc9	-	NT	NT	
loc10	-	NT	NT	
loc11	-	NT	NT	
loc12/lpf1	Yes	Increase	Increase*	[168, 170, 178, 179]
loc13/lpf2	Yes	Increase/no phenotype	Increase*	[170, 178, 179]

loc14	-	NT	NT	
ecp/mat	Yes	Increase	NT	[180, 182]
hcp	Yes	Increase	NT	[183]

Increase = the fimbrial locus increases adherence

No phenotype = the fimbrial locus does not influence adherence under the conditions tested

NT = Not tested

- = has not been tested or does not form a surface structure under the conditions tested

* An *lpf1/2* double mutant has reduced colonization compared to WT EHEC in infant rabbits and lambs

Chapter Two: EutR is a direct regulator of virulence

Part of this chapter has been adapted from "EutR is a direct regulator of genes that contribute to metabolism and virulence in enterohemorrhagic *Escherichia coli* O157:H7"

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[1]D.E.C. and [2]L.A.G. work is not shown.

Abstract

Ethanolamine (EA) metabolism is a trait associated with enteric pathogens, including enterohemorrhagic *Escherichia coli* O157:H7 (EHEC). EHEC causes severe bloody diarrhea and hemolytic uremic syndrome. EHEC encodes the EA utilization (*eut*) operon that allows EHEC to metabolize EA and gain a competitive advantage when colonizing the gastrointestinal tract. The *eut* operon encodes the transcriptional regulator EutR. Genetic studies indicated that EutR expression is induced by EA and vitamin B_{12} and that EutR promotes expression of the *eut* operon; however, biochemical evidence for these interactions has been lacking. We performed primer extension assays, electrophoretic mobility shift assays (EMSAs), and measured transcript levels to elucidate a mechanism for EutR gene regulation. These studies demonstrated that genes in the *eut* operon are significantly increased only when EHEC is grown with both EA and B_{12}, and that expression of *eutS* requires the presence of EutR. EutR contributes to expression of the locus of enterocyte effacement (LEE) in an EA-dependent manner. We performed EMSAs to examine EutR activation of the LEE. The results demonstrated that EutR directly binds the regulatory region of the *ler* promoter. These results present the first mechanistic description of EutR gene regulation and reveal a novel role for EutR in EHEC pathogenesis.

Introduction

The ability of a pathogen to colonize a host and cause disease requires coordinated expression of genes that mediate nutrient acquisition, as well as genes involved in virulence [241]. Ethanolamine (EA) is abundant in the gastrointestinal (GI) tract due to the turnover of host enterocytes and commensal microbes, as well as through the host diet [217, 242, 243]. EA can serve as a source of carbon and/or nitrogen for enteric pathogens, and the ability to metabolize EA gives enteric pathogens a competitive advantage when colonizing the GI tract [224, 225]. Indeed, genes encoding EA metabolism are found in diverse bacterial pathogens, including enterohemorrhagic *Escherichia coli* O157:H7 (EHEC), enteropathogenic *E. coli* (EPEC), *Salmonella*, *Clostridium*, *Listeria*, and *Enterococcus* [163, 223, 242, 244].

Recent studies demonstrated that EA is present in the mammalian intestine at concentrations that support growth of EHEC and *Salmonella* and that EA utilization by these pathogens confers a growth advantage over indigenous microbes [224, 225]. In EHEC and *Salmonella*, the genes that encode EA metabolism are located within the EA utilization (*eut*) operon. The *eut* operon includes 17 genes that allow transport and breakdown of EA, as well as production of a carboxysome-like structure that contains toxic breakdown products of EA metabolism [222, 245-251]. The *eut* operon also encodes the transcriptional regulator EutR, which promotes expression of the *eut* operon in response to EA and vitamin B_{12} [222, 252].

EHEC is a food-borne pathogen that causes bloody diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome, which may be fatal [253]. In the colon, EHEC forms attaching and effacing (AE) lesions, which are a hallmark of EHEC

disease. The AE lesion is characterized by the destruction of microvilli and the rearrangement of the cytoskeleton to form a pedestal-like structure that cups the bacterium, thereby allowing intimate attachment of EHEC to host enterocytes [62, 254, 255]. Most of the genes involved in AE lesion formation are encoded within a chromosomal pathogenicity island called the locus of enterocyte effacement (LEE) [57]. The LEE contains five major operons that encode a type III secretion system (T3SS) and effector proteins that are translocated into the host epithelial cell through the bacterial T3SS [62, 67, 254-259]. Ler (LEE-encoded regulator) is encoded within *LEE1* and is the master regulator of this pathogenicity island [59, 60, 98, 260].

The goal of this study was to examine the mechanism of EutR-dependent gene regulation. Here, we establish a direct role for EutR in promoting expression of genes that are critical to EHEC pathogenesis. Furthermore, we begin to elucidate the mechanism of EutR as a transcription factor for virulence genes.

Materials and Methods

Strains, plasmids, growth conditions, and recombinant DNA techniques

All strains and plasmids used in this study are listed in Table A2.1. Standard methods were used to perform plasmid purification, PCR, ligation, restriction digests, transformations, and gel electrophoresis. The nonpolar *eutR* deletion strain MK37 (*ΔeutR*) was constructed using λ-red mutagenesis. Plasmid pMK53 was constructed by amplifying the *eutR* gene from EHEC strain 86-24 using AccuTaq polymerase (Sigma) with primers EutRexp_F1 and EutRexp_R1 and cloning the

resulting PCR product into the NcoI/Sbf1 cloning sites of vector pMAL-c5X. The oligonucleotide primers are listed in Table A2.2

RNA extraction and qRT-PCR

Cultures of 86-24 and MK37 were grown in LB medium at 37°C overnight and then diluted 1:100 in Dulbecco's modified Eagle's medium (DMEM) and grown at 37°C. Where indicated, EA (Sigma) was added at a final concentration of 5 mM, and vitamin B_{12} (cyanocobalamin; Sigma) was added at a final concentration of 150 nM. RNA from three biological replicate cultures of each strain was extracted using the RiboPure Bacteria RNA isolation kit (Ambion). The primers used in the quantitative real-time PCR (qRT-PCR) assays are listed in Table A2.2. qRT-PCR was performed in a one-step reaction using an ABI 7500 sequence detection system (Applied Biosystems).

Data were collected using the ABI Sequence Detection 1.2 software (Applied Biosystems). All data were normalized to levels of *rpoA* and analyzed using the comparative cycle threshold (C_T) method [261]. The expression levels of the target genes under the various conditions were compared using the relative-quantification method [261]. Real-time data are expressed as the changes in expression levels compared to the wild- type (WT) levels. Statistical significance was determined by Student's t-test.

Reverse transcriptase PCR (RT-PCR)

SuperScipt II reverse transcriptase (Invitrogen) and random primers were

used to create cDNA from RNA samples. The cDNA was used for PCR with gene-specific primers (Table A2.2). Genomic DNA was used as a positive control, and a reaction without reverse transcriptase was used as a negative control.

Purification of EutR

In order to purify the maltose-binding protein (MBP)-tagged EutR protein, *Escherichia coli* strain BL-21 (DE3) containing pMK53 was grown at 37°C in LB with glucose (0.2% final concentration) and ampicillin (100 μg/ml) to an optical density at 600 nm ($O.D._{600}$)of 0.5, at which point IPTG was added to a final concentration of 0.3 mM and allowed to induce overnight at 18°C. Cells were harvested by centrifugation at 4,000 X *g* for 20 min and then resuspended in 25 ml column buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA) and lysed by homogenization. The lysed cells were centrifuged, and the lysate was loaded onto a gravity column (Qiagen) with amylose resin. The column was washed with column buffer and then eluted with column buffer containing 10 mM maltose. Fractions containing purified proteins were confirmed by SDS-PAGE and Western analysis.

Electrophoretic mobility shift assays (EMSAs)

To determine the direct binding of EutR to target promoters, electrophoretic mobility shift assays (EMSAs) were performed using the purified EutR-MBP and PCR-amplified DNA probes (Table A2.2), as previously described [46]. The DNA probes were then end labeled with (γ-^{32}P) ATP (Perkin-Elmer) using T4 polynucleotide kinase (NEB) following standard procedures [262]. End-labeled

fragments were run on a 6% polyacrylamide gel, excised, and purified using the Qiagen PCR purification kit.

EMSAs were performed by adding increasing amounts of purified EutR protein to end-labeled probe (10 ng) in binding buffer (500 μg ml^{-1} BSA (NEB), 50 ng poly(dI-dC), 60 mM HEPES, pH 7.5, 5 mM EDTA, 3 mM dithiothreitol (DTT), 300 mM KCl, and 25 mM $MgCl_2$), 1 mM EA, and 150 nM B_{12} and incubated for 20 min at 37°C. Stop Solution (USB) or 1% Ficoll solution was added to the reaction mixtures immediately before loading the samples on the gel. The reaction mixtures were electrophoresed for approximately 6 h at 150 V on a 6% polyacrylamide gel, dried, and imaged with a PhosphorImager (Molecular Dynamics).

Primer Extension

Primer extension analysis was performed as previously described [46]. Briefly, *ler* and *eutS* specific reverse primers (Table A2.2) were end-labeled as described above. A total of 40 μg of RNA, isolated from strain 86-24, was used to generate cDNA using the Primer Extension System— AMV Reverse Transcriptase kit (Promega) for the *eutS* primer extension samples. For *ler* primer extension samples, 40 μg total of RNA, isolated from strain 86-24 with the plasmid pVS23, was used to generate cDNA. The resultant cDNA was precipitated, electrophoresed on a 6% polyacrylamide-urea gel next to a sequencing reaction (Affymetrix). Amplified genomic DNA from strain 86-24 was used to generate the sequencing ladder using primers listed in Table A2.2 for the *ler* and *eutS* promoters.

Results

The presence of EA and B_{12} is necessary for expression of the eut operon

Genetic studies in *Salmonella* revealed that EutR, encoded in the *eut* operon (Figure 2.1A), promotes expression of genes necessary for EA metabolism in the presence of EA and B_{12} [252]. We confirmed the necessity of EA and B_{12} for induction of the *eut* operon by performing qRT-PCR and RT-PCR analyses using RNA extracted from WT EHEC grown in presence and absence of EA and B_{12}. Expression of *eutS,* a putative carboxysome structural protein and the first gene in the *eut* operon, was increased in the presence of EA and B_{12} and was unchanged in the presence of EA only compared to expression in the absence of EA and B_{12}. Expression of *eutS* in the presence of B_{12} was decreased in comparison to expression in the absence of EA and B_{12} (Figures 2.1B and 2.2). These data indicate that EA and B_{12} are required to induce expression of the *eut* operon. RNA from different technical replicates was used for qRT-PCR and RT-PCR analyses. Differences in *eutS* expression in the presence of B_{12} are due to human error and should be repeated to confirm that there is no change in *eutS* expression in the presence of B_{12} compared to the absence of EA and B_{12}.

EutR is necessary for induction of the eut operon

We examined the ability of EA and B_{12} to induce *eutS* expression in the absence of EutR. Expression of *eutS* was significantly decreased in the *ΔeutR* strain in the presence and absence of EA and/or B_{12} compared to WT grown in the absence of EA and B_{12} (Figures 2.1B and 2.2). These data corroborate studies from Roof and

Roth indicating that EutR is required to promote expression of genes necessary for EA metabolism [252].

eutS is transcribed from a typical σ^{70} promoter

To further characterize *eutS* expression and map the putative promoter region, we performed primer extension analyses. Primer extension analysis was performed using cDNA synthesized from RNA grown in the presence and absence of EA and B_{12}. The primer extension results demonstrate that expression of *eutS* was increased in the presence of EA and B_{12} compared to the absence of EA and B_{12} (Figure 2.3). The results reveal one transcriptional start site in the *eutS* promoter (Figure 2.3), which we mapped to approximately 45 base pairs upstream of the translation start site. The -10 sequence, TTTGTT, had three mismatches from the σ^{70} consensus sequence, TATAAT, and the -35 sequence, TTTAAA, had two mismatches from the consensus sequence, TTGACA (Figure 2.3). The -10 and -35 promoter sequences are separated by 13 nucleotides (Figure 2.3). The EutR consensus binding sequence is shown in bold (Figure 2.3) [110]. There is no match to alternative sigma factor consensus sequences such as σ^{32}, σ^{54}, and σ^{28}. Site directed mutagenesis of the putative *eutS* promoter should be performed to verify the σ^{70} consensus sequences.

EutR regulates transcription of the LEE through direct interaction

Previous studies demonstrated that EutR affects expression of the LEE pathogenicity island, as well as EHEC's ability to form AE lesions on epithelial cells

[109]. Here, we performed EMSAs to address whether these effects were due to direct regulation of *LEE1/ler* by EutR. We generated a probe harboring the entire regulatory region of *ler.* These data indicated that EutR was able to bind and shift the radiolabeled probe comprising the *ler* promoter regions and that EutR did not bind to the negative-control *amp* promoter (Figure 2.4), indicating a direct interaction between EutR and its target genes.

To further characterize *ler* expression in the presence of EA and B_{12}, we performed primer extension analyses using cDNA synthesized from RNA grown in the presence and absence of EA and B_{12}. The primer extension results demonstrate transcription of *ler* from the EHEC proximal promoter (Figure 3.5), which was downstream of the EutR binding sequence. We observed multiple bands consistent with previous studies [59, 106]. This banding pattern is likely an artifact and could be alleviated by using a different reverse primer for primer extension or by using 5' RACE to map the transcription start site.

EA does not regulate EPEC virulence

To determine whether EA plays a role in modulating virulence gene expression in EPEC, we performed qRT-PCR and measured expression of *eutR* and *ler* in the presence and absence of EA and B_{12}. Between EHEC and EPEC, *ler* is 99% identical with 0 mismatches in the *ler* coding sequence, 8 gaps and 1 mismatch in the 500 basepairs upstream of the *ler* translation start site. The EHEC EutR consensus binding sequence is found in the *ler* promoter in EPEC. Between EHEC and EPEC, *eutR* is 98% identical with 10 mismatches in the *eutR* coding sequence, 5

of which are located in the DNA binding motif. However, at the protein level there are only 3 amino acids differences between EPEC and EHEC EutR, and none of the changes are located in the DNA binding motif. There is only 1 basepair change and no gaps in the 500 basepairs upstream of *eutR* compared between EPEC and EHEC. Transcription of *ler* and *eutR* was unchanged when EPEC was grown to early or mid log (Figure 2.6). Additional data from the lab indicate that EPEC is unable to utilize EA as a carbon or nitrogen source (data not shown). These findings indicate that EA does not function as a virulence cue in EPEC.

Discussion

EA is a breakdown product of phosphatidylethanolamine (PE), a major component of both mammalian and bacterial cell membrane lipids [263, 264]. PE enhances the ability of both EHEC and EPEC to bind to epithelial cells [265, 266], and the ability to metabolize EA has been associated with virulence in several pathogens, including *Salmonella*, *Listeria*, and *Enterococcus* species [226, 251, 267-269]. Bertin *et al.* recently showed that EHEC gains a growth advantage in the mammalian GI tract by metabolizing EA as a nitrogen source [224]. The ability of EHEC to metabolize EA may contribute to EHEC's low infectious dose; as little as 50 to 100 CFU is sufficient to allow EHEC to colonize the colon and cause disease [24]. Interestingly, EPEC, which colonizes the small intestine, has a much higher infectious dose. The genome sequence of EPEC indicates a large phage insertion between the *eutB* and *eutC* genes, which encode the ammonia lyase [270]. We determined that EPEC does not upregulate expression of *eutR* or *ler* in the presence

of EA and B_{12} (Figure 2.6). These data suggest that there may be different selective pressures in the environments of the large and small intestine. Previous studies showed that EA-dependent virulence gene regulation did not depend on EA metabolism [109].

The *eut*-encoded transcriptional regulator EutR is responsible for EA-dependent virulence gene regulation in EHEC [109]. This study aimed to investigate the molecular mechanisms by which EutR activates expression of the *eut* genes that are involved in EA metabolism, as well as expression of *LEE1-ler*, which is important for AE lesion formation. EutR was first identified and described by Roof and Roth [252] through the generation of transposon mutations in the *eut* operon. In their study, Roof and Roth determined that EA and B_{12} increased expression of EutR and that this increased expression acted as part of a feedback loop required for transcription of the entire *eut* operon. Typically, AraC/XylS-type transcriptional regulators are positive transcriptional activators that respond to a ligand and directly contact target DNA sequences to promote gene expression [29]. The EMSAs confirm that EutR directly contacts the *ler* promoter (Figure 2.4). Additional studies are necessary to fully elucidate the mechanism underlying EutR as a transcription factor. Because EutR is an activator of transcription and binds upstream of the promoter regions in *eutS* and *ler* (Figures 2.3 and 2.5), EutR may function by recruiting RNA polymerase to promote transcription [271], as opposed to relieving H-NS repression to increase expression of the *LEE* [272]. This hypothesis could be tested by performing *in vitro* transcription experiments with EutR in the presence and absence of EA and B_{12} to determine if EutR is actively promoting transcription

[273]. *In vitro* transcription experiments can also be performed with EutR and the repressor H-NS to determine if EutR counteracts H-NS repression to promote transcription. Additional experiments could look at the interplay of EutR with RNA polymerase in the presence and absence of EA and B_{12} by performing a bacterial two-hybrid assay [274]. These experiments would determine how EutR activates target gene transcription and if EA and B_{12} influence EutR interaction with RNA polymerase.

Our data provide new mechanistic information regarding EutR activation of the *eut* operon. We demonstrated that both EA and B_{12} are required to promote transcription of *eutS* (Figures 2.1 and 2.2). In addition to promoting expression of the *eut* operon, EutR is the major regulator involved in EA-dependent regulation of *ler* [109]. Expression of *ler* is significantly decreased in the Δ*eutR* strain of EHEC compared to wild-type EHEC, and the Δ*eutR* strain also displays diminished ability to form AE lesions on epithelial cells [109]. We address the nature of this regulation in this study. The EMSA data indicate that EutR binds directly to the *ler* (*LEE1*) promoter (Figure 2.4), suggesting a novel function for EutR as a direct regulator of genes critical for EHEC pathogenesis. Additional studies are needed to determine the binding kinetics of EutR to *ler* and *eutS*, which will provide information in regards to preferential binding of EutR to target DNA sequences.

Altogether, these data present the initial steps in understanding the mechanisms by which EA contributes to EHEC pathogenesis through the transcriptional regulator EutR. EutR responds to EA and B_{12} to promote expression of the *eut* operon. This may allow EHEC to compete with the indigenous microbiota

for nutrients and aid in EHEC colonization of the host. Additionally, EutR directly activates expression of *ler*, ultimately enabling EHEC to intimately attach to host epithelial cells and establish infection. The findings presented in this study may shed light on how other pathogens sense environmental cues in the GI tract to coordinate virulence gene expression and also highlight the notion that signaling networks involved in regulation of fundamental processes (i.e., metabolism) are often intricately linked to bacterial virulence [241, 275]. Additional studies are needed to fully elucidate the mechanism and global role of EutR gene regulation, the role of EA metabolism versus EA regulation of virulence genes, and the role of EA signaling *in vivo*. Future studies could examine a more global role for EutR by performing ChIP-seq to identify global DNA binding regions for EutR. Previous studies indicate that EHEC can utilize EA to grow in bovine intestinal contents; however, it is unclear how EA metabolism contributes to growth in a nutrient rich environment [224]. To further explore the interplay between EA metabolism and the regulation of virulence traits, we could perform competition experiments using an *eutR* deletion strain that cannot sense EA, and an *eutB* deletion strain that cannot metabolize EA. A recent study in *Salmonella* Typhimurium demonstrated that *Salmonella* utilizes EA to coordinate metabolism and virulence [228]. EA metabolism is important for *Salmonella* colonization of the GI tract and EutR signaling is important during systemic infection [228]. Key differences between *Salmonella* and EHEC are that EHEC is not an intracellular pathogen, does not disseminate, and cannot utilize EA as a carbon source, which *Salmonella* can. Our competition studies will determine the influence of EA metabolism and signaling to

EHEC colonization and virulence. Identifying the role of EutR *in vivo* is challenging due to the lack of an animal model to recapitulate all clinical manifestations of EHEC disease [146, 276]. Despite the limitations of EHEC infection models, the surrogate pathogen *Citrobacter rodentium* could be used to study the role of EutR during infection. These experiments would determine the role of EutR signaling *in vivo.*

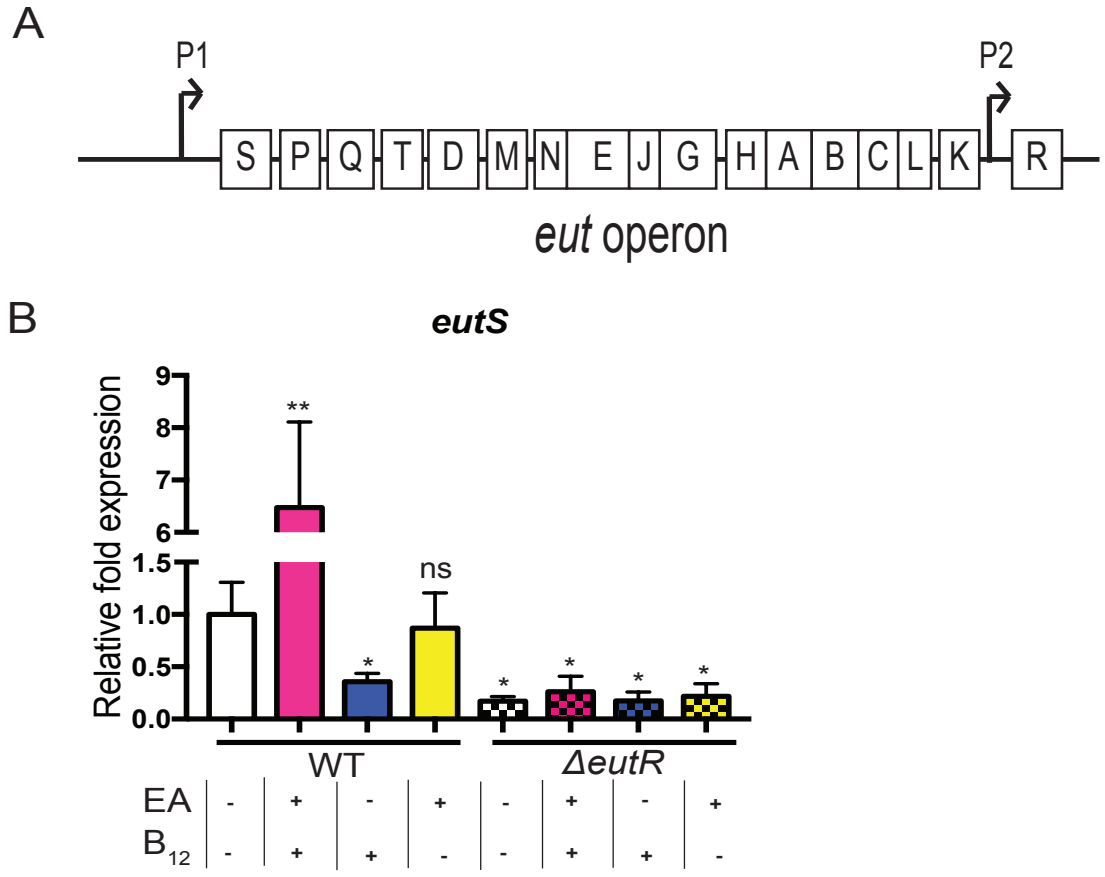


Figure 2.1 Expression of *eutS*, the first gene in the EA utilization operon (*eut*). (A) Schematic representation of the *eut* operon. (B) qRT-PCR of *eutS* from WT 86-24 anaerobically grown in DMEM in the presence and absence of EA and B_{12}, as indicated. n=3; error bars represent the geometric mean ±SD. Statistical significance was determined by a Student's *t* test and is shown relative to WT 86-24 grown in the absence of EA and B_{12}.*, P≤ 0.05; **, P≤ 0.005; ns = not significant.

Figure 2.2 Expression of *eutS*. RT-PCR of *eutS* from WT 86-24 anaerobically grown in DMEM in the presence and absence of EA and B_{12}, as indicated. Genomic DNA (gDNA) was used as a positive control and a reaction without RT was used as a negative control.

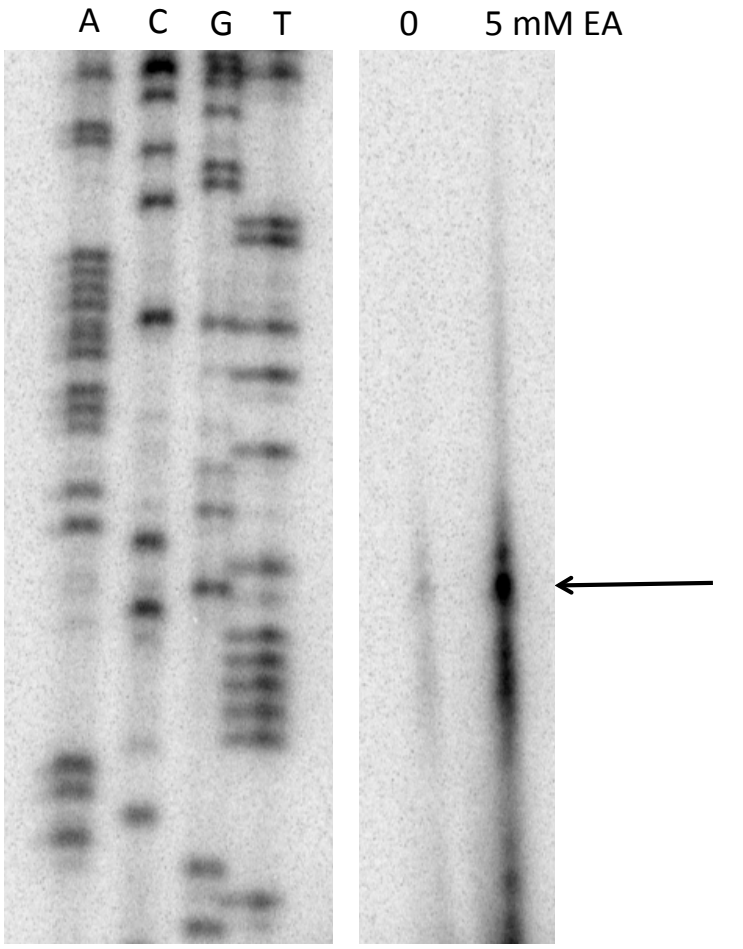


Figure 2.3 *eutS* promoter region. Primer extension assay of *eutS*. Lanes 1-4 show the *eutS* sequencing ladder. Lanes 5 and 6 show *eutS* cDNA made from RNA grown in the absence and presence of EA, respectively. The arrow represents the transcription start site of *eutS*. The promoter sequence of *eutS* is shown with the transcription start site and the predicted -10 and -35 regions. The bolded region is the EutR binding consensus sequence.

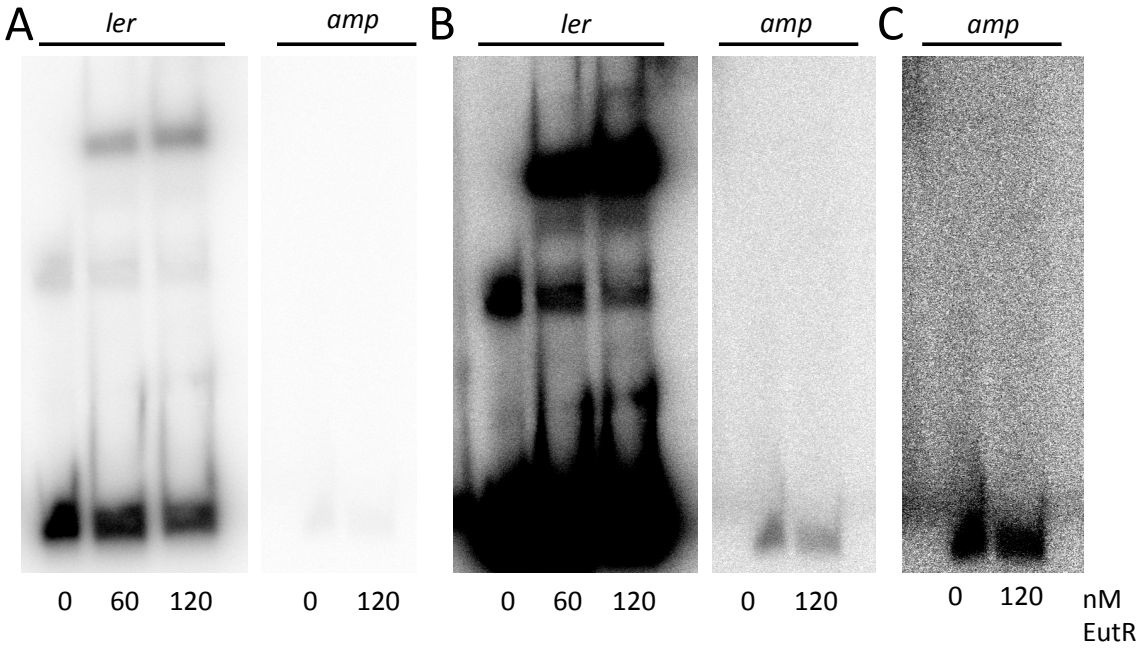
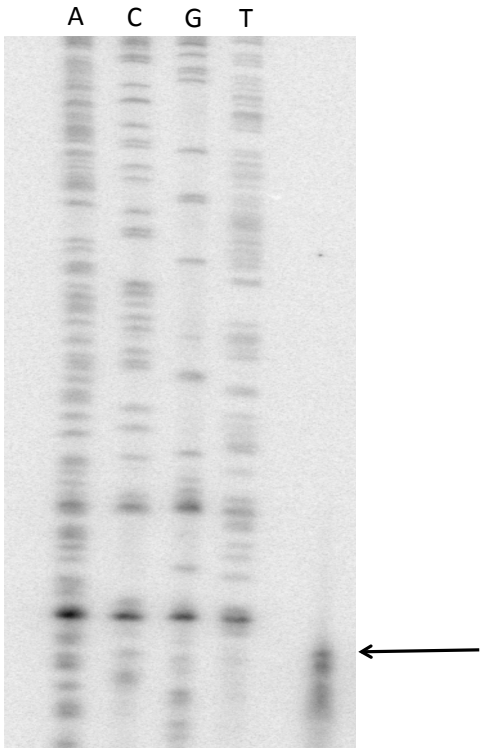


Figure 2.4 EutR directly binds to the *ler* promoter region to activate LEE expression (A) EMSA of the *ler* promoter and *amp* negative-control promoter region with EutR::MBP. (B) Darker exposure of the EMSA from (A). (C) Even darker exposure of the *amp* promoter region from (A).



ler promoter region

AAGG**T**G**GTT**G**TTT**GA**T**GAAATAGATGTGTCCTAATTTGATAGATAAACGTTATCTC
ACATAATTTATATCA TTTGATTAATTGTTGGTCCTTCCTGATAAGGTCGCTAATAG
-35 -10 +1

Figure 2.5 *ler* promoter region. Primer extension assay of *ler*. Lanes 1-4 show the *ler* sequencing ladder. Lanes 5 and 6 show *ler* cDNA made from RNA grown in the absence and presence of EA, respectively. The arrow represents the transcription start site of *ler*, which maps to the proximal promoter region [211]. The promoter sequence of *ler* is shown with the transcription start site and -10 and -35 regions. Bolded region is the EutR binding consensus sequence.

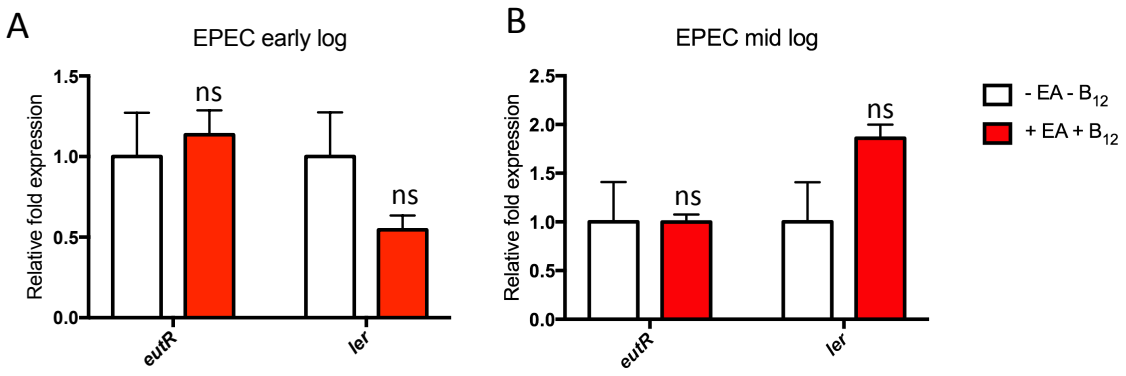


Figure 2.6 Expression of *eutR* and *ler* in WT EPEC E2348/69. qRT-PCR of *eutR* and *ler* from WT 86-24 in the presence and absence of EA and B_{12}, as indicated, to (A) early log and (B) mid log. n=3; error bars represent the geometric mean ±SD. Statistical significance shown relative to WT 86-24 grown in the absence of EA and ns = not significant.

Chapter Three: The ETT2-encoded regulator EtrB modulates virulence

Part of this chapter has been adapted from "The ETT2-encoded regulator EtrB modulates enterohemorrhagic *Escherichia coli* virulence gene expression"

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[1]G.G.W. and [2]M.J.W. analyzed the microarray data

Abstract

Enterohemorrhagic *Escherichia coli* O157:H7 (EHEC) is a foodborne pathogen that causes bloody diarrhea and hemolytic uremic syndrome throughout the world. A defining feature of EHEC pathogenesis is the formation of attaching and effacing (AE) lesions on colonic epithelial cells. Most of the genes that code for AE lesion formation, including a type three secretion system (T3SS) and effectors, are carried within a chromosomal pathogenicity island called the locus of enterocyte effacement (LEE). In this study, we report that a putative regulator, which is encoded in the cryptic *E. coli* type three secretion system 2 (ETT2) locus and herein renamed EtrB, plays an important role in EHEC pathogenesis. The *etrB* gene is expressed as a monocistronic transcript, and EtrB autoregulates expression. We provide evidence that EtrB directly interacts with the *ler* regulatory region to activate LEE expression and promote AE lesion formation. Additionally, we mapped the EtrB regulatory circuit in EHEC to determine a global role for EtrB. EtrB is regulated by the transcription factor QseA, suggesting that these proteins comprise a regulatory circuit important for EHEC colonization of the GI tract.

Introduction

Enterohemorrhagic Escherichia coli O157:H7 (EHEC) is a foodborne pathogen that causes severe bloody diarrhea, which may be associated with complications, including hemolytic uremic syndrome [253]. A defining feature of EHEC infection is the formation of attaching and effacing (AE) lesions on colonic enterocytes. AE lesions are characterized by intimate attachment of EHEC to epithelial cells, rearrangement of the actin cytoskeleton and effacement of the microvilli [62, 254, 255]. Most of the genes that mediate AE lesion formation are carried with the locus of enterocyte effacement (LEE) pathogenicity island [57]. The LEE is comprised of five major operons, named *LEE1-LEE5* [59, 61, 277], which encode a type three secretion system (T3SS) (*E. coli* type three secretion system-1 (ETT1)) [254], an adhesin (intimin) [255] and its receptor (Tir) [62], and secreted effectors [67, 256-259]. The LEE also encodes regulatory proteins, including the LEE1-encoded regulator Ler that activates expression of all of the LEE genes [59] as well as GrlA and GrlR, which positively and negatively influence LEE expression, respectively [100, 101, 215]. Additionally, the LEE-encoded T3SS translocates effector proteins encoded outside of the LEE that are also important for virulence [68, 70, 87, 100, 278-281].

In addition to the LEE-encoded T3SS, EHEC carries another locus that encodes a nonfunctional T3SS, named *E. coli* T3SS-2 (ETT2) [163, 229], and which shares homology to the *Salmonella* T3SS-1 [206]. The ETT2 pathogenicity island encodes five predicted or characterized transcription factors. A study by Zhang *et al.,* showed that two of these, EtrA and EivF, repress LEE expression and adherence

to epithelial cells, whereas YgeH displayed no regulatory phenotype [124]. The other ETT2-encoded putative transcription factors YqeI and YgeK, herein renamed EtrB, have not been characterized.

EHEC controls expression of virulence traits through complex regulatory circuits that are responsive to metabolites, host hormones, and bacterial signaling molecules, in addition to other environmental cues [109, 113, 114, 121, 236, 282-286]. For example, expression of the transcription factor QseA is induced through bacterial cell signaling as well as by ethanolamine, an abundant metabolite in the intestine [109, 214]. QseA is a LysR-type regulator that activates LEE expression by directly binding the *ler* promoter as well as promoting *grlA* transcription [46, 106, 214, 215]. Additionally, QseA controls expression of genes encoded in several O-islands, which are regions of the genome not carried in *E. coli* K-12 strains [163, 229]. Importantly, QseA positively regulates *etrB* expression [46]. Because ETT2-encoded proteins have been shown to influence virulence and because genetic data indicate that *etrB* is part of the QseA regulatory cascade, we hypothesized that EtrB plays a role in modulating EHEC virulence gene expression. Our findings indicate that EtrB activates LEE expression, not only through direct regulation, but also by repressing expression of *eivF* and *etrA*. Moreover, we mapped the EtrB regulon and report that EtrB also modulates expression of genes encoding distinct functions, including the non-LEE encoded effector NleA, a fimbrial adhesin, an sRNA, and maltose and tryptophan metabolism.

Materials and Methods

Strains, plasmids, growth conditions, and recombinant DNA techniques

Strains and plasmids used in this study are listed in Table A3.1. Standard methods were used to perform plasmid purification, PCR, ligation, restriction digests, transformations, and gel electrophoresis. Luria-Burtani (LB) broth (Invitrogen) or Dulbecco's modified Eagle's medium (DMEM; Invitrogen) was used to grow bacteria. For experiments with the *qseA* deletion strain (VS145), bacteria were grown overnight in LB broth and then diluted 1:100 in low-glucose DMEM and grown at 37°C, aerobically to late-exponential growth phase ($O.D._{600}$ of 1.0). For all other experiments bacteria were grown overnight in LB broth, diluted 1:100 in low-glucose DMEM and grown for 6 h statically at 37°C under a 5% CO_2 atmosphere. Streptomycin was added to overnight cultures of EHEC at a final concentration of 50 μg/ml. Overnight cultures of strains carrying pGEN, pDL01 (*etrB* in pGEN), or pDL03 (*etrB* promoter region in pGEN-luxCDABE) contained ampicillin to a final concentration of 100 μg/mL. The nonpolar EHEC 86-24 *etrB* mutant (DL01) was constructed using λ-red mutagenesis [287]. The mutant was complemented with *etrB* under the endogenous promoter cloned into pGEN-MCS [288] (Addgene MTA) using the restriction enzymes HindIII and NcoI (NEB). When complement data are shown, the WT and Δ*etrB* strains contain empty vector controls. Strains and plasmids were confirmed by DNA sequencing. Primers used to generate the *etrB* deletion and complement strains are listed in Table A3.2.

Measurement of etrB expression

The plasmid pGEN-luxCDABE [288] was used to create the *etrB* expression plasmid named pDL03. For this, approximately 332 base pairs of the etrB promoter region was inserted upstream of *luxCDABE* using the restriction enzymes PmeI and SnaBI (NEB). Luminescence was measured using a VICTOR Wallac luminometer (Perkin-Elmer). Luminescence was corrected for $O.D._{600}$ for each condition. Statistical significance was determined by a Student's t-test.

RNA extraction and qRT-PCR

RNA purification and quantification of RNA transcription was performed as described previously [228]. RNA was extracted from three biological replicate cultures of each strain/condition using the RiboPure Bacteria RNA isolation kit (Ambion). The amplification efficiency and template specificity of each of the primer pairs (Table A3.2) were validated and reaction mixtures were prepared as previously described [233]. Quantitative real-time PCR (qRT-PCR) was performed using a one-step reaction with an ABI 7500-FAST sequence detection system (Applied Biosystems). Data were collected using the ABI Sequence Detection 1.2 software (Applied Biosystems). All data were normalized to levels of *rpoA* and analyzed using the comparative cycle threshold (C_T) method [261]. Target gene expression levels were compared by the relative-quantification method [261]. Statistical significant was determined by a Student's t-test.

Reverse transcriptase PCR (RT-PCR)

SuperScipt II reverse transcriptase (Invitrogen) and random primers were used to create cDNA from RNA samples. The cDNA was used for PCR with gene-specific primers (Table A3.2). Genomic DNA was used as a positive control, and a reaction without reverse transcriptase was used as a negative control.

Fluorescent actin staining (FAS) assay

FAS assays were performed as described previously [289]. Briefly, overnight bacterial cultures were grown in LB at 37°C and then diluted 1:100 to infect HeLa cells. Infected HeLa cells were grown on coverslips for 6 h at 37°C with 5% CO_2. Subsequently, the coverslips were washed, fixed with formaldehyde, then the membranes were permeabilized with 0.2% Triton-X and stained with fluorescein isothiocyanate-labeled phalloidin to visualize actin. Bacteria and HeLa cell nuclei were stained with propidium iodide. AE lesions formed by each strain were enumerated for at least 400 HeLa cells in each experiment. Two independent experiments with three biological replicates of each condition were performed. Statistical significant was determined by a Student's t-test.

Secreted protein and whole-cell lysate immunoblotting

Secreted proteins were collected as previously described [254]. Secreted proteins from culture supernatants were separated from bacterial cells using centrifugation and filtration. SDS-PAGE and immunoblotting were performed as previously described [262]. Whole-cell lysates were prepared by separating

bacterial cells from supernatants, re-suspending in 1X PBS, and boiling. Samples were subjected to immunoblotting with rabbit polyclonal antiserum to EspA and visualized with enhanced chemiluminescence (BioRad). RpoA (Neoclone) was visualized as a loading control for whole-cell lysates. Coomassie blue staining was used to visualize bovine serum albumin (BSA) loading controls. Expression of EspA was quantified from three replicate samples using ImageJ and normalized to BSA. Expression levels are shown relative to WT. Three independent experiments were performed.

Purification of EtrB and QseA

The EtrB protein was fused with the maltose-binding protein (MBP) using the pMAL-C5x vector (NEB) with the restriction enzymes NcoI and SbfI (NEB) to create pDL02. E. coli strain BL21 (DE3) containing pDL02 was grown at 37°C in LB with glucose (0.2% final concentration) and ampicillin (100 μg/mL) to an $O.D._{600}$ of 0.5. IPTG was added to a final concentration of 0.3 M and protein expression was induced overnight at 16°C. Cells were then pelleted by centrifugation at 4,000 g for 10 min and resuspended in column buffer (20 mM Tris-HCL, 200 mM NaCl, 1 mM EDTA). The cells were lysed with an emulsiflex. The lysed cells were centrifuged at 4°C and the supernatant was loaded onto a gravity column (Qiagen) with amylose resin. The column was washed with column buffer and protein was eluted from the column using column buffer containing 10 mM maltose. Fractions containing purified EtrB were confirmed by SDS-PAGE and Western analysis. The His-tagged QseA protein from plasmid pMK08 was purified as previously described [46].

Briefly, the *E. coli* strain BL21 (DE3) containing pMK08 was grown to an $O.D._{600}$ of 0.5 and then induced with 0.4 M IPTG for 3 h. Cells were lysed as described above and purification was performed using gravity columns (Qiagen) with nickel beads. The column was washed with nickel wash buffer (50 mM $NaPO_4$, 300 mM NaCl, 20 mM imidazole) and protein was eluted using nickel wash buffer containing 250 mM imidazole.

Electrophoretic mobility shift assays (EMSAs)

EMSAs were performed using the purified EtrB-MBP, QseA-His, and PCR-amplified DNA probes (Table A3.2), as previously described [110]. DNA probes were end-labeled using T4 polynucleotide kinase (NEB) with (^{32}P) ATP (Perkin-Elmer) [262]. End-labeled probes were purified using the Invitrogen NucAway Spin Columns. EMSAs were performed by adding increasing amounts of purified QseA or EtrB protein to end-labeled probe in binding solution (500 µg/mL BSA, 50 ng poly(dI-dC), 60 mM HEPES pH 7.5, 5 mM EDTA, 3 mM dithiothreitol (DTT), 300 mM KCl, and 25 mM $MgCl_2$) and incubated at room temperature for 25 minutes. A 1% Ficoll solution was added to the reaction mixtures immediately before loading the samples on the gel. The reactions were electrophoresed on a 6% polyacrylamide gel for approximately 6 h at 150 V, dried, and imaged with a PhosphorImager (Molecular Dynamics). Bands were quantified using ImageQuant software as indicated.

Primer Extension

Primer extension analysis was performed as previously described [46]. Briefly, primer etrB_PE_R (Table A3.2) was end-labeled as described above. A total of 40 μg of RNA, isolated from strain 86-24, was used to generate cDNA using the Primer Extension System— AMV Reverse Transcriptase kit (Promega). The resultant cDNA was precipitated, electrophoresed on a 6% polyacrylamide-urea gel next to a sequencing reaction (Affymetrix). Amplified genomic DNA from strain 86-24 was used to generate the sequencing ladder using primers etrB_prom_F1 and etrB_PE_R for the *etrB* promoter.

Microarray

Affymetrix 2.0 *E. coli* gene arrays were used to compare gene expression of strain 86-24 to that of DL01 (Δ*etrB*) as previously described [109]. The RNA processing, labeling, hybridization, and slide-scanning procedures were performed as described in the Affymetrix Gene Expression technical manual. Data analyses from the array were performed as previously described [290]. The Affymetrix GeneChip Command Console Software (AGCC) was used to obtain the output from scanning a single replicate of the Affymetrix GeneChip *E. coli* Genome 2.0 array for each of the biological conditions, following the manufacturer's instructions. Data were normalized using Robust Multiarray analyses (RMA), and the resulting data were compared to determine genes whose expression was increased or decreased in response to the presence of *etrB*.

Results

Identification and characterization of etrB.

The gene *etrB* (*ygeK*, MG1655 genome; *Z4176*, EDL933 genome; *ECs712*, Sakai genome [163, 229]) is carried in the ETT2 pathogenicity island (Figure 3.1A) and is predicted to encode a 16 kDa protein that shares homology to NarL-type transcription factors, including the *Salmonella* regulatory protein SsrB [206]. The *etrB* open reading frame (ORF) is present in several *E. coli* strains; however, bioinformatics analyses indicate that etrB is a pseudogene in nonpathogenic *E. coli* K-12 strains [206]. In pathogenic EHEC strains, the potential functionality of EtrB is less clear. The Sakai and EDL933 annotated genome sequences predict different ORFs, with etrB encoding a 663 bp gene in Sakai or a 447 bp gene in EDL933 [163, 229]. We study EHEC pathogenesis using the strain 86-24, which was isolated from a patient suffering hemorrhagic colitis [149] and has been used in several EHEC animal studies [291-298]. The ETT2 genetic locus is located between the *araE* and *glyU* backbone (conserved) genes in EHEC. To begin to characterize *etrB* in strain 86-24, we performed RT-PCR to determine whether *etrB* was expressed as part of a transcript with the adjacent up- or downstream genes (depicted in Figure 3.1B). For this, we used cDNA synthesized from RNA that was purified from WT EHEC grown statically for 6 h in DMEM. No PCR products were obtained in reactions that included primers specific for the flanking genes (Figure 3.1B and C); however, a PCR product was visible when primers specific for *etrB* were used in the reaction (Figure 3.1C). These findings indicate that *etrB* is expressed in EHEC strain 86-24, but that it is not co-transcribed with the immediate up- or downstream genes.

To further characterize *etrB* expression and map the promoter region, we performed primer extension analyses. For this, we designed a primer approximately 50 base pairs downstream from the etrB translational start codon. Then, primer extension analysis was performed using cDNA synthesized from RNA. The primer extension results revealed one transcriptional start site in the *etrB* promoter (Figure 3.2), which we mapped to approximately 44 base pairs upstream of the translational start site. These data are consistent with the annotation of the EDL933 genome. These data suggest a -10 sequence, TGTAAC, and the - 35 sequence, CTGAAG, which contain two and three mismatches from the σ^{70} consensus sequences (TATAAT and TTGACA), respectively, and which are separated by 15 nucleotides (Figure 3.2A). There is no match to alternative sigma factor consensus sequences such as σ^{32}, σ^{54}, and σ^{28}. Site directed mutagenesis of the putative *etrB* promoter should be performed to verify the σ^{70} consensus sequences.

To begin to characterize EtrB expression, we examined whether EtrB autoregulates transcription. For this, we generated a deletion of *etrB*. The deletion of etrB did not impact EHEC growth rate in our experimental conditions, as the wild type (WT) and *ΔetrB* strains reached similar O.D.s after 6 h of static growth in DMEM (WT $O.D._{600}$ = 0.841 +/- 0.008; *ΔetrB* $O.D._{600}$ = 0.842 +/- 0.004). Then, we transformed the WT and the *ΔetrB* strains with a plasmid containing the etrB promoter fused to the *luxCDABE* gene cluster that encodes bacterial luciferase [299]. Expression of *etrB*::*lux* was significantly decreased in the ΔetrB strain compared to WT (Figure 3.2B), indicating that EtrB positively autoregulates expression.

Proteins belonging to the NarL-family bind DNA to regulate transcription [47]. Therefore, we performed EMSAs to investigate whether EtrB binds its own promoter to regulate transcription. For this, we constructed a plasmid that expresses a fusion protein in which the C terminus of MBP was fused to the N terminus of EtrB. EMSAs indicated that EtrB directly binds its promoter to regulate expression (Figure 3.3A). To confirm specificity of binding, we performed EMSAs with purified MBP alone as well as competition EMSAs. Purified MBP did not bind the *etrB* promoter (Figure 3.3A). Moreover, EtrB binding was outcompeted by the addition of unlabeled *etrB* probe, whereas no competition was observed when increasing amounts of cold *kan* probe was added (Figure 3.3B). Altogether, these findings indicate that *etrB* is expressed and encodes a functional protein that controls its own expression. Accordingly, we renamed *ygeK etrB* (ETT2 transcriptional regulator B) following the nomenclature of Zhang *et al.* [124].

QseA activates etrB expression

We originally identified *etrB* in a microarray study as being activated by QseA [46]. Here, we confirmed that QseA influences *etrB* transcription and tested whether this was through direct interaction (Figure 3.4A). First, we performed qRT-PCR analyses using RNA extracted from WT, the *ΔqseA*, or the *qseA* (*qseA*+) complemented strain. Transcription of etrB was significantly decreased in *ΔqseA* strain compared to WT, and complementation of the *ΔqseA* strain restored *etrB* expression to WT levels (Figure 3.4B). To determine whether QseA influenced *etrB* expression directly, we performed EMSAs. We observed a shift with the addition of

purified QseA to radiolabeled *etrB* promoter DNA; however, no shift was observed using the negative control *kan* DNA (Figure 3.4C). Quantification of radiolabeled DNA confirmed that the amount of shifted *etrB* DNA correlated with increasing amounts of QseA added to the reaction (Figure 3.4D). Collectively, these data indicate that *etrB* is a direct target of QseA regulation.

Expression of *qseA* is increased in the presence of EA, in an EutR-dependent manner [109]. Because of the interaction between EutR and *qseA*, we hypothesized that EutR may contribute to *etrB* expression. To test this hypothesis, we performed qRT-PCR. The data reveal that *etrB* expression is similar in the presence or absence of EA (Figure 3.5). Levels of *etrB* were unchanged in the *ΔeutR* strain compared to WT and were unchanged in the *ΔeutR* strain in the presence and absence of EA (Figure 3.5), indicating that EutR has no effect on *etrB* expression.

EtrB activates LEE transcription

To determine whether EtrB plays a role in directing virulence gene expression in EHEC, we performed qRT-PCR and measured expression of one gene in each LEE operon (Figure 3.6A), as well as expression of the *stx2a* gene, which encodes Shiga toxin. Transcription of *ler*, encoded in *LEE1*, as well as transcription of *grlA* and *LEE2-LEE5* was significantly decreased in the *ΔetrB* strain compared to WT (Figure 3.6B); however, no differences in *stx2a* expression were measured (Figure 3.6C). Additionally, trans complementation with *etrB* on a low-copy plasmid nearly restored LEE mRNA and protein to WT levels (Figure 3.6D-F). Levels of *escC*

were higher in the *etrB* complement strain compared to the *ΔetrB* strain but not restored to WT levels (Figure 3.6D).

To functionally test the impact of EtrB on LEE expression, we assessed AE lesion formation. For this, we performed a FAS assay [289] and determined the number of pedestals formed on epithelial cells by WT EHEC, the *ΔetrB* strain, or the *etrB* complemented strain. In agreement with the LEE expression data, we measured significantly fewer pedestals when HeLa cells were infected with the *ΔetrB* strain compared to cells infected with WT EHEC, and the *etrB* plasmid was able to complement the Δ*etrB* strain (Figure 3.7A and B). This suggests that the increase in *escC* mRNA levels and the increase in EspA protein levels *etrB* complement strain compared to the *ΔetrB* strain are sufficient to restore to WT levels.

To determine whether EtrB directly regulates LEE expression, we performed EMSAs. For these experiments, we generated a probe containing the entire *ler* regulatory region. EtrB shifted the radiolabeled ler DNA, but not the negative-control amp promoter (Figure 3.8A). To ensure specificity of binding, we performed EMSAs using MBP alone as well as competition EMSAs as described for the *etrB* promoter. In these assays, no shift was observed with MBP alone (Figure 3.8A). Additionally, the unlabeled *ler* probe competed for EtrB binding at a ratio of 1:1 (labeled probe to unlabeled probe) (Figure 3.8B); however, the negative-control *kan* probe exhibited no competition for binding (Figure 3.8B). These findings indicate a specific and direct interaction between EtrB and the *ler/LEE1* regulatory region.

To further verify previous data demonstrating that EA does not influence *etrB* expression (Figure 3.5), we assessed how EA affects EtrB regulation of the LEE. We evaluated LEE expression by measuring EspA levels through Western blot analysis and determined that expression of EspA was significantly lower in the *ΔetrB* strain compared to WT in the presence or absence of EA (Figure 3.9). Overall, these data indicate that the EtrB regulatory circuit is independent of the EA regulatory circuit.

EtrB transcriptome analyses

To investigate the global role of EtrB in EHEC gene regulation, we performed transcriptome analyses using the Affymetrix *E. coli* 2.0 gene microarrays. These data revealed that EtrB functions to both positively and negatively influence gene expression in EHEC. For example, 46 genes probe sets were decreased and 70 probes sets were increased in the Δ*etrB* strain compared to WT (using a ≥ 2-fold change in expression as the cut off for differentially regulated probes). Genes regulated by EtrB included virulence factors, such as the LEE genes and non-LEE encoded effectors, adhesins and ETT2-encoded genes, genes important for metabolism, and the non-coding RNA, RyeA/SraC.

We confirmed a subset of transcripts that were differentially regulated in the array. NleA is an effector encoded outside of the LEE, but which is secreted through the LEE- encoded T3SS. In EHEC, NleA has diverse functions and has been shown to disrupt tight junctions of epithelial cells, inhibit protein secretion, and modulate the host immune response [70, 300, 301]. Moreover, an *nleA* deletion strain is

attenuated during murine infection, highlighting its importance to EHEC pathogenesis [70]. EtrB positively influences *nleA* expression, as *nleA* transcription was decreased in the Δ*etrB* strain compared to WT (Figure 3.10A). NleA expression and secretion is at least partly dependent on Ler [90], thus, the decrease of NleA in the Δ*etrB* strain may be an indirect result of EtrB-dependent regulation of LEE expression.

Besides activating expression of virulence factors, EtrB positively influences transcription of genes involved in metabolism and post-transcriptional gene expression. For example, genes involved in tryptophan and maltose utilization were decreased in the *ΔetrB* strain (Figure 3.10B and C). Additionally, transcript levels of the sRNA RyeA/SraC were decreased in the *ΔetrB* strain compared to WT (Figure 3.10D). RyeA/SraC is present as a 270 bp RNA during exponential growth, and is processed to a shorter 150 bp RNA during stationary phase [302-304]. These findings suggest that RyeA/SraC plays a role during stress response; however, the biological role of this sRNA is not known.

EHEC encodes 16 fimbrial loci, which mediate attachment to epithelial cells. The fimbrial locus 11 (*loc11*) belongs to the chaperone-usher family of adhesins. These types of fimbriae typically are composed of a chaperone, an usher, and a major fimbrial subunit, and may also include additional minor subunits [305]. The microarray data indicated that EtrB represses expression of *loc11*. This locus contains seven ORFs, all of which have been shown to be expressed and co-transcribed [166]; therefore, we confirmed the microarray data by measuring expression of the first gene in this operon, *Z4498*, which is predicted to encode the

major fimbrial subunit. Expression of *Z4498* was significantly increased in the *ΔetrB* strain compared to WT EHEC (Figure 3.11A), and complementation restored expression to WT levels (Figure 3.11B). Collectively, these data suggest that EtrB is important for coordinating expression of fimbriae- and LEE-mediated adherence.

The ETT2 pathogenicity island carries 35 ORFs that are predicted to encode effectors, components of a T3SS and transcription factors, as well as pseudogenes [163, 206, 229]. The microarray data indicated that EtrB represses expression of ORFs encoded in the ETT2 pathogenicity island. For example, the expression of the ORFs *ygeI* and *yqeK* were significantly increased in the *ΔetrB* strain compared to WT (Figure 3.11C). Additionally, expression of the ETT2-encoded regulators *eivF* and *etrA* were increased in the *ΔetrB* strain (Figure 3.11C).

Discussion

The ETT2 locus is present in the majority of *E. coli* strains; however, many of the ETT2 gene clusters carry mutations and deletions, suggesting that the ETT2 T3SS is not functional [206]. Despite this fact, deletions of ETT2-encoded regulatory or structural proteins impact virulence in EHEC as well as in meningitis-causing *E. coli* strains [209, 306, 307]. However, no biochemical evidence of how ETT2-encoded genes affect pathogenesis has been reported. EtrB belongs to the NarL-family of transcription factors, which can function independently or function as part of a two-component system (TCS) [48, 49]. The *etrB* gene is not encoded adjacent to a putative histidine kinase, which is typical for a cognate TCS [308]. A previous study predicted that EtrB might function as an orphan response regulator and

demonstrated that purified EtrB could be phosphorylated *in vitro* [309]; however, the physiological relevance of this was not further examined. Our studies indicate that EtrB is able to bind target DNA in the absence of phosphorylation (Figures 3.3 and 3.8), suggesting that EtrB acts as an independent transcription factor.

We mapped the EtrB regulon, and our data revealed that EtrB plays a broad role in EHEC gene expression, affecting expression of genes important for virulence, metabolism, and post-transcriptional gene expression (Figures 3.6, 3.10, 3.11, and summarized in Figure 3.12). Specifically, EtrB activates LEE expression through direct interaction with the *LEE1* regulatory region (Figure 3.8). This is distinct from the regulatory influence of EtrA and EivF, which repress LEE expression [124]. Interestingly, *etrA* and *eivF* expression was increased in the Δ*etrB* strain (Figure 3.11), suggesting that EtrB functions to promote AE lesion formation not only by direct interaction with the *LEE1* regulatory region but also by repressing expression of negative regulators of the LEE.

Finally, we provide biochemical evidence showing that *etrB* is a direct regulatory target of QseA. Therefore, we propose that QseA and EtrB function in a coherent feed-forward loop (FFL) [310]. This FFL has important implications for the regulatory dynamics of LEE expression. For example, the coordination of multiple regulators might act to amplify environmental cues and promote AE lesion formation. Additionally, a previous study demonstrated that QseA negatively regulates its own transcription [216]. In this model, as QseA levels decrease due to autoregulation, EtrB could still act to promote LEE expression, thereby prolonging expression to ensure efficient colonization of the GI tract. Overall, this study has

identified EtrB as an important regulator of gene expression in EHEC and provides a mechanistic understanding as to how ETT2-encoded regulators influence bacterial pathogenesis.

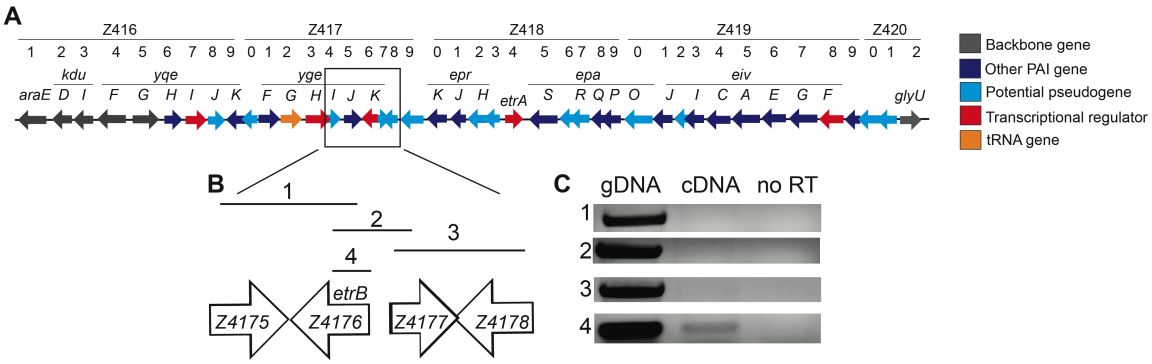


Figure 3.1 *etrB* expression. (A) Schematic representation of the ETT2 genetic locus located between the *araE* and *glyU* backbone genes in EHEC. (B) Inset of *etrB* and adjacent genes. Lines with numbers indicate amplified regions and correspond to PCR reactions shown in (C). (C) RT-PCR of *etrB* and adjacent genes. Genomic DNA (gDNA) was used as a positive control and a reaction without RT was used as a negative control.

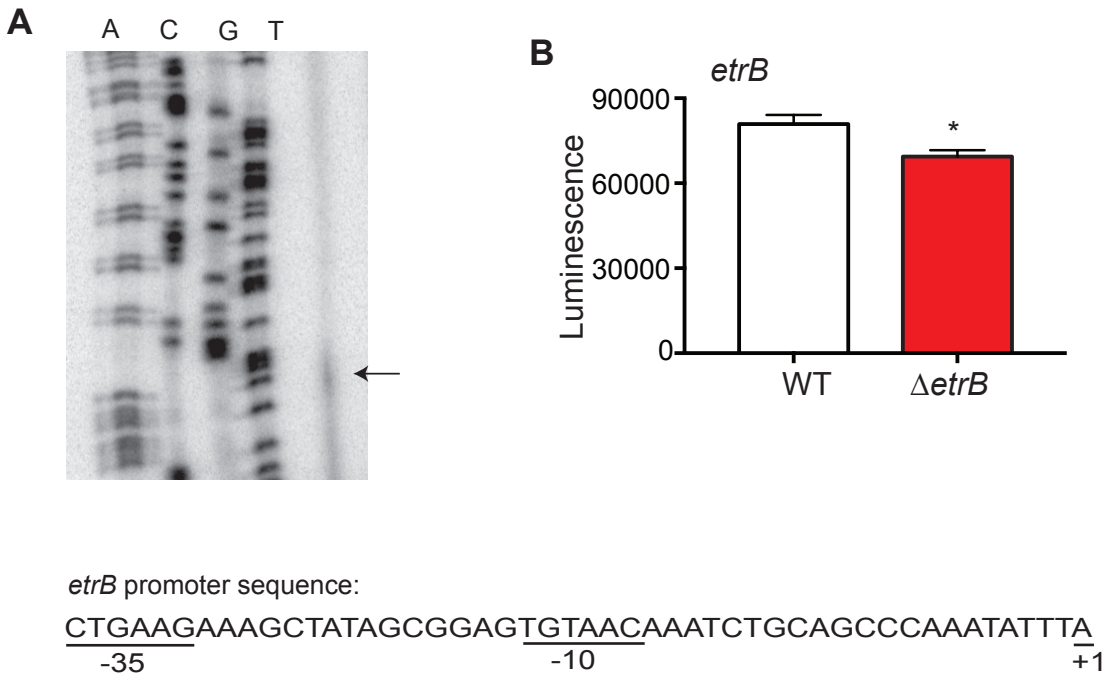


Figure 3.2 *etrB* promoter and autoregulation. (A) Primer extension assay of *etrB*. Lanes 1-4 show the *etrB* sequencing ladder. The arrow represents the transcription start site of *etrB*. The promoter sequence of *etrB* is shown with the predicted transcription start site and -10 and -35 regions. (B) Expression of *etrB* in WT 86-24 or the Δ*etrB* strain transformed with the *etrB::lux* reporter. n =3; error bars represent the geometric mean ±SD. *, P≤ 0.05.

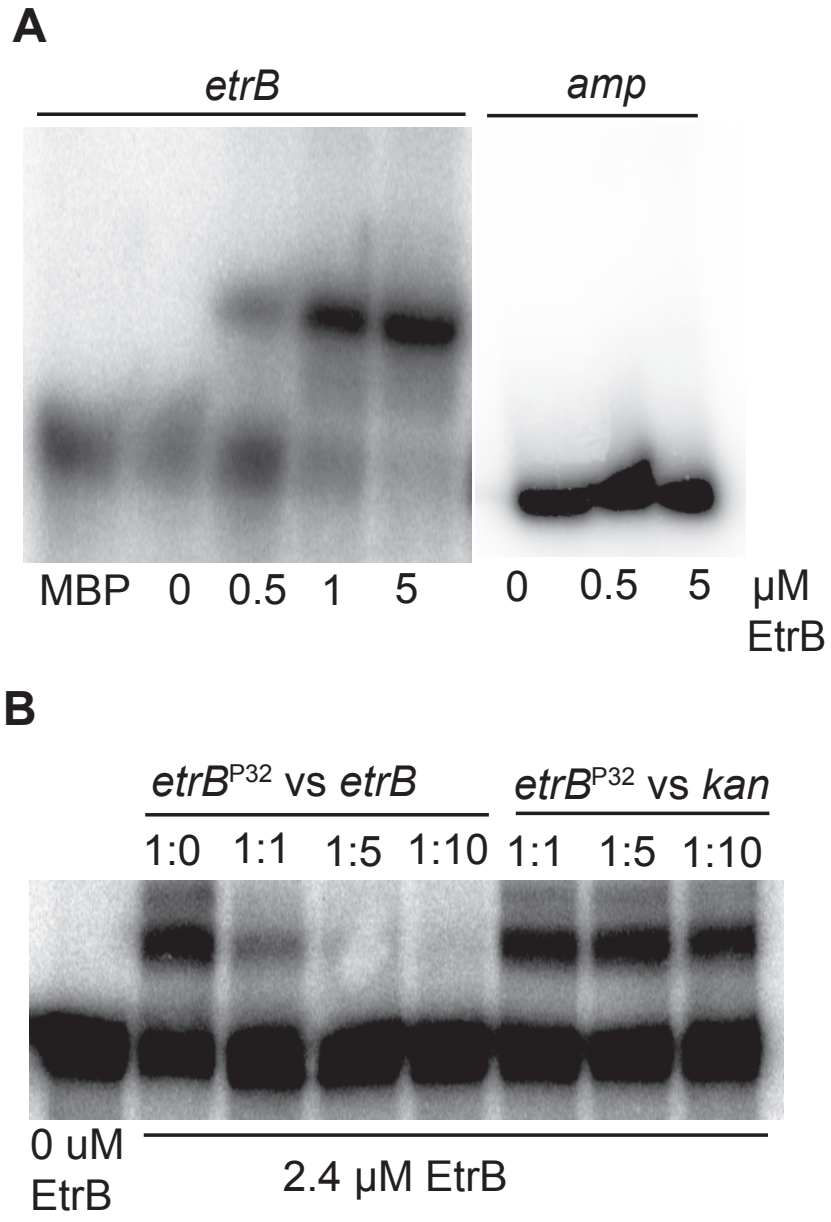


Figure 3.3 *etrB* directly autoregulates expression. (A) EMSA of the *etrB* promoter and *amp* negative-control promoter region with MBP or EtrB::MBP. (B) Competition assays with EtrB. The assay was performed with increasing amounts of the unlabeled *etrB* probe or the unlabeled *kan* probe as a negative-control.

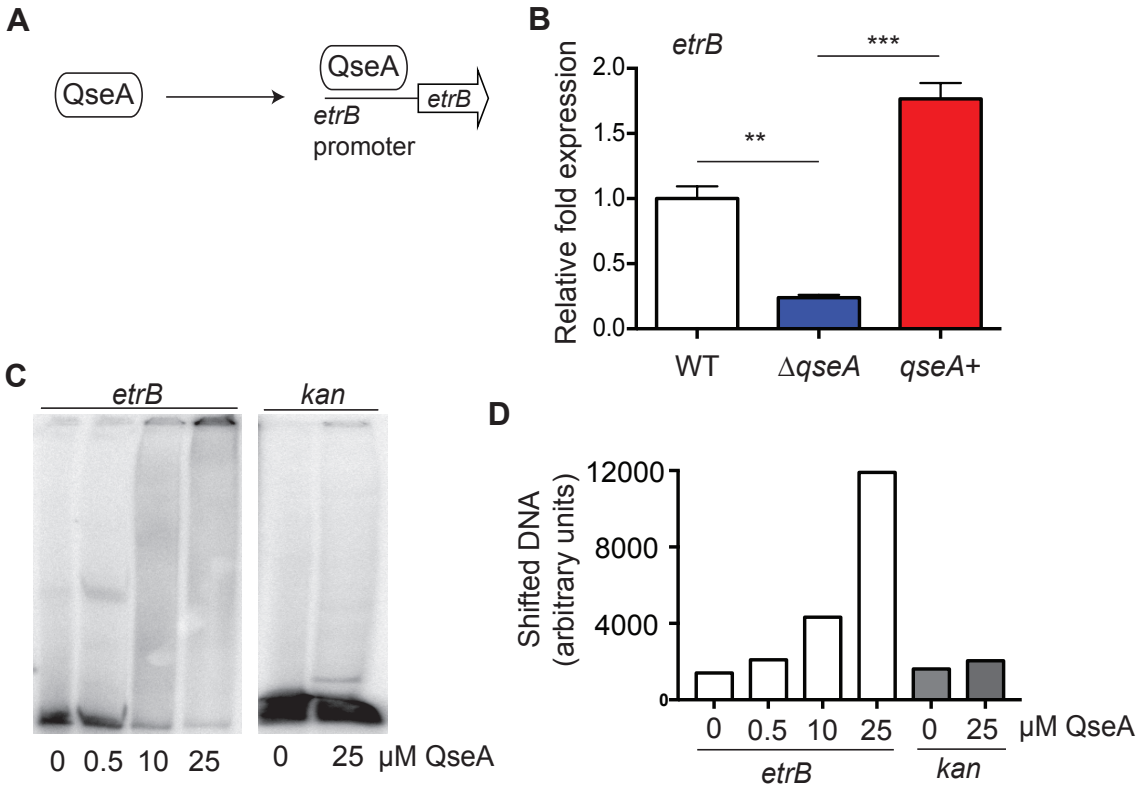


Figure 3.4 QseA activates *etrB* expression. (A) Schematic of model being tested. (B) qRT-PCR of etrB expression in WT 86-24, the Δ*qseA* strain, and *qseA* complemented strain (*qseA*+). n=3; error bars represent the geometric mean ±SD. **, P≤ 0.005; ***, P≤ 0.0005. (C) EMSA of the *etrB* promoter and the *kan* negative-control promoter regions with QseA protein. (D) Quantification of shifted *etrB* or *kan* DNA depicted in (C).

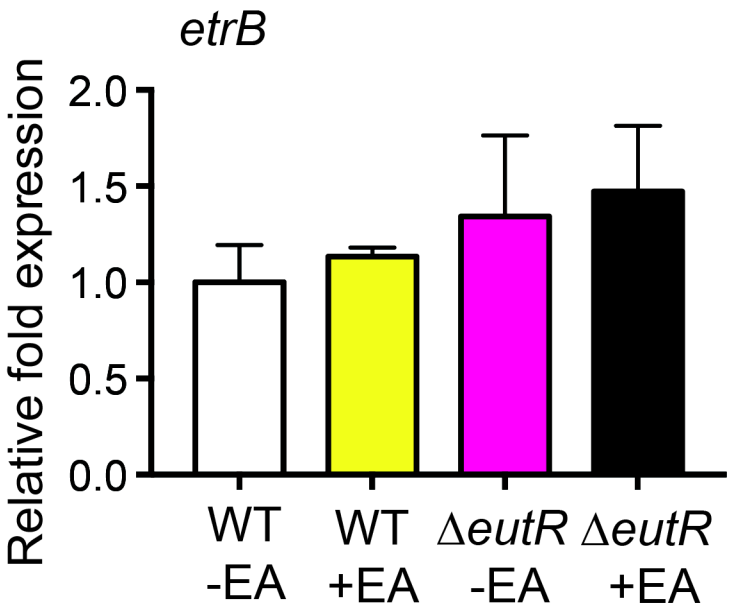


Figure 3.5 *etrB* expression is not influenced by EA or EutR. qRT-PCR of *etrB* in WT 86-24 and the Δ*eutR* strain in the presence and absence of EA.

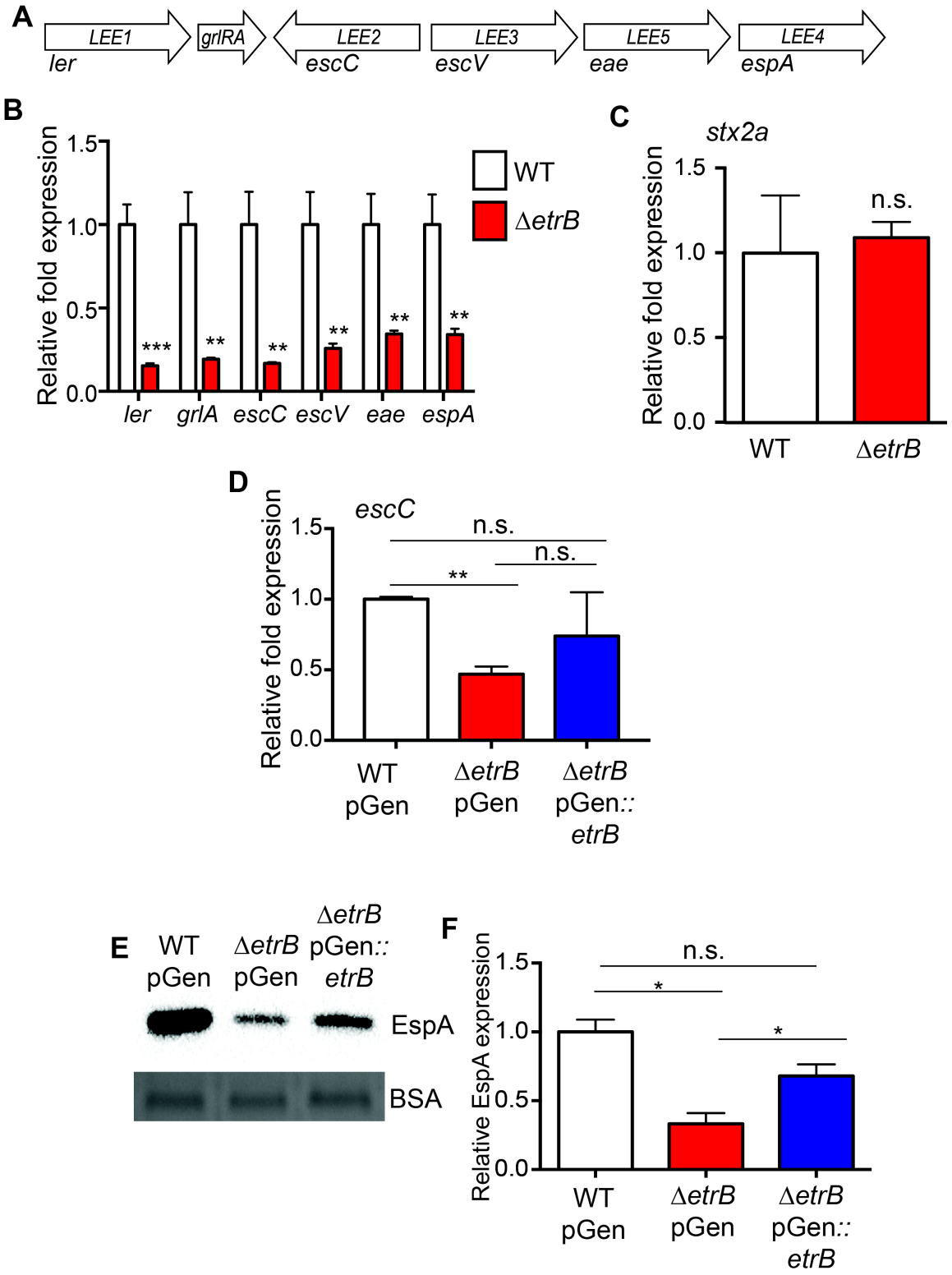


Figure 3.6 EtrB activates expression of the LEE. (A) Schematic of the LEE pathogenicity island. (B) qRT-PCR of *ler, grlA, escC, escV, eae,* and *espA* in WT 86-24

and the Δ*etrB* strains. (C) Expression of *stx2a* by qRT-PCR in WT 86-24 and the Δ*etrB* strains. (D) Expression of *escC* by qRT-PCR in WT 86-24 transformed with an empty vector control, the Δ*etrB* strain transformed with an empty vector control, and the Δ*etrB* strain complemented with *etrB*. (E) Representative Western blot of the LEE-encoded EspA secreted protein in WT 86-24 transformed with an empty vector control, the Δ*etrB* strain transformed with an empty vector control, and the Δ*etrB* strain complemented with *etrB*. BSA, bovine serum albumin is shown as a loading control. (F) Quantification of EspA expression from three independent assays from EHEC strains as described in (E). *, P≤ 0.05; **, P≤ 0.005; ***, P≤ 0.0005; ns = not significant.

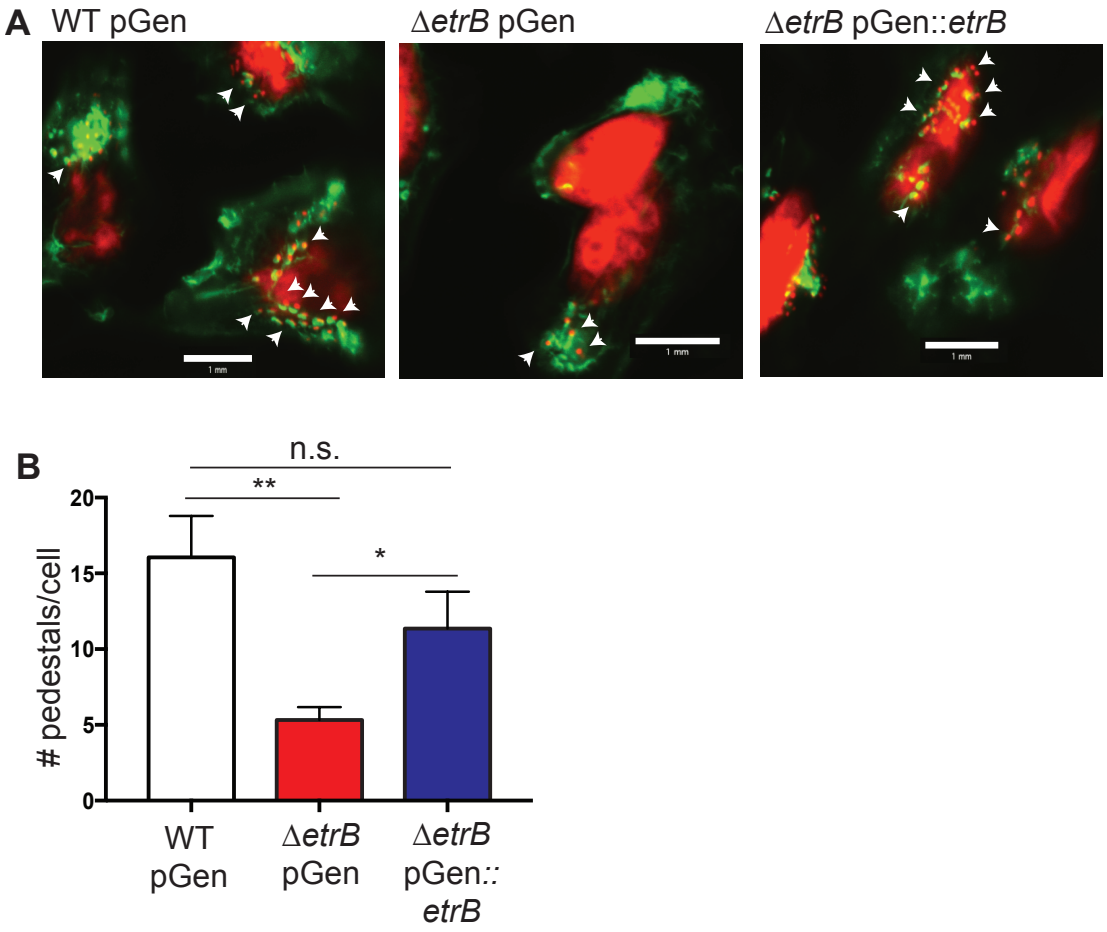


Figure 3.7 EtrB activates AE lesion formation (A) FAS assay with WT 86-24 transformed with an empty vector control, the Δ*etrB* strain transformed with an empty vector control, and the Δ*etrB* strain complemented with *etrB*. HeLa nuclei and bacteria were stained red with propidium iodide, and HeLa cell actin cytoskeleton was stained green with FITC-phalloidin. AE lesions are observed as punctate green structures associated with bacterial cells and are indicated by arrowheads. Scale bar = 1 mM. (B) Number of AE lesions per HeLa cell. Statistical significance shown relative to WT 86-24 unless otherwise indicated. *, P≤ 0.05; **, P≤ 0.005; ***, P≤ 0.0005; ns = not significant.

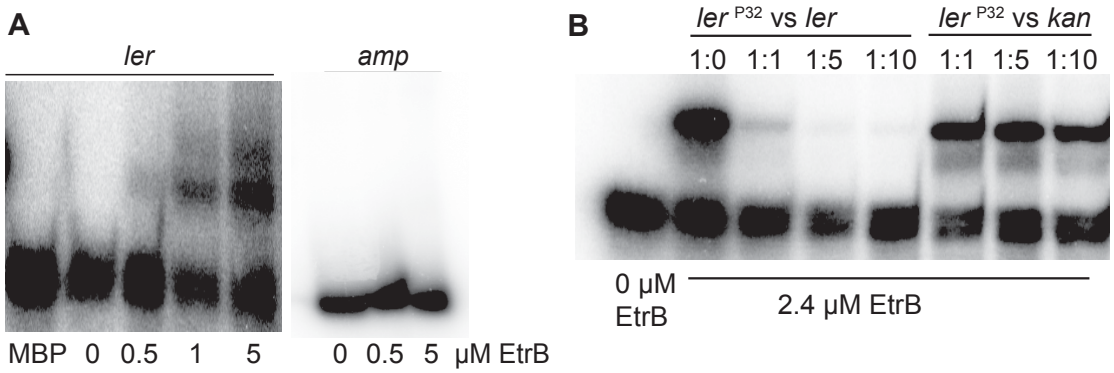


Figure 3.8 EtrB directly binds to the *ler* promoter region to activate LEE expression (A) EMSA of the *ler* promoter and *amp* negative-control promoter region with MBP or EtrB::MBP. (B) Competition assays with EtrB. The assay was performed with increasing amounts of the unlabeled *ler* probe or the unlabeled *kan* probe as a negative-control.

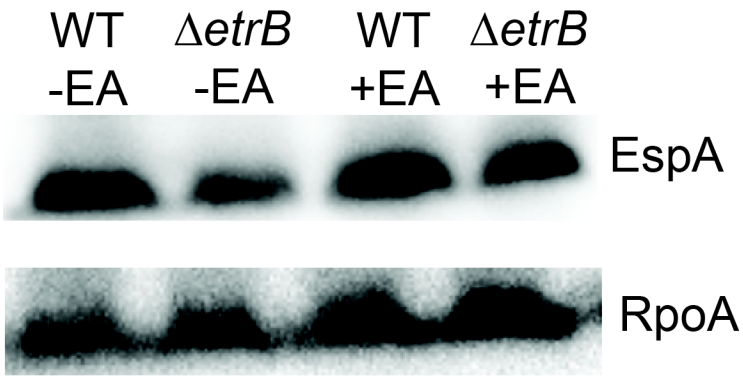


Figure 3.9 EA does not affect EtrB regulation of EspA. Representative Western blot of the LEE-encoded EspA secreted protein in WT 86-24 and the Δ*etrB* strain in the presence and absence of EA. RpoA is shown as a loading control.

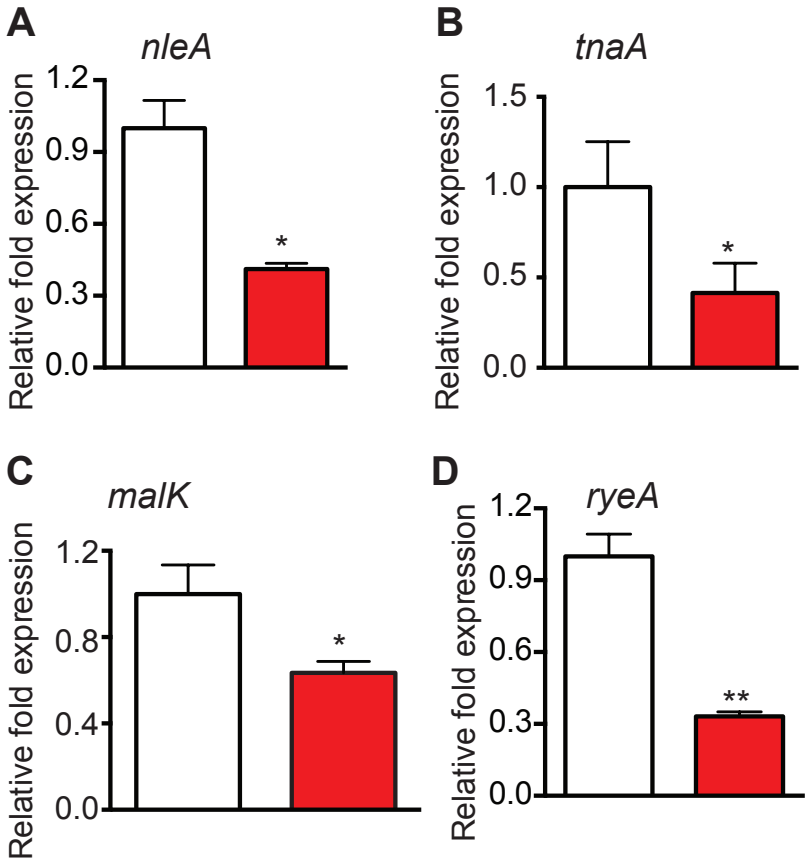


Figure 3.10 EtrB positive targets of regulation. (A-D) qRT-PCR of *nleA, tnaA, malK,* and *ryeA* in WT 86-24 and the Δ*etrB* strain. n=3; error bars represent the geometric mean ±SD. Statistical significance shown relative to WT 86-24. *, P≤ 0.05; **, P≤ 0.005.

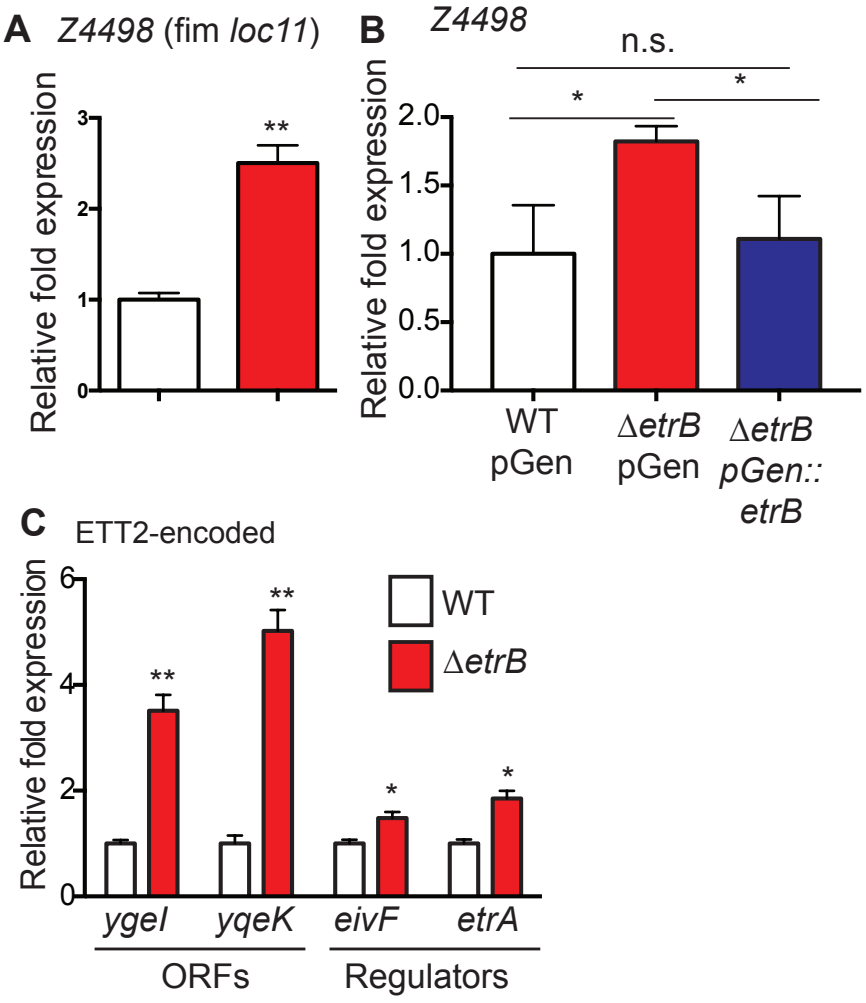


Figure 3.11 EtrB negatively regulates transcription of fimbrial locus 11 and ETT2-encoded genes. (A) qRT-PCR of *Z4498* in WT 86-24 and the Δ*etrB* strain. (B) qRT-PCR of *Z4498* in WT 86-24 transformed with an empty vector control, the Δ*etrB* strain transformed with an empty vector control, and the Δ*etrB* strain complemented with *etrB*. (C) qRT-PCR of ETT2-encoded genes *ygeI, yqeK, eivF,* and *etrA* in WT 86-24 and the Δ*etrB* strain. n=3; error bars represent the geometric

mean ±SD. Statistical significance shown relative to WT 86-24 unless otherwise indicated. *, P≤ 0.05; **, P≤ 0.005; ns = not significant.

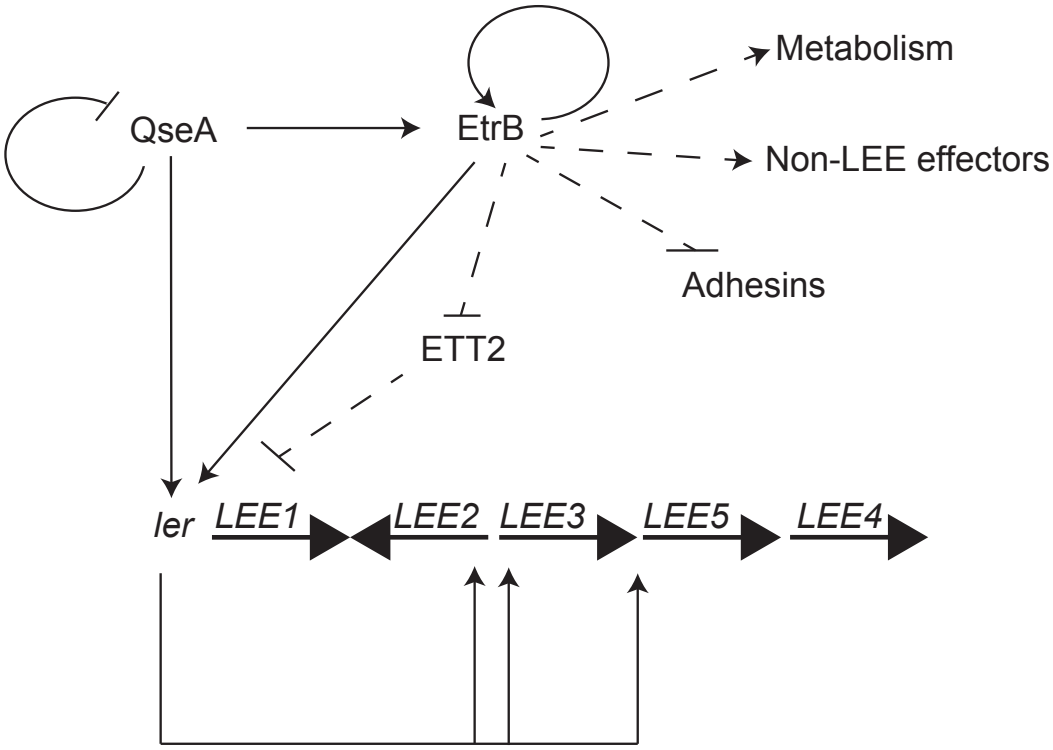


Figure 3.12 Model of the EtrB regulatory circuit. Lines with arrows indicate positive regulation; lines with bars indicate negative regulation. Solid lines represent direct interactions that have been biochemically defined; hatched lines represent interactions that occur indirectly or that have not been shown to bind biochemically to the target.

Chapter Four: Fimbriae influence the expression of virulence traits

Part of this chapter has been adapted from "A role for enterohemorrhagic *Escherichia coli* O157:H7 fimbriae in colonization" (*in preparation)*

Deborah H. Luzader, Laura A. Gonyar[1], and Melissa M. Kendall. 2016.

[1]L.A.G. assisted with operon mapping of the *erf1* locus and animal infections

Abstract

Fimbriae are proteinaceous surface structures that mediate bacterial attachment to a surface. Thus, fimbriae play an essential role in pathogen colonization by adhering a bacterium to the host tissue. The foodborne pathogen enterohemorrhagic *Escherichia coli* O157:H7 (EHEC) encodes 16 fimbrial loci that may play a role in the establishment of EHEC infection. The metabolite ethanolamine (EA) induces the expression of 15 fimbriae in EHEC. We provide evidence that Erf1 and Erf2 have redundant functions in mediating early attachment events *in vitro*, but Erf1 and Erf2 each play a critical role in colonization of the murine gastrointestinal (GI) tract. We demonstrate that Erf1 and Erf2 fimbriae, but not fimbrial locus 3, increases the formation of attaching and effacing (AE) lesions, an important virulence trait in EHEC. This study then sought to characterize the mechanism by which two EA-regulated fimbriae, Erf1 and Erf2, influence AE lesion formation through regulation of EHEC virulence gene expression. Interestingly, the expression of single components of the *erf1* and *erf2* fimbrial loci were sufficient to regulate the expression of virulence traits, suggesting that formation of Erf1 and Erf2 fimbrial surface structures is not necessary to regulate AE lesion formation. These findings reveal a crucial role for fimbriae in bacterial pathogenesis by demonstrating that fimbriae function as more than just adhesins by influencing virulence gene expression and suggest a novel mechanism for fimbrial regulation of virulence traits.

Introduction

Pathogens must attach to host cells to establish infection in the gastrointestinal (GI) tract. This process is necessary for bacterial pathogens to colonize the host by preventing bacterial displacement due to physical stresses [311]. Fimbriae are surface-exposed structures that play an essential role in pathogenesis by anchoring bacteria to host tissue [312, 313]. This is an important process for pathogens as fimbriae-mediated adhesion precedes the activation of further virulence traits such as type three secretions systems (T3SS), effector proteins, and toxins [314]. The precise coordination of these virulence traits is necessary for pathogens to cause disease. However, it is unknown whether fimbriae have additional roles in pathogenesis and if fimbriae directly contribute to the expression of other virulence factors.

Enterohemorrhagic *Escherichia coli* O157:H7 (EHEC) is a foodborne pathogen that causes severe hemorrhagic colitis and hemolytic uremic syndrome (HUS) [253]. EHEC has a very low infectious dose, with ingestion of less than 100 colony-forming units sufficient to cause disease [24, 315]. The low infectious dose of EHEC combined with patient treatment being limited to supportive care, emphasize the need to study EHEC to understand mechanisms of virulence. EHEC encodes 16 fimbrial loci, suggesting fimbriae are an important virulence determinant in EHEC [163, 229]. However, the involvement of fimbriae in EHEC pathogenesis is not well understood, due to a lack of understanding of environmental cues that promote EHEC fimbriae expression *in vitro* [162, 164, 165, 230].

A well-defined trait of EHEC virulence is the formation of attaching and

effacing (AE) lesions [62, 254, 255]. AE lesions involve effacement of the microvilli and intimate attachment of EHEC to the host epithelium by a receptor-mediated process. EHEC injects effector proteins to manipulate the host cytoskeleton and form an actin-rich pedestal-like structure under the bacterium. The formation of AE lesions requires the locus of enterocyte effacement (LEE) [4]. The LEE encodes a T3SS [254], secreted effectors [67, 256-259], the adhesin intimin (*eae*) [255], and the translocated intimin receptor (*tir*) [62].

EHEC also produces Shiga toxin, which is responsible for the deadly complications associated with EHEC [127]. Shiga toxin is absorbed systemically and binds to the receptor Gb3 on kidney epithelial cells where it inhibits protein synthesis [140, 145, 253, 316]. This leads to the development of HUS [127].

Ethanolamine (EA), an abundant metabolite in the GI tract, is a signal that EHEC utilizes to sense the host environment and regulate the expression of virulence traits [109, 110, 166]. EA is present in the GI tract from the diet and from the breakdown of the membrane component phosphatidylethanolamine (PE) derived from host and bacterial cells [219, 224, 317]. AE lesion formation, Shiga toxin production, and fimbriae are increased when EHEC is grown in the presence of EA [109, 110, 166]. Genes encoded in fimbrial locus 9 (*Z3276-Z3279*; *yeh*) and fimbrial locus 2 (*Z0146-Z0142*; *yad*), herein respectively renamed EA-regulated fimbriae (*erf1* and *erf2*) were significantly increased when EHEC was grown in the presence of EA [166]. Interestingly, preliminary studies suggest that Erf1 and Erf2 affect LEE expression in EHEC; however, the mechanism underlying this regulation in EHEC is unknown. The goal of this study was to determine the function of Erf1

and Erf2 in EHEC pathogenesis and the mechanism underlying Erf1 and Erf2 regulation of EHEC virulence traits.

We provide evidence that although Erf1 and Erf2 do not mediate adhesion *in vitro*, both fimbriae influence EHEC colonization of the GI tract. Our findings indicate that the formation of Erf1 and Erf2 fimbrial surface structures is not required to mediate virulence gene expression and suggest a novel mechanism for fimbriae in the regulation of virulence traits.

Materials and Methods

Strains and Plasmids

All strains and plasmids used in this study are listed in Table A4.1. Luria-Bertani (LB) and Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) were used to grow bacteria. Bacteria were grown overnight in Luria-Bertani Broth and then diluted 1:100 in low-glucose DMEM supplemented with 10 mM EA and 150 nM vitamin B_{12} (Sigma) unless otherwise indicated and grown for 6 h statically at 37°C in 5% CO_2. Streptomycin was added to overnight cultures of EHEC at a final concentration of 50 μg/ml. Recombinant DNA and molecular biology techniques were performed as described previously [109]. Construction of nonpolar EHEC 86-24 *erf1* (LG01), *erf2* (LG02), *Z3279*, *Z3278*, *Z3276*, *Z3277-Z3276*, *Z3278-Z3277* mutants was achieved using λ-red mutagenesis [287]. The mutants were complemented with the entire *erf1* (*ZZ3276-Z3279)* or *erf2* (*Z0146-Z0152*) fimbrial locus, including endogenous promoters, cloned into pGEN-MCS (Addgene MTA) [288]. The mutants were also complemented with different genes from the *erf1*

(*ZZ3276-Z3279)* or *erf2* (*Z0146-Z0152*) fimbrial locus in pBAD24 or pBAD33 [318]. Primers used are listed in Table A4.2. As controls, the *erf1* and *erf2* mutants were also transformed with empty vectors.

Operon analysis by RT-PCR

WT EHEC was grown as described above. RNA was extracted with the RiboPure kit (Ambion) and was reverse transcribed using SuperScript II reverse transcriptase (Invitrogen). Random primers were used to create cDNA from RNA samples and cDNA amplified by PCR with different primer sets listed in Table A4.2. Genomic DNA was used as a positive control, and a reaction without reverse transcriptase was used as a negative control. PCR and gel electrophoresis were performed using standard methods.

Quantitative Real-Time RT-PCR

Primer validation and qRT-PCR were performed as described previously [109] using primers listed in Table A4.2. Briefly, RNA was extracted from three biological replicates, and qRT- PCR was performed using a one-step reaction with an ABI 7500-FAST sequence detection system (Applied Biosystems). Data were collected using the ABI Sequence Detection 1.2 software (Applied Biosystems). All data were normalized to levels of *rpoA* and analyzed using the comparative cycle threshold (C_T) method [261]. Target gene expression levels were compared by the relative-quantification method and data are shown as changes in expression levels compared to the WT levels [261]. Statistical significance was determined by a

Student's t-test.

Adhesion assay

Adhesion assays were performed as previously described [254]. Briefly, EHEC strains were grown as described above. Caco-2 cells were washed twice with 1X phosphate-buffer saline (PBS) before infection and placed in low-glucose DMEM supplemented with 10 mM EA and 150 nM vitamin B_{12}. EHEC strains were diluted to approximately 1.0 X 10^9 CFU/ml to infect the Caco-2 cells. Caco-2 cells were incubated with the bacteria for 3 h at 37°C in 5% CO_2. After infection, cells were washed, the HeLa cells were lysed, and bacterial cells plated to determine CFU/ml. The results of six replicates were averaged, and statistical significance was determined by a Student's t-test.

Fluorescent actin staining (FAS) assay

FAS assays were performed as previously described [289] and essentially as described above, with the following modifications. Briefly, bacterial cultures were grown overnight anaerobically in LB at 37°C and were diluted 1:100 to infect HeLa or Caco-2 cells. Infected HeLa or Caco-2 cells were grown on coverslips for 6 h at 37°C with 5% CO_2 with the cells being washed after 3 hours of infection. Subsequently, the coverslips were washed, fixed with formaldehyde, permeabilized with 0.2% Triton X, and treated with fluorescein isothiocyanate-labeled phalloidin to visualize actin accumulation. Propidium iodide was added to stain the bacteria and epithelial cell nuclei. AE lesions formed by each strain were enumerated for at

least 150 HeLa or Caco-2 cells in each experiment. Statistical significance was determined by a Student's t-test.

Western Blot Analysis

Secreted proteins were harvested as previously described [254]. Bacteria were grown as described above and supernatants were isolated and concentrated using Ambicon 10K concentrators. Whole-cell lysates were collected and cells were gently lysed by boiling. All samples were subjected to 10% SDS-PAGE, transferred to a polyvinylidene difluoride membrane. The membranes were probed with an anti-EspA, anti-Stx2a (Santa Cruz Biotechnology), anti-His (Cell Signaling Technologies) or anti-RpoA (Neoclone). RpoA was visualized as a loading control for whole-cell lysates. Coomassie blue staining was used to visualize bovine serum albumin (BSA) loading controls. Relative expression of EspA was quantified from three replicate samples using ImageJ and normalized to the loading control (BSA or RpoA for secreted proteins or WCL, respectively). Expression levels are shown relative to WT. At least three independent experiments were performed.

Primer Extension

Primer extension analysis was performed as previously described [46]. Briefly, primer yehD_cDNA_R1 (Table A4.2) was end-labeled using (γ-^{32}P)-ATP. A total of 40 μg of RNA, isolated as described above from strain 86-24, was used to create cDNA with the Primer Extension System— AMV Reverse Transcriptase kit (Promega). The resultant cDNA was precipitated, run on a 6% polyacrylamide-urea

gel and visualized by autoradiography. The primer extension reaction as run adjacent to a sequencing reaction (Affymetrix). Amplified genomic DNA from 86-24 was used to generate the sequencing ladder using primers yehD_prom_F1 and yehD_cDNA_R1for the *erf1* promoter.

Purification of EutR

To purify the maltose-binding protein (MBP)-tagged EutR protein [110], *Escherichia coli* strain BL-21 (DE3) containing pMK53 was grown at 37°C in LB with glucose (0.2% final concentration) and ampicillin (100 μg/ml) to an optical density at 600 nm ($O.D._{600}$) of 0.5, at which point IPTG was added to a final concentration of 0.3 mM and allowed to induce overnight at 18°C. Cells were harvested by centrifugation at 4,000 X *g* for 20 min and then resuspended in 25 ml column buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA) and lysed by homogenization. The lysed cells were centrifuged, and the lysate was loaded onto a gravity column (Qiagen) with amylose resin. The column was washed with column buffer and then eluted with column buffer containing 10 mM maltose. Fractions containing purified proteins were confirmed by SDS-PAGE and Western analysis, and the protein concentration was determined using the Bradford assay.

Electrophoretic mobility shift assays (EMSAs)

EMSAs were performed using the purified EutR-MBP and PCR-amplified DNA probes (Table A4.2) as previously described [110]. DNA probes were end-labeled using T4 polynucleotide kinase (NEB) with (^{32}P) ATP (Perkin-Elmer) [262]. End-

labeled probes were purified using the Invitrogen NucAway Spin Columns. EMSAs were performed by adding increasing amounts of purified EutR protein to end-labeled probe in binding solution (500 µg/mL BSA, 50 ng poly(dI-dC), 60 mM HEPES pH 7.5, 5 mM EDTA, 3 mM dithiothreitol (DTT), 300 mM KCl, and 25 mM $MgCl_2$) and incubated at room temperature for 25 minutes. A 1% Ficoll solution was added to the reaction mixtures immediately before loading the samples on the gel. The reactions were electrophoresed on a 6% polyacrylamide gel for approximately 6 h at 150 V, dried, and imaged with a PhosphorImager (Molecular Dynamics). Bands were quantified using ImageQuant software as indicated.

Animal Experiments

All experiments were approved by the Institutional Animal Care and Use Committee at the University of Virginal School of Medicine. 5-6 week old, male, CD-1 mice were given streptomycin- treated water (5g/L) 24 hours prior to infection [319]. Mice were inoculated with 2 x 10^8 CFU of each indicated strain (total dose 4 x 10^8 CFU) [320]. Fecal pellets were collected daily and CFUs were enumerated by plating homogenates on MacConkey agar. A Δ*lacZ* EHEC strain was used as a surrogate for WT to allow for differentiation between strains grown on MacConkey agar. The *lacZ* deletion does not affect the ability of EHEC to colonize the GI tract [121]. The ratio of recovered WT to mutant bacteria was normalized using the ratio in the inoculum to determine the competitive index. A one-sample t-test was used to determine statistical significance from the expected value of 1. Comparisons to determine significance between competitive index values was assessed by an

unpaired t-test. Colonization of the intestinal compartments was performed as previously described [321]. For enumeration of bacteria in intestinal compartments, a 3 cm section of the colon as measured from the cecum was excised. The colon was open and rinsed thoroughly with 1X PBS. The mucus layer was removed by gently scraping with a spatula. The mucus layer was diluted in 200 μL, and serial dilutions were plated on MacConkey agar. After removing the mucus layer, the epithelium was weighed, homogenized, and bacterial counts were determined as described above.

Results

Characterization of erf1 and erf2

Before determining the mechanism underlying fimbrial regulation of virulence traits, we further characterized the expression of the *erf1* and *erf2* fimbrial loci. The *erf1* locus (y*eh*; *Z3276-Z3279*, EDL933 genome; *ECs2914-ECs2917*, Sakai genome) encodes a putative chaperone-usher fimbriae. In a previous study, expression of the major subunit (*yehD, Z3279*) was the most highly induced fimbrial gene in the presence of EA compared to the absence [166]. We decided to characterize *erf1* because of the high induction of *erf1* in the presence of EA. In addition, preliminary data from the lab suggested that *erf1* is involved in EHEC pathogenesis by regulating expression of the *LEE* (Gonyar, unpublished). To begin to characterize *erf1*, we performed RT-PCR to verify that all genes encoded in the locus were expressed and to determine if the upstream gene, *yehE* (*Z3280,* EDL933 genome; *ECs2918*, Sakai genome), an open-reading frame of unknown function, is

expressed as part of the locus. Annotations of the *erf1* fimbrial locus sometimes include *yehE* [164] and other times do not include *yehE* [165]. For RT-PCR, we used cDNA synthesized from RNA that was purified from WT EHEC grown statically for 6 h in DMEM in the presence of EA. No PCR product was obtained in reaction that included primers specific for the flanking gene *yehE* (Figure 4.1A and B); however, PCR products were visible when primers specific for the *erf1* locus were used in the reaction (Figure 4.1A and B), indicating that *erf1* is expressed as one transcript. These findings demonstrate that all components of the *erf1* locus are expressed and suggests that *erf1* could produce a functional fimbriae.

To characterize *erf1* expression and map the promoter region, we performed primer extension analyses. For this, we designed a primer approximately 50 base pairs downstream from the major subunit (*yehD, Z3279)* translational start codon. Primer extension analysis was performed using cDNA synthesized from RNA grown as previously described. The primer extension analyses revealed two transcriptional start sites in the *erf1* promoter designated P1 and P2 for the distal and proximal transcription start sites, respectively (Figure 4.2). The P1 distal transcription start site mapped to approximately 39 base pairs upstream of the translation start site. For P2, the -10 sequence, TTTGTT, had three mismatches from the σ^{70} consensus sequences TATAAT, and the -35 sequence, TTTACA, had one mismatch from the consensus TTGACA, with a 27-base-pair spacer (Figure 4.2). The proximal P2 transcription start site is located 30 base pairs upstream of the translation start site. The P2 -10 sequence, CAGACT, had three mismatches from the consensus sequence,

and the -35 sequence, GAGAAA, had three mismatches from the consensus sequence, with a 17-base-pair spacer (Figure 4.2).

A previous study of EHEC fimbriae demonstrated that the *erf2* (*yad*; *Z0146-Z0152*, EDL933 genome; *ECs0139-ECs0145*, Sakai genome) major subunit (*yadN, Z0146*) was the most highly expressed fimbrial gene in the presence of EA of the 16 fimbrial loci [166]. Unpublished data from the Kendall lab suggested that *erf2* is involved in EHEC pathogenesis (Gonyar, unpublished). Because of the high induction of *erf2* and the influence of *erf2* on EHEC pathogenesis, we characterized the chaperone-usher fimbrial locus *erf2*. Previous data from the Kendall lab demonstrated that all the components of the *erf2* locus are expressed in three transcriptional units (Gonyar, unpublished). Expression of the *erf2* major subunit (*yadN, Z0146)* in the presence of EA is EutR-dependent [166]. We performed EMSAs to investigate whether EutR is directly regulating *erf2* expression. EutR did not shift the radiolabeled *erf2* DNA or the negative-control *amp* promoter, but did shift the positive control *ler* promoter (Figure 4.3A and B). These findings indicate that although *erf2* expression is EutR-dependent, EutR does not directly regulate the expression of *erf2*.

Role of Erf1 and Erf2 in colonization of the murine GI tract

To determine if Erf1 and Erf2 impact host colonization *in vivo*, we performed murine infection experiments. We used a murine colonization model of EHEC infection to measure the relative fitness the *Δerf1* and *Δerf2* strains compared to WT. We performed *in vivo* competition assays by infecting mice with WT *ΔlacZ* and the

Δerf1 or *Δerf2* mutant strain at a ratio of 1:1 and measured colonization by bacterial shedding in the feces. When infected with WT and the *Δerf1* strain, significantly more WT than *Δerf1* were being shed in the feces from day 5 post infection to the day of euthanasia (Figure 4.4A). At the day of euthanasia, the competitive indices of WT and *Δerf1* compared in the feces and the colon or in the feces and the cecum were not significantly different (Figures 4.5 and 4.6A). This data validates our use of fecal shedding as a measure of EHEC colonization of the murine GI tract and indicate that Erf1 contributes to EHEC colonization of the murine GI tract.

We sought to further characterize the contribution of Erf1 to EHEC colonization by assessing EHEC colonization in the lumen, mucus, and epithelial layer of the colon. We hypothesized that Erf1 influences adhesion of EHEC to the murine epithelium, and that we would observe more severe differences in colonization between WT and the *Δerf1* strain in the colonic epithelium as compared to the lumen. To assess colonization of the colonic compartments, we performed competition experiments with WT and *Δerf1* to determined the bacterial burden and competitive indices in the colonic mucus layer and the colonic epithelium at 9 days post infection. This time point was chosen to evaluate colonic compartment colonization when the mutant was recovered at significantly lower levels than WT. In both the colonic mucus and epithelium, significantly less *Δerf1* than WT was recovered (Figure 4.6A). There was no difference in the competitive indices between the colonic mucus and epithelium, the whole cecum and feces, the colonic mucus and the feces, or the epithelium and the feces (Figure 4.6A), indicating that the ratio of WT to *Δerf1* was consistently observed in the lumen, mucus, and tissue

of the colon and cecum. Thus, the higher proportion of WT compared to the *Δerf1* strain was consistently observed in the lumen, mucus layer, and tissue of the colon at 9 days post infection. These data suggest that our initial hypothesis regarding the influence of Erf1 on adhesion is incorrect, as expression of Erf1 increases colonization over the *Δerf1* strain at all environments of the GI tract, including the mucus and epithelial layer.

When mice were infected with WT and the *Δerf2* strain, there was a trend towards an increase in colonization by WT compared to *Δerf2* at 9 days post infection (Figure 4.4B). The lack of significance is likely due to one mouse that was highly colonized with the *Δerf2* strain compared to WT and has a CI value of 20.9 at 9 days post infection. It is difficult to determine if this mouse is an outlier in comparison to the group, as we did not assume an even standard deviation. If we assume normal or Gaussian distribution of our values, we can calculate significant outliers using Grubb's test, also known as the extreme studentized deviante method. Analyzing the data assuming a Gaussian distribution or a lognormal distribution we determine that the CI value of 20.9 at 9 days post infection is significantly different from the other 9 data points (p value < 0.01; $Z=2.5832$, $N=10$). To support our hypothesis that the colonization of this one mouse was an outlier, we will have to repeat the *Δerf2*/WT competition infections in more mice to obtain a larger sample size.

We observed that at days 1 and 3 post infection, *Δerf2* was shed at significantly higher levels than WT (Figure 4.4B) in our competition infections. At days 5, 7 and 9 post infection, WT and *Δerf2* was shed at similar levels (Figure 4.4B).

This initial increase in *Δerf2* compared to WT could indicate that Erf2 hinders early colonization of the murine GI tract because the *Δerf2* strain is colonizing the GI tract more effectively than WT. Alternatively, the *Δerf2* strain could have altered expression of other fimbrial loci that may aid in initial colonization of the murine GI tract. The increased shedding of *Δerf2* at early time-points could be due to a decreased adherence of *Δerf2,* causing *Δerf2* to be shed more easily. At the day of euthanasia, there was a slight difference in fecal shedding compared to colon colonization at 12 days post infection that appeared to be due to colonization differences in one mouse (Figure 4.5C). However, the competitive indices of WT and *Δerf2* in the feces and the colon at 14 days post infection, or in the feces and the cecum at 9 days post infection were similar (Figures 4.5D and 4.6D), indicating that the difference observed in colon colonization compared to fecal shedding in one mouse at 12 days post infection was likely due to human error (Figure 4.5C).

To further understand the contribution of Erf2 to EHEC colonization, we determined the competitive indices in the colonic mucus and colonic epithelium at 9 days post infection. WT colonized the cecum and feces at significantly higher levels than *Δerf2* (Figure 4.6B) and there was no statistically significant difference in CI values from the colonic mucus ($p = 0.0665$) and the colonic epithelium ($p = 0.0719$). There was no difference in relative numbers of WT and *Δerf2* between the mucus and colonic epithelium, the mucus and feces, the colonic epithelium and feces, or the cecum and feces (Figure 4.6B). This indicates that Erf2 does not influence EHEC localization within the colon and suggests that Erf2 contributes to overall colonization of EHEC at early and later stages of infection. It is interesting to note

that the CI values in the mucus, colonic epithelium, and feces appear to be distributed in two clusters. While all conditions in replicate experiments were held the same, one experiment contained mostly high CI values of around 0.7-1.5 CI and the other experiment contained lower CI values of 0.45-0.7. A larger sample size is needed to determine the influence of Erf2 on EHEC colonization at later stages of infection.

Erf1 and Erf2 do not influence early adherence events, but mediate AE lesion formation

We investigated whether Erf1 and Erf2 influence EHEC adhesion to epithelial cells. Data from the Kendall lab demonstrated that Erf1 and Erf2 did not influence EHEC adhesion to HeLa cells, a cervical epithelial cell line commonly used to assess EHEC pathogenesis (Gonyar, unpublished); however, the relevance of EHEC adhesion to HeLa cells for human pathogenesis is unclear. We investigated if Erf1 and Erf2 play a role in EHEC adhesion to Caco-2 cells, a colonic epithelial cell line that undergoes enterocyte differentiation [322]. We did not observe any difference in adhesion to Caco-2 cells in the *Δerf1* and *Δerf2* strains compared to WT (Figure 4.7)

Previous data determined that Erf1 and Erf2 influence AE lesion formation, as the *Δerf1* and *Δerf2* strains formed significantly fewer AE lesions on HeLa cells compared to WT (Gonyar, unpublished). This previous work also demonstrated that an *erf1* and *erf2* complement plasmid, which contains the fimbrial locus under the control of the endogenous promoter, restores gene expression to WT levels in the

Δerf1 and *Δerf2* strains (Gonyar, unpublished). We further investigated the functionality of the *erf1* and *erf2* complements to restore EHEC AE lesion formation by performing a fluorescein actin staining (FAS) assay by infecting HeLa cells with WT, the *Δerf1* and *Δerf2* mutant strains, and the *erf1* and *erf2* complement strains. In agreement with previous data, we observed that *Δerf1* and *Δerf2* formed significantly fewer AE lesions on HeLa cells than WT (Figure 4.8A and B). The *erf1* complement strain showed no difference in AE lesions on HeLa cells as compared to WT and formed significantly more AE lesions when compared to the *Δerf1* strain (Figure 4.8A and B). Similarly, the *erf2* complement strain showed no difference in AE lesion formation on HeLa cells as compared to WT and formed significantly more AE lesions as compared to the *Δerf2* mutant strain (Figure 4.8A and B).

To further determine the influence of Erf1 and Erf2 on AE lesion formation, we performed FAS assays with Caco-2 cells, a cell line that is more directly relevant to human epithelial cells. Similar to AE lesion formation on HeLa cells, we observed that the *Δerf1* and *Δerf2* strains formed significantly fewer AE lesions on Caco-2 cells than WT (Figure 4.9A and B). These data suggest that while Erf1 and Erf2 do not influence fimbriae-mediated adherence, both fimbrial loci promote AE lesion formation.

Erf1 and Erf2 do not influence in vitro growth

Because previous data from the Kendall lab observed that *erf1* and *erf2* regulate global gene expression in EHEC, we investigated if *erf1* and *erf2* influence EHEC growth (Gonyar, unpublished). Although no genes associated with

metabolism came up in a microarray comparing WT 86-24, *Δerf1*, and *Δerf2*, we wanted to eliminate growth differences as a possible explanation for differences in AE lesion formation. We grew WT 86-24, *Δerf1*, and *Δerf2* in a rich medium and measured growth over time by optical density (O.D.). We did not observe any growth difference from WT in the *Δerf1* or *Δerf2* strains when EHEC was grown aerobically (Figure 4.10A) or anaerobically (Figure 4.10B). These data indicate that any differences observed between WT and the *Δerf1* or *Δerf2* strain are not due to growth defects of the mutant strains.

Erf1 and Erf2 influence LEE expression and Shiga toxin expression

Previous data from the Kendall lab demonstrate that both Erf1 and Erf2 influence virulence gene expression (Gonyar, unpublished). In the *Δerf1* strain, expression of *ler (LEE1), grlA, escC (LEE2), escV (LEE3), eae (LEE5),* and *espA (LEE4)* were significantly decreased compared to WT (Gonyar, unpublished). Expression of *escC (LEE2)* and *espA (LEE4)* was significantly decreased in the *Δerf2* strain compared to WT (Gonyar, unpublished). In addition, expression of *stx2a* was significantly decreased in the *Δerf2* strain (Gonyar, unpublished). These data indicate that Erf1 and Erf2 influence EHEC virulence through the regulation of virulence gene expression and suggest that Erf1 and Erf2 act through distinct signaling pathways.

To assess if expression of the *erf1* fimbrial locus must be induced to modulate gene expression of the LEE, we assessed expression of the LEE in WT and the *erf1* mutant strain in the absence of EA. Expression of *ler (LEE1), grlA, escC (LEE2), escV*

(LEE3), eae (LEE5), and *espA (LEE4)* were unchanged in the *Δerf1* strain compared to WT when EHEC was grown in the absence of the signaling molecule EA (Figure 4.11A). Western blot analysis of the *LEE4* encoded secreted protein EspA showed that protein levels were unchanged in the *Δerf1* strain (Figure 4.11B). Overall this suggests that regulation of the LEE by Erf1 requires robust expression of the fimbrial locus, which is induced in the presence of EA [166].

Fimbrial locus in Citrobacter rodentium does not influence LEE expression

The natural-murine AE-lesion forming, pathogen *Citrobacter rodentium* is a commonly used surrogate for EHEC infection. *C. rodentium* does contain a fimbrial locus (*ROD_22311-ROD_22341*, ICC168 genome) that was identified as similar to the *erf1* fimbrial locus based on gene organization in the locus and fimbrial class, as both EHEC *erf1* and the *C. rodentium* fimbrial locus are chaperone-usher γ_4 fimbriae. However, regulation of the LEE by *C. rodentium*-encoded fimbrial locus is not conserved (Figure 4.12A and B). This is likely due to a high degree of sequence dissimilarity [323]. At the protein level the major subunit, chaperone, usher, and minor subunit of EHEC and *C. rodentium* were 68%, 77%, 79%, and 50% identical; however, at the sequence level the fimbrial loci from EHEC and *C. rodentium* could not be aligned using BLAST (Basic Local Alignment Search Tool).

loc3 does NOT influence virulence gene expression

Next, we wanted to assess if fimbrial regulation of EHEC virulence traits is conserved among other EHEC fimbriae. We decided to investigate fimbrial locus 3

(*sfm*; *Z0686-Z0693*, EDL933 genome; *ECs0592-ECs0597*, Sakai genome), which encodes a putative chaperone-usher fimbriae. Expression of the major subunit (*sfmA*, *Z0686*) was significantly increased during growth with EA compared to growth without EA [166]. Because there is no data concerning the role of *loc3* in EHEC, we characterized *loc3* by RT-PCR to confirm that all the genes necessary to form a functional fimbriae are expressed. RT-PCR demonstrated that *loc3* is expressed as four transcripts: *Z0686-Z0688* (major subunit and chaperone), *Z0689* (usher), *Z0690-Z0691* (tip adhesin and minor subunit), and *Z0693* (regulator) (Figure 4.13A and B). These data demonstrate that all components of *loc3* are expressed and suggests that Loc3 forms a functional fimbriae structure.

Next, we wanted to explore the influence of *loc3* on expression of the *LEE* because of the previously determine role of *erf1* and *erf2* on LEE expression. We performed qRT-PCR on representative genes encoded in the LEE and saw no difference in expression when comparing WT to the *Δloc3* strain (Figure 4.14A). This lack of LEE regulation was further confirmed by Western blot analysis of EspA, a LEE-encoded protein (Figure 4.14B). We also assessed if Loc3 has a role in Shiga toxin expression by measuring transcription. Transcription of *stx2a* was unchanged in the *Δloc3* strain compared to WT (Figure 4.14C), further indicating that Loc3 does not regulate the expression of virulence genes. These data demonstrate that not all EHEC fimbriae can modulate virulence gene expression in EHEC.

Global role of Erf1 and Erf2 in EHEC gene regulation

To further determine the role of Erf1 and Erf2 in EHEC gene regulation, Laura Gonyar performed a microarray with WT EHEC and the *Δerf1* and *Δerf2* mutant strains. Our collaborators analyzed the microarray data and we verified expression of select genes by qRT-PCR. We chose to validate the expression of transcriptional regulators because we hypothesized that these transcriptional regulators may be involved in modulating virulence gene expression in response to Erf1 and Erf2 expression. Expression of *pyrI*, a regulatory subunit of aspartate carbamoyltransferase, was significantly increased in the *Δerf1* strain compared to WT (Figure 4.15A). The Ecp fimbrial locus regulator, *ecpR*, was significantly decreased in expression in *Δerf1*, as was the major Ecp fimbrial subunit, *yagZ* (Figure 4.15A). Expression of *chaB*, predicted regulatory of cation transport, and *nadR,* a transcriptional repressor and kinase, were significantly increased in the *Δerf2* strain compared to WT (Figure 4.15B). The regulator *ecpR* of the Ecp fimbrial locus, was significantly decreased in expression in *Δerf2* as compared to WT (Figure 4.15B). Expression of *fliA*, sigma factor σ^{28}, which controls expression of genes involved in cell motility, is significantly increased in the *Δerf2* strain compared to WT (Figure 4.15B). These data demonstrate that Erf1 and Erf2 influence the expression of genes encoded outside of the *LEE* and further suggest that Erf1 and Erf2 act through distinct signaling pathways because they exhibit different patterns of global gene expression.

Investigation of potential regulatory intermediates to Erf1 and Erf2 regulation of the LEE

One potential mechanism for Erf1 and/or Erf2 regulation of the LEE is through a signaling intermediate or intermediates, which would modulate LEE expression in response to Erf1 and/or Erf2. We hypothesized that the fimbriae may be signaling through a two-component system, a mechanism that has been demonstrated to regulate gene expression in other pathogens [324, 325]. Two-component systems are composed of a histidine kinase that is located in the inner membrane and a response regulator that resides in the cytoplasm [326]. The histidine kinase is regulated by an environmental stimulus and then activates the response regulator to promote or inhibit gene expression. Two-component systems are typically autoregulated, such that when the response regulator is activated, it promotes expression of its own gene and its partner histidine kinase [327]. To test the hypothesis that Erf1 and/or Erf2 is modulating gene expression through a two-component system, we assessed the expression of various components of different two-component systems, under the assumption that activation of any two-component system would lead to differential expression of the genes encoding that system. We measured expression of *baeR*, the response regulator of BaeRS which is involved in response to envelope stress and is auto-regulated, *cpxP*, the negative regulator of CpxRAP a system involved in envelope stress, and *uhpA* and *uhpB*, the response regulator and histidine kinase of UhpABC which is involved in the regulation of hexose phosphate uptake [328, 329]. Expression of *baeR* was unchanged in both the *Δerf1* and *Δerf2* strains compared to WT (Figure 4.16A and

B). In *Δerf1*, expression of *cpxP* was unchanged, but expression of *uhpA* and *uhpB* were significantly decreased compared to WT (Figure 4.16A). In *Δerf2*, expression of *uhpA* and *uhpB* were unchanged, but expression of *cpxP* was significantly increased (Figure 4.16B). Expression of *casA*, a component of the Cas complex, which is upregulated by BaeR, was unchanged in the *Δerf2* strain compared to WT (Figure 4.16B) [330]. In addition, protein levels of RpoS, a sigma factor σ^{38} that regulates genes in response to stress, were unchanged in the *Δerf1* and *Δerf2* strains (Figure 4.16C and D). Overall this data suggest that Erf1 regulation of the LEE does not involve BaeRS, CpxRAP, and RpoS pathways; however based on expression data, UhpABC could be a regulatory intermediate for Erf1-mediated LEE regulation. In regards to Erf2 regulation of the LEE, it is likely not occurring through UhpABC, BaeRS, CpxRAP or RpoS.

Because of the decrease in expression of *uhpA* and *uhpB* in the *Δerf1* strain, we investigated the potential function of UhpABC in regulation of the LEE. We performed qRT-PCR of the LEE-encoded genes *ler* and *espA* in WT and *ΔuhpABC* strains and observed that expression was unchanged (Figure 4.17A). Protein levels of the secreted protein EspA were unchanged between WT and *ΔuhpABC* (Figure 4.17B). Expression of Shiga toxin, *stx2a*, was unchanged in *ΔuhpABC* compared to WT (Figure 4.17C). This indicates that UhpABC does not regulate the expression of virulence genes and is not the regulatory intermediate linking Erf1 expression to LEE regulation.

Not all components of Erf2 are necessary to regulate virulence gene expression

Previous studies have demonstrated that fimbriae and flagella can function as mechanosensors to induce virulence traits in pathogens [324, 331, 332]. To determine if the Erf2 fimbrial structure is necessary to regulate the expression of virulence traits, we complemented the *Δerf2* strain with the usher alone (*htrE*) or the chaperone and major subunit (*yadN* and *ecpD*), both of which will be unable to form Erf2 fimbrial surface structures. We evaluated EspA protein levels and determined that expression of secreted and cellular levels of EspA were significantly decreased in the *Δerf2* strain, but was complemented by expression of pBAD24:*ChpSub* (pBAD24::*Chaperone Major Subunit*, *yadN* and *ecpD)* and pBAD24:*Usher* (pBAD24::*Usher*, *htrE*) (Figure 4.18A and B). As we can make similar conclusions from secreted and cellular levels of EspA, we use cellular levels of EspA in future experiments. This indicates that the entire Erf2 fimbrial locus is not necessary to regulate expression of the LEE and suggests that there may be two pathways to mediating LEE expression, one through the usher alone and another through the chaperone and major subunit.

We next wanted to test whether the usher (*htrE*) or the chaperone and major subunit (*yadN* and *ecpD*) impacted Shiga toxin expression. As previously described, expression of Stx2a was increased in *Δerf2* in comparison to WT (Figure 4.18C). Complementation of *Δerf2* with pBAD24:*ChpSub* (pBAD24::*Chaperone Major Subunit*, *yadN* and *ecpD)* did not change Stx2a levels as compared to *Δerf2* (Figure 4.18C). However, complementation of *Δerf2* with pBAD24:*Usher* (pBAD24::*Usher*,

htrE) reduced Stx2a to WT levels (Figure 4.18C). These results suggest that the entire Erf2 fimbrial locus is not necessary to regulate Shiga toxin expression.

The entire Erf1 fimbrial locus is not necessary to regulate expression of the LEE

Previous data from the Kendall lab demonstrated that expression of the LEE could be complemented in *Δerf1* by expression of *erf1* on a low-copy number plasmid, under the control of its endogenous promoter (Gonyar, unpublished; Figure 4.6). To determine if the entire Erf1 fimbrial locus is necessary to regulate the expression of virulence traits, we complemented the *Δerf1* strain with the usher (*yehB*), the chaperone alone (*yehC*), or the chaperone and major subunit (*yadN* and *ecpD*). We also included an inducible *erf1* complement plasmid, which contains the entire *erf1* locus under the control of an inducible promoter. Expression of secreted EspA was decreased in *Δerf1* compared to WT, but was complemented back to WT levels by expression of pBAD24:*ChpSub* (pBAD24::*Chaperone Major Subunit*, *yehDC)*, pBAD24:*Usher* (pBAD24::*Usher*, *yehB*), and pBAD24::*erf1* (entire *erf1* locus, *yehDCB* and *Z3276*) (Figure 4.19A). Similarly, intracellular levels of EspA were decreased in *Δerf1* compared to WT, but was complemented back to WT levels by expression of pBAD24:*Chp* (pBAD24::*Chaperone*, *yehC)*, pBAD24:*Usher* (pBAD24::*Usher*, *yehB*), and pBAD24::*erf1* (entire *erf1* locus, *yehDCB* and *Z3276*) (Figure 4.19B). Further analysis of these complement strains by qRT-PCR demonstrated that partial complementation is observed in with the pBAD24:*ChpSub* (pBAD24::*Chaperone Major Subunit*, *yehDC)* and pBAD24:*Usher* (pBAD24::*Usher*, *yehB*) complement strains (Figure 4.19C and D). Expression of *ler* was significantly decreased in the

Δerf1 strain compared to WT and was increased in expression by complementation with pBAD24:*ChpSub* (pBAD24::*Chaperone Major Subunit*, *yehDC)*, pBAD24:*Usher* (pBAD24::*Usher*, *yehB*), and pBAD24::*erf1* (entire *erf1* locus, *yehDCB* and *Z3276*) as compared to *Δerf1* (Figure 4.19C). However, expression of *ler* was still significantly decreased in all the pBAD24 *Δerf1* complement strains as compared to WT, with pBAD24::*erf1* still two fold decreased in *ler* expression compared to WT (Figure 4.19C). Similar expression was observed with *espA* and the complement vectors. Expression of *espA* was significantly decreased in the *Δerf1* strain compared to WT and was increased in expression by complementation with pBAD24:*ChpSub* (pBAD24::*Chaperone Major Subunit*, *yehDC)*, pBAD24:*Usher* (pBAD24::*Usher*, *yehB*), and pBAD24::*erf1* (entire *erf1* locus, *yehDCB* and *Z3276*) as compared to *Δerf1* (Figure 4.19D). In the pBAD24:*ChpSub* (pBAD24::*Chaperone Major Subunit*, *yehDC)* and pBAD24:*Usher* (pBAD24::*Usher*, *yehB)* complements, expression of *espA* was still significantly decreased compared to WT, but was restored to WT levels in the pBAD24::*erf1* complement (Figure 4.19D). These data suggest that regulation of the LEE is not occurring through the 5' UTR region of *erf1,* a mechanism of gene regulation seen in *Salmonella Typhimurium* [333]. We can conclude this based on the complement data observed with the pBAD24::*erf1* (entire *erf1* locus, *yehDCB* and *Z3276*) vector which contains the entire *erf1* locus under the control of an inducible promoter, and because the *Δerf1* strain still contains the 5' UTR and 48 base pairs of the first gene in the locus, *yehD* (major subunit, *Z3279*). Additionally, these data suggest that expression of the entire Erf1 locus is not necessary to fully regulate LEE

expression as expression of just the Erf1 usher and expression of the Erf1 chaperone and major subunit restored LEE expression in the *Δerf1* strain.

Next, we wanted to determine if transport of the Erf1 usher and chaperone to the periplasm were necessary to regulate LEE expression. We deleted the putative signal sequence of the Erf1 usher and chaperone to investigate if the accumulation of fimbrial proteins in the periplasm or outer membrane is involved in regulation of the LEE. Typically fimbrial proteins contain N-terminal signal sequences that allow transport of proteins from the cytoplasm to the periplasm through the Sec secretory pathway [334, 335]. These signal sequences are typically around 20 amino acids and contain an N-terminal region that is positively charged, a middle region of hydrophobic residues, and then a more polar region containing the cleavage site [336-340]. Based on these typical components of a signal sequence, we estimated the cleavage site of the Erf1 chaperone and Erf1 usher to be around 22 amino acids or 66 base pairs downstream the ATG start codon. We constructed complement vectors for the chaperone and usher alone without the putative signal sequence and measured EspA levels. Expression of EspA was increased back to WT levels in the pBAD24:*ChpNoSS* (pBAD24::*Chaperone no signal sequence*, *yehC* missing the first 66 base pairs*)* and pBAD24:*UshNoSS* (pBAD24::*Usher no signal sequence*, *yehB* missing the first 66 base pairs) (Figure 4.19E). These data demonstrate that the first 66 base pairs of both the Erf1 chaperone and Erf1 usher are not required to regulate LEE expression, suggesting that trafficking of the chaperone and usher to the periplasm may not be necessary to regulate LEE expression.

To determine if transcription of the Erf1 chaperone or usher is sufficient to regulate expression of the LEE, we constructed complement vectors in pBAD33, a low copy number plasmid that contains an inducible promoter and is lacking a ribosomal binding site [318]. As mentioned in the introduction, the 5' UTR of an mRNA controls fimbrial expression in *S.* Typhimurium [333], therefore we hypothesized that non-coding RNAs encoded within the Erf1 or the mRNA could be modulating expression of the LEE [341]. Genes cloned into the pBAD33 vector should be transcribed but not translated. To verify the lack of translation from the pBAD33 vector, we created a pBADmychis::*usher* vector containing a His tagged Erf1 usher (*yehB*) and we created a pBAD33::*usher*-mychis vector containing the same *usher*-mychis sequence from pBADmychis::*usher*. We observed translation of the Erf1 usher from the pBADmychis vector as measured by detectable his-tagged usher protein by Western blot (Figure 4.20A). We did not observe any translation from the pBAD33 vector, as measured by the lack of usher protein (Figure 4.20A). This indicates that no translation from the pBAD33 vector is occurring and validates our use of the pBAD33 vector to investigate the influence of RNA on gene expression. Next we determined how transcription of the Erf1 chaperone and usher influences LEE expression. We observed that EspA as complemented back to WT levels by expression of pBAD33:*Chp* (pBAD33::*Chaperone*, *yehC)* and pBAD33:*Usher* (pBAD33::*Usher*, *yehB*) in the *Δerf1* strain (Figure 4.20B and C). However, the reproducibility of this pBAD33 complement data is unclear. When we introduced a pBAD33 vector with a random DNA sequence (*eutB*, Z*3277*) in *Δerf1*, we observed complementation of EspA back to WT levels in about 50% of the experiments

(Figure 4.20D and E). Because of the inconsistency of the inducible vectors, we decided to look at single fimbrial gene deletions in the Erf1 locus. Utilizing deletion strains will remove the possibility of inconsistencies in our data being due to vector copy number or due to induced expression.

To determine what components of the Erf1 locus are necessary to regulate LEE expression, we created single deletions of the major subunit (*Δmajor*, *yehD*, *Z3279*) and minor subunit (*Δminor, Z3276)*. We observed no difference in EspA expression in the *Δmajor* and *Δminor* strains compared to WT (Figure 4.21A-C) indicating that expression of the entire Erf1 fimbrial locus is not necessary to regulate the LEE. Next, we created a chaperone deletion strain (*Δchp*, *yehC, Z3278*), which should be unable to form an Erf1 fimbrial structure. We observed that EspA expression was unchanged in *Δchp* as compared to WT and expressed significantly more EspA than the *Δerf1* strain (Figure 4.21D and E). Similarly, we created a *Δusher* strain and determined that EspA expression was unchanged in the *Δusher* strain compared to WT (Figure 4.21F and G).

Next, we investigated if the deletion of two of the four genes in the *erf1* locus influences EspA expression. We created double deletion strains of the usher and minor subunit (*ΔushΔminor, yehB* and *Z3276, Z3276-Z3277*) and of the chaperone and usher (*ΔchpΔush, yehCB, Z3277-Z3278*) both of which should be unable to form Erf1 fimbrial surface structures. We measured expression of the major subunit (*yehD*, *Z3279*) and the chaperone (*yehC*, *Z3278*) by qRT-PCR in WT and the *ΔushΔminor* deletion strain and determined that expression of *yehD* and *yehC* was unchanged in *ΔushΔminor* compared to WT (Figure 4.22A), indicating that any

differences observed in the *ΔushΔminor* strain are not due to differential expression of *yehD* and *yehC*. Expression of EspA in *ΔushΔminor* was 1.5 fold increased over EspA expression in WT (Figure 4.22B and D). EspA expression in *ΔchpΔush* was 2 fold increased when compared to WT (Figure 4.22C and D). These data further demonstrate that expression of an Erf1 surface structure is not necessary to regulate the LEE and suggest that deletion of the Erf1 usher causes increased production of the LEE-encoded protein EspA.

We observed that expression of just the Erf1 major and minor subunit, in the *ΔchpΔush* deletion strain, is sufficient to regulate LEE expression. We wanted to determine if the converse is also sufficient, so if expression of just the chaperone and usher regulate LEE expression. We created the *ΔmajorΔminor* deletion strain (*yehD* and *Z3276*, *Z3279* and *Z3276*) and determined that EspA expression was unchanged in *ΔmajorΔminor* compared to WT (Figure 4.22E and F). Overall these data indicate that formation of an Erf1 fimbrial structure is not necessary to regulate the LEE and suggest that multiple, redundant mechanisms may exist within the Erf1 locus to regulate expression of the LEE.

Discussion

Contribution of Erf1 and Erf2 to EHEC colonization of the murine GI tract

In our murine model of EHEC infection, we observed a consistent decrease in shedding of the *Δerf1* strain in comparison to WT EHEC (Figure 4.4), indicating that the *Δerf1* strain is less fit than WT. Competitive indices in the colon and the cecum were comparable to fecal shedding, indicating that CFUs enumerated from fecal

shedding reflects EHEC organ colonization (Figures 4.5 and 4.6). Additionally, competitive indices in the colonic mucus layer and the colonic epithelium were comparable (Figure 4.6), indicating that Erf1 influences overall EHEC colonization. Less adherent bacteria can be cleared from the GI tract by the flow of the luminal contents, which would lead to a decrease in colonization and thus bacterial shedding in the feces over time.

We did not observe any growth differences between WT EHEC, *Δerf1*, and *Δerf2* strains under several *in vitro* conditions (Figure 4.10), suggesting that differences in colonization of *Δerf1* and *Δerf2* compared to WT EHEC are not due to growth differences in the murine GI tract. One possibility for the decreased fitness of *Δerf1* compared to WT could be differential expression of other fimbriae or virulence genes in the *Δerf1* strain that make it less fit to colonize the murine GI tract. Nine fimbrial loci are decreased in the *Δerf1* strain compared to WT (Gonyar, unpublished). Decreased expression of these fimbrial loci in the *Δerf1* strain could influence EHEC adhesion and thus EHEC colonization of the murine GI tract. These fimbriae could target surface molecules expressed in the murine GI tract that are absent from HeLa or Caco-2 cells, as we see no fimbriae-mediated attachment defect in the *Δerf1* and *Δerf2* strains *in vitro* to HeLa or Caco-2 cells (Figure 4.9; Gonyar, unpublished). We could create fimbrial locus deletion strains in these Erf1-regulated fimbriae and assess EHEC adhesion. These experiments would determine if any Erf1-regulated fimbriae contribute to fimbrial-mediated adhesion in EHEC.

AE lesion formation has not been demonstrated in murine models of EHEC infection [342], and differences observed in AE lesion formation in *Δerf1* and *Δerf2*

compared to WT EHEC may not contribute to colonization differences observed in this study. However, differential regulation of LEE-encoded T3SS, secreted effectors, intimin, or other fimbrial adhesions could influence EHEC colonization of the murine GI tract independent of AE lesion formation. Studies of EHEC infected mice determined that expression of the *LEE5*-encoded adhesin *eae*, otherwise known as intimin, is crucial for EHEC colonization of the murine GI tract [320]. EHEC intimin binds β1 integrins or nucleolin on the surface of murine enterocytes [81-83, 320]. To test if intimin binding to murine enterocytes is decreased in the *Δerf1* strain, we could perform competition experiments. First we could assess if *Δeae* is shed at lower levels than WT in a competition experiment. If *Δeae* displays a colonization defect, we could perform a competition experiment with *Δeae* and *ΔeaeΔerf1* to determine if Erf1 modulation of intimin (*eae*) is affecting EHEC colonization. We expect to observe no difference in the CI values in a *Δeae* and *ΔeaeΔerf1* competition experiment, indicating that Erf1 influences EHEC murine colonization through regulation of intimin. Additional future studies could perform competition experiments as described above using a GFP-expression WT EHEC strain and a RFP-expressing *Δerf1* strain. We could then examine adherence patterns by fluorescent microscopy. Alternatively, we could perform single infections of WT and the *Δerf1* strain to determine if colonization differences are still observed. If there is decreased colonization of the *Δerf1* strain compared to WT, samples of the colon can be taken to examine adherence patterns by transmission electron microscopy. Overall these experiments would determine how Erf1 affects EHEC colonization of the murine GI tract. Regardless of the mechanism, expression of Erf1 is crucial for

EHEC colonization of the murine GI tract, a phenotype that has not been demonstrated for any other characterized EHEC fimbriae.

The *Δerf2* strain displayed increased initial colonization of the murine GI tract compared to WT and then a trend towards a decrease in colonization at 9 days post infection (Figure 4.4). Competitive indices in the colon and the cecum were comparable to fecal shedding, indicating that CFUs enumerated from fecal shedding reflects EHEC organ colonization (Figures 4.5 and 4.6). These data overall indicate that Erf2 expression influences EHEC colonization. The expression of two fimbrial loci were significantly decreased and two fimbrial loci were 2 fold increased, with high variability, in the *Δerf2* strain compared to WT (Gonyar, unpublished). Differences in colonization could be due to differential expression of other fimbriae or virulence traits in the *Δerf2* strain that aid in the initial colonization of the murine GI tract as described above for *Δerf1*.

Influence of Erf1 and Erf2 on EHEC virulence

EHEC contains 16 fimbrial loci, however very little is known about their role and function in EHEC virulence. Fimbriae are thought to mediate bacterial attachment to epithelial cells, other bacterial cells, or to both [313, 343]. Because of the redundant function of some fimbriae, it is difficult to assess their role in colonization and bacterial pathogenesis. The fimbrial loci *erf1*, *erf2*, and *loc3* express all the necessary components to form functional fimbriae through the chaperone-usher pathway (Figures 4.1 and 4.13; Gonyar, unpublished); however, we did not observe any adherence defect in the *erf1* and *erf2* mutant strains (Figure 4.7;

Gonyar, unpublished). This suggests that there are EHEC-encoded fimbriae that perform redundant adherence functions. Another possibility is that the receptors Erf1 and Erf2 adhere to on epithelial cells are not present in our system. Previous studies of Caco-2 cells demonstrated that Caco-2 monolayers do not express all the components of an apical membrane [344-346] and gene expression in Caco-2 cells can be highly variable in response to small differences in culture media [347]. Additionally, surface receptors have variable expression on Caco-2 cells influencing EHEC accessibility to Caco-2 surface receptors [348]. To overcome this obstacle we could assess EHEC adhesion to HT-29 cells, another epithelial cell line that was derived from colonic enterocytes in a patient with colorectal carcinoma, and T-84 cells, an epithelial cell line that was derived from a metastatic site in the lung in a patient with colorectal carcinoma. Similar to Caco-2 cells, T-84 cells exhibit tight junctions and desmosomes when confluent. T-84 cells also express many receptors on the cell surface [349]. However, T-84 and HT-29 cells are both immortalized, highly-passaged cell lines and we may see no difference in EHEC adhesion due to Erf1 and Erf2 target ligands not being expressed. Another method we could use to assess the contribution of Erf1 and Erf2 to EHEC adhesion is to perform assays with primary intestinal epithelial cells [350]. These primary epithelial cells could be derived from the colon of humans or mice to create enteroids or 'mini-guts' that contain crypt, villi, and lumen-like domains [351]. Human-derived enteroids could be used to assess how Erf1 and Erf2 influence EHEC adhesion to primary epithelial cells. In addition, murine-derived enteroids could be used to determine how Erf1 and Erf2 influence EHEC colonization of the murine GI tract. Together, these studies

will investigate if Erf1 and Erf2 influence fimbrial-mediated adhesion to epithelial cells.

Despite the lack of an adherence phenotype in the cell lines tested, both Erf1 and Erf2, but not Loc3, activate AE lesion formation through regulation of the LEE by distinct signaling pathways (Figures 4.8, 4.9, 4.14; Gonyar, unpublished). Our data suggest that this defect in AE lesion formation in the *Δerf1* and *Δerf2* strains is not due to a decrease in initial EHEC adherence that then leads to a defect in AE lesion formation, as we saw no difference in adhesion in any cell lines tested (Figure 7; Gonyar, unpublished). Additionally, expression of LEE-encoded genes necessary for the formation of AE lesions are decreased in the *Δerf1* and *Δerf2* strains, suggesting that Erf1 and Erf2 modulate expression of the LEE to influence EHEC AE lesion formation (Figures 4.8, 4.9, 4.18, 4.19, 4.20, 4.21; Gonyar, unpublished).

Mechanism(s) by which Erf1 and Erf2 modulate virulence gene expression

We hypothesize that Erf1 action of the LEE is through the master regulator Ler, as we see a decrease in expression of all 5 LEE operons in the *Δerf1* strain compared to WT (Gonyar, unpublished). Erf1-mediated regulation of the LEE required EA, indicating that increased expression of *erf1* is necessary [166]. There are a few examples in the literature regarding fimbrial regulation of gene expression. In *Salmonella* Typhimurium, the 5' untranslated region (UTR) of a fimbrial locus controls the expression of an alternative fimbrial locus [333]. Both the *Δerf1* and *Δerf2* mutants contain the 5' UTR and contain between 20-30 base pairs of the first gene in the locus, therefore the 5' UTR is present and transcribed.

Additionally, the *Δerf1* strain can be complemented to back to WT with an inducible vector containing the *erf1* locus but not the *erf1* 5' UTR (Figure 4.19). Overall, this suggests that Erf1 and Erf2 do not regulate virulence gene expression through their 5' UTR. Some fimbrial loci encode regulatory proteins that can act on genes located outside of the fimbrial locus, such as PapX from uropathogenic *E. coli* and MrpJ from *Proteus mirabilis* [352, 353]. No regulators are encoded in *erf1* and *erf2* leading us to hypothesize that Erf1 and Erf2 indirectly regulate gene expression and require one or more signaling intermediates.

Erf1 and Erf2 may modulate gene expression by ligand binding. EHEC, as well as other pathogens such as *Pseudomonas aeruginosa* and *Bacillus subtilis*, use mechanical stress generated from host cell contact to regulate gene expression [324, 331, 332, 354]. In many of these examples, pathogens use contact-dependent mechanisms to regulate gene expression through a two-component system or a cytosolic regulatory protein [324, 325, 355, 356]. Regulation of the LEE does not require the formation of an Erf1 fimbrial structure, as single and double mutants that would be unable to form an Erf1 surface structure do not decrease expression of the LEE (Figure 4.21). Multiple, redundant mechanisms may exist within the Erf1 locus to regulate expression of the LEE because deletion of the major and minor subunit as well as deletion of the chaperone and usher does not decrease LEE expression (Figure 4.21). To better understand what components of Erf1 regulate the LEE, future studies could examine LEE expression in triple deletion strains where the erf1 promoter is expressing only one gene from Erf1. For example, create a *ΔmajorΔushΔminor* deletion strain that only expresses the Erf1 chaperone. These

experiments would determine if single components of Erf1 regulate LEE expression and would allow more targeted approaches to assess the contribution of single Erf1 components to LEE regulation.

Based on the data presented, it is unclear if the regulatory activity of Erf1 is through transcription, translation, or translocation of the Erf1 components. As mentioned above, previous studies observed that fimbrial mRNA regulates gene expression [333, 341]. Additionally, in some pathogens cytoplasmic chaperones interact with regulatory proteins to activate gene expression [357-359]. To test if transcription of *erf1*, or if transcription of certain genes encoded in *erf1*, is sufficient to regulate LEE expression site-directed mutagenesis could be performed to create a premature stop codon in the *erf1* gene of interest. Deletion of the putative signal sequence in the *erf1* gene of interest on the chromosome can be done to assess if regulation of the LEE requires just translation of *erf1* or if translocation to the periplasm or outer membrane is required.

Erf2 increased expression of genes only in *LEE2* and *LEE4*, which encode many of the T3SS structural genes [360]. Regulation of only *LEE2* and *LEE4* could be a mechanism for EHEC to increase adherence to the epithelium in a T3SS-dependent manner. Complement data suggests that expression of the entire Erf2 fimbrial locus is not necessary to regulate expression of *LEE4*, and that there are two potential regulatory pathways in Erf2 to regulate LEE expression: through expression of the major subunit and chaperone, or through expression of the usher alone (Figure 4.18). Additional studies are needed to verify which components of Erf2 are sufficient to regulate *LEE2* and *LEE4* expression and to determine how *erf2*

expression regulates *LEE2* and *LEE4.* To investigate this, we could create single Erf2 component strains and perform site-directed mutagenesis as outlined for Erf1 in the two preceding paragraphs. Overall, these experiments would determine how Erf2 regulates expression of the LEE.

In addition to regulation of the LEE, Erf2 decreases Shiga toxin expression, which may aid in LEE-mediated EHEC adherence (Fig 4.18; Gonyar, unpublished). Expression of the Erf2 usher alone is sufficient to decrease Shiga toxin expression (Fig. 4.18). Further studies are needed to examine the role of CpxRAP in Erf2 regulation of virulence traits expression of *cpxP*, a repressor of CpxRA [361, 362], was two fold increased in the *Δerf2* strain (Fig. 4.16B). CpxRA is involved in the regulation of fimbriae in *E. coli* species by both promoting and inhibiting fimbriae expression and assembly [363-366]. Additionally, CpxR regulates expression of the master regulation of virulence genes in *Shigella* [367], further suggesting that CpxRA may be involved in repressing Shiga toxin expression in WT EHEC in response to Erf2. Additional experiments could assess the expression of *cpxP, cxpA*, and *cpxR*, in WT EHEC, the *Δerf2* strain, and the *Δerf2* pBAD24:*Usher* (pBAD24::*Usher*, *htrE*) to determine if repression of *cpxRA* is occurring in the *Δerf2* strain. Expression of Shiga toxin in a *Δerf2ΔcpxRAP* strain could be investigated to determine if the increase in Shiga toxin expression in the *Δerf2* strain is due to decreased levels CpxRA. These experiments would assess if the TCS CpxRA is a involved in Erf2 regulation of Shiga toxin production.

The involvement of CpxRA in regulation of the LEE is unclear. Two papers published in 2016 report conflicting data in regards to the involvement of CpxRA in

EHEC. One study reports that CpxRA indirectly represses expression of the LEE in response to environmental stimuli [368], while the other reports that CpxRA indirectly activates expression of the LEE [369]. CpxP represses activity of CpxRA [361, 362], suggesting that CpxRA levels are decreased in the *Δerf2* strain. Erf2 regulation of the LEE may involve signaling through CpxRA if CpxRA activates expression of the LEE [369]. To determine the involvement of the CpxRA TCS, we would first need to investigate if Cpx influences LEE expression by creating a *ΔcpxRA* strain. If CpxRA activates expression of the LEE, we could determine if Erf2 regulation of the LEE is involved in the same signaling pathway by creating a *Δerf2ΔcpxRA* to assess if there are additive affects on LEE expression.

Altogether, these data further describe the mechanism by which fimbriae regulation the expression of virulence traits in EHEC. Signaling pathways involving fimbriae could represent a way for EHEC to determine its spatial orientation in the GI tract and may be a strategy for EHEC to increase the expression of virulence traits when EHEC is in close proximity or in contact with the host epithelium. Further studies are needed to determine the mechanism by which fimbriae influence gene expression.

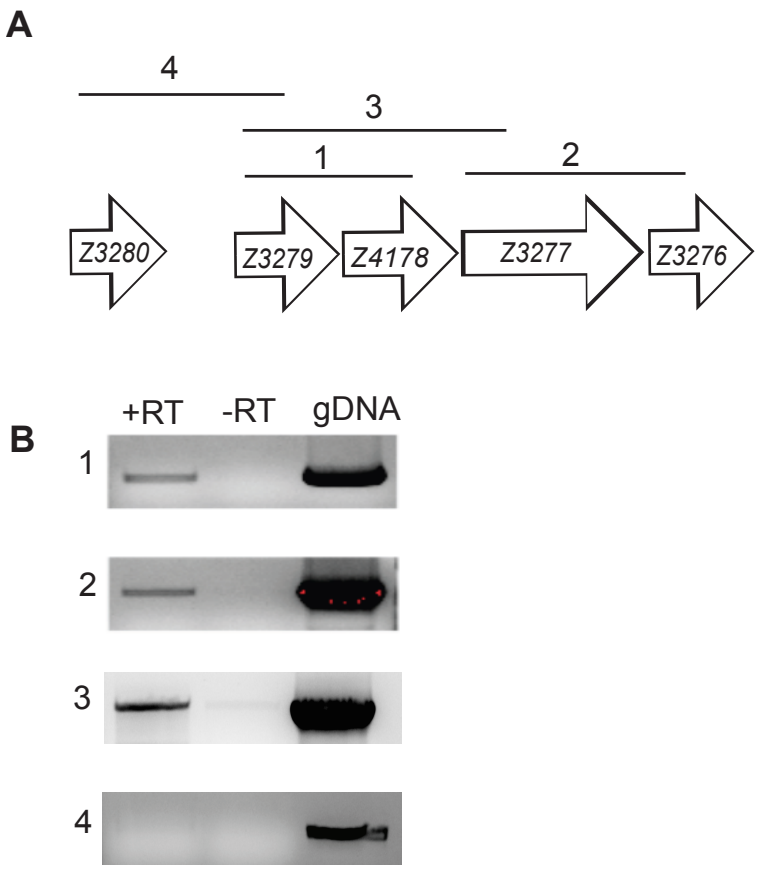
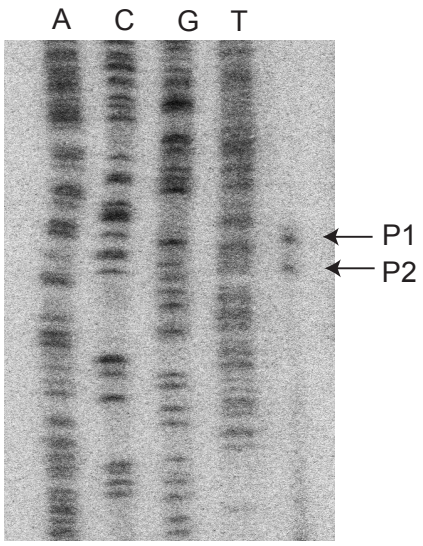


Figure 4.1 *erf1* expression. (A) Schematic representation of the *erf1* (*Z3276-Z3279, yeh*) genetic locus and the adjacent gene *yehE* (*Z3280*) in EHEC. The gene *yehE* (*Z3280*) encodes an unknown ORF, *Z3279* encodes the Erf1 major subunit, *Z3278* encodes the Erf1 chaperone, *Z3277* encodes the Erf1 usher, *Z3276* encodes therErf1 minor subunit. (B) RT-PCR of the *erf1* genetic locus and the adjacent gene *Z3280* (*yehE*). Genomic DNA (gDNA) was used as a positive control and a reaction without RT was used as a negative control. Laura Gonyar assisted with these experiments.



erf1 promoter sequence:

P1: TTTACATTGCGGCAACCTTTTTGATGTGAGAAATTTGTTTCCAAGAGATACAGA
-35 -10 +1

P2: GAGAAATTTGTTTCCAAGAGATACAGACTCTTAACA
-35 -10 +1

Figure 4.2 *erf1* promoter. Primer extension assay of *erf1*. Lanes 1-4 show the *erf1* sequencing ladder. The arrows represent the proximal (P2) and distal (P1) transcription start sites of *erf1*. The promoter sequences of *erf1* are shown with the predicted transcription start sites and -10 and -35 regions.

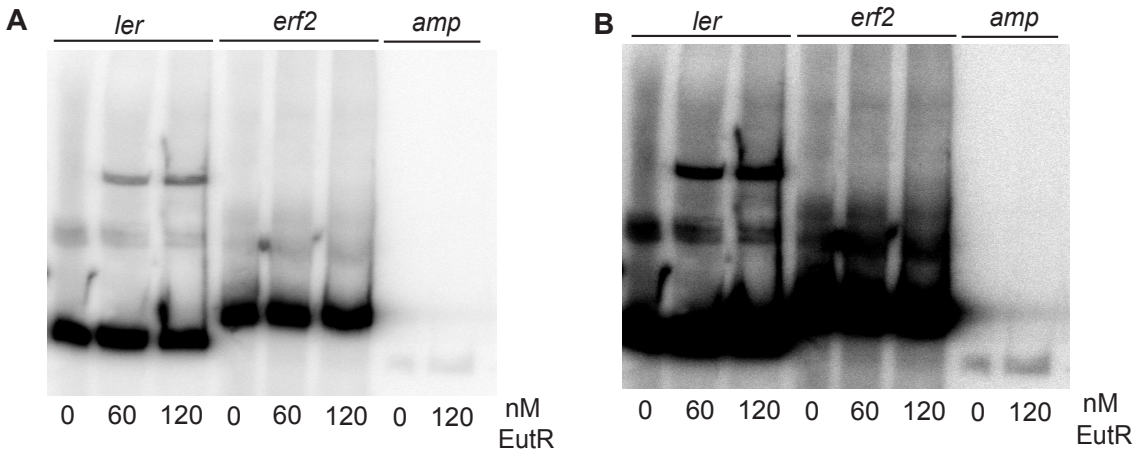


Figure 4.3 EutR does not bind to the *erf2* promoter (A) EMSA of the *ler, erf2* and *amp* promoter region with EutR::MBP. Binding to *amp* is shown as a negative control (B) Darker exposure of the EMSA from (A).

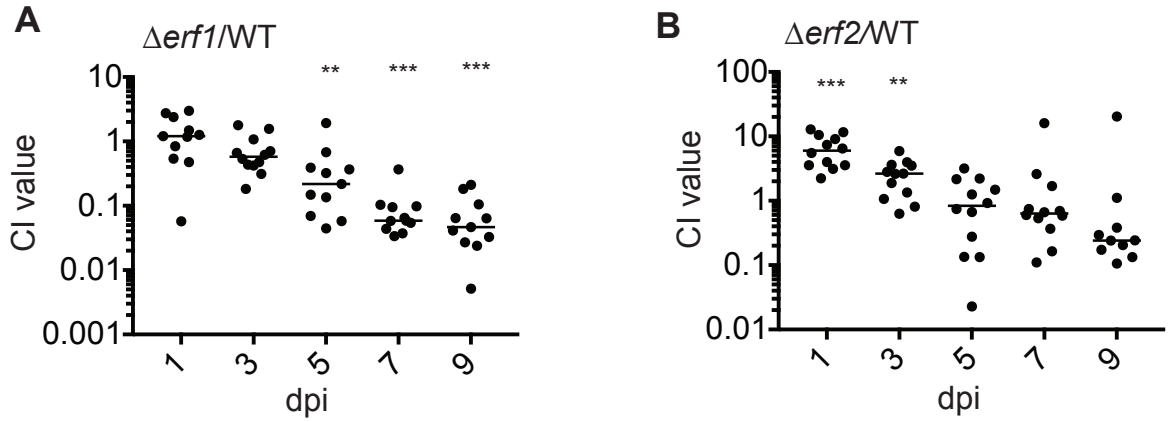


Figure 4.4 Erf1 and Erf2 influence EHEC colonization of the murine GI tract. (A) Colonization fitness of Erf1 assessed by calculating the competitive indices (CI) of WT and Δ*erf1* shed in the feces at the indicated days post infection (dpi). A one-sample t-test was used to determine significance comparing to an expected value of 1. A total of 12 mice were infected in three independent experiments. Lines represent the median. (B) Colonization fitness of Erf2, measured as described for (A). **, P≤ 0.005; ***, P≤ 0.0005. Laura Gonyar assisted with these experiments.

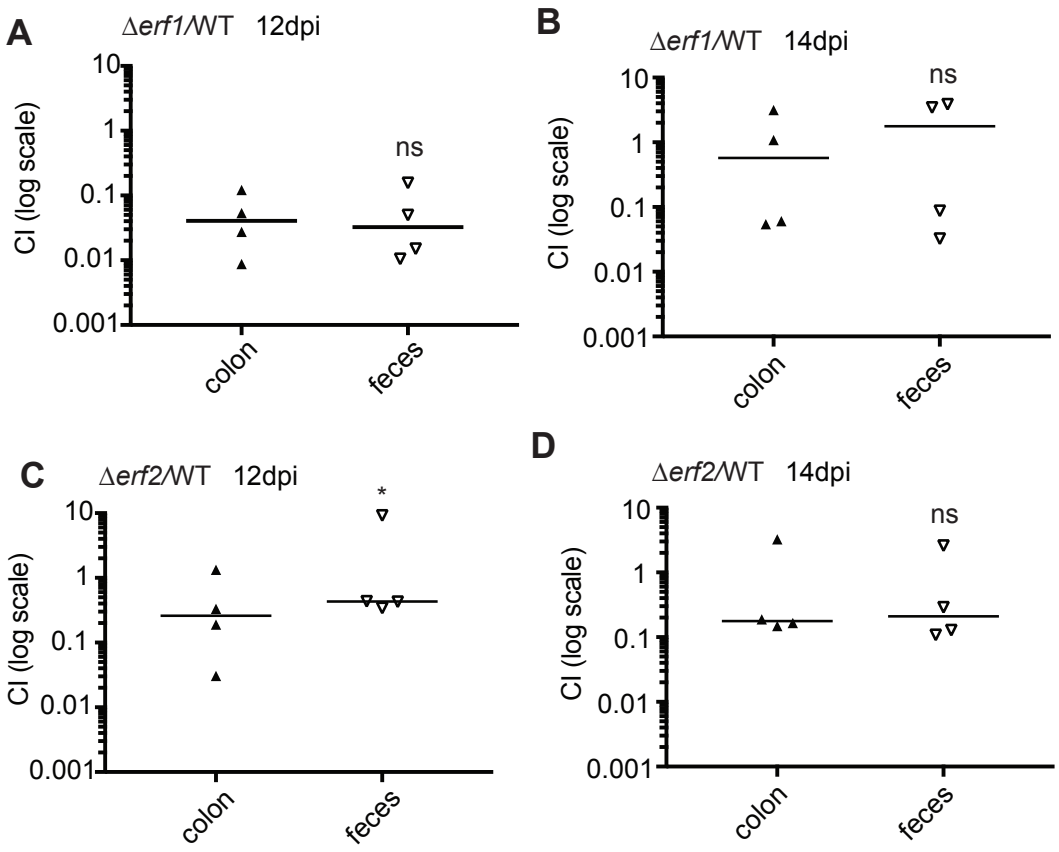


Figure 4.5 Bacterial burden comparison between fecal shedding and colon colonization. Competitive indices (CI) were compared on the day of euthanasia between the colon and the feces. CI of Δ*erf1*/WT at (A) 12 days post infection (dpi) and (B) 14 dpi. CI of Δ*erf2I*/WT at (C) 12 dpi and (D) 14 dpi. Colon and feces CI values were compared using an unpaired t-test and an even standard deviation was not assumed. Lines represent the median. *, P≤ 0.05. ns= not significant. Laura Gonyar assisted with these experiments.

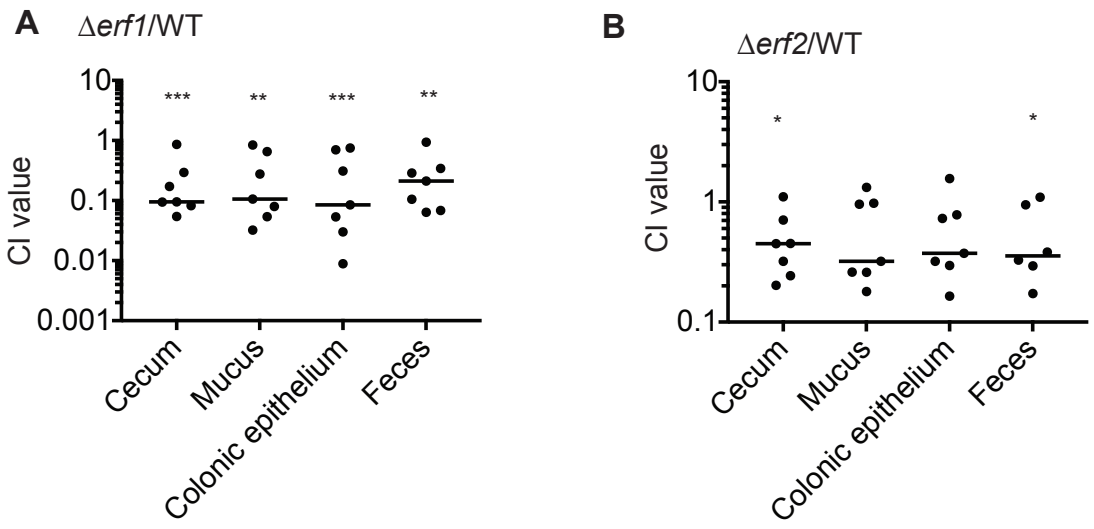


Figure 4.6 Colonization of colonic intestinal compartments. (A) CI of Δ*erf1*/WT at 9 dpi in the total cecum, colon mucus, colonic epithelium, and feces. (B) CI of Δ*erf2*/WT at 9 dpi in the total cecum, colon mucus, colonic epithelium, and feces. the total cecum and feces. A one-sample t test was used to determine significance comparing to an expected value of 1, and significance is displayed as: *, P≤ 0.05; **, P≤ 0.005; ***, P≤ 0.0005. CI values of the cecum, mucus, epithelium, and feces CI were compared using an unpaired t-test and an even standard deviation was not assumed. When CI values in each compartment were compared, all comparisons were not significant for Δ*erf1*/WT and Δ*erf2*/WT experiments.

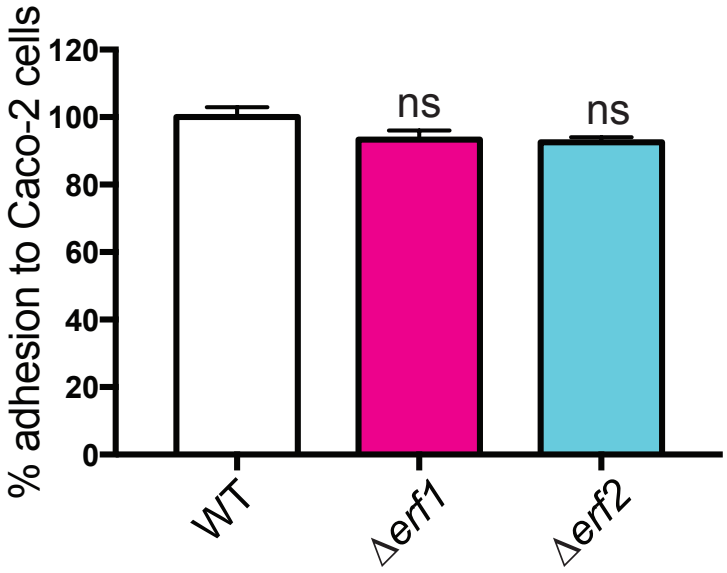


Figure 4.7 Erf1 and Erf2 do not influence EHEC adhesion to Caco-2 cells. Quantification of total adherence of WT 86-24, Δ*erf1*, and Δ*erf2* to Caco-2 cells after 3 hours of infection. N=4; error bars represent the geometric mean ±SD. ns = not significant.

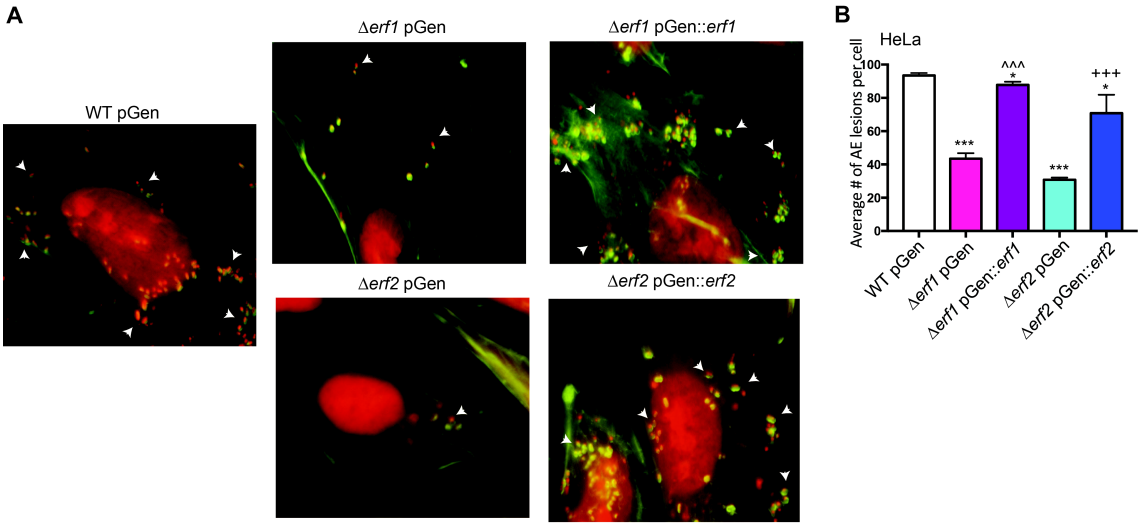


Figure 4.8 Erf1 and Erf2 activate AE lesion formation to HeLa cells. (A) FAS assay with WT 86-24 transformed with an empty vector control, the Δ*erf1* and Δ*erf2* strains transformed with an empty vector control, the Δ*erf1* strain complemented with *erf1*, and the Δ*erf2* strain complemented with *erf2*. HeLa nuclei and bacteria were stained red with propidium iodide, and HeLa cell actin cytoskeleton was stained green with FITC-phalloidin. AE lesions are observed as punctate green structures associated with bacterial cells and are indicated by arrowheads. (B) Number of AE lesions per HeLa cell. Statistical significance relative to WT 86-24 as: *, P≤ 0.05; ***, P≤ 0.0005; ns = not significant. Statistical significance relative to Δ*erf1* as: ^^^, P≤ 0.0005. Statistical significance relative to Δ*erf2* as: $^{+++}$, P≤ 0.0005.

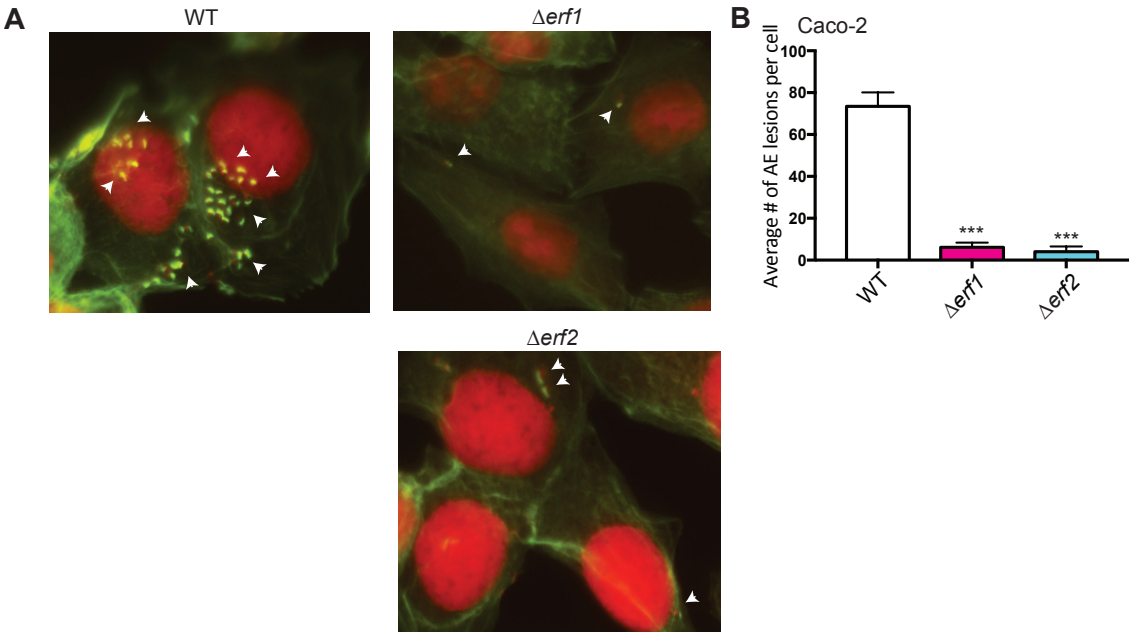


Figure 4.9 Erf1 and Erf2 activate AE lesion formation to Caco-2 cells. (A) FAS assay with WT 86-24 and the Δ*erf1* and Δ*erf2* strains. Caco-2 nuclei and bacteria were stained red with propidium iodide, and Caco-2 cell actin cytoskeleton was stained green with FITC-phalloidin. AE lesions are observed as punctate green structures associated with bacterial cells and are indicated by arrowheads. (B) Number of AE lesions per Caco-2 cell. Statistical significance relative to WT 86-24 as: ***, P≤ 0.0005.

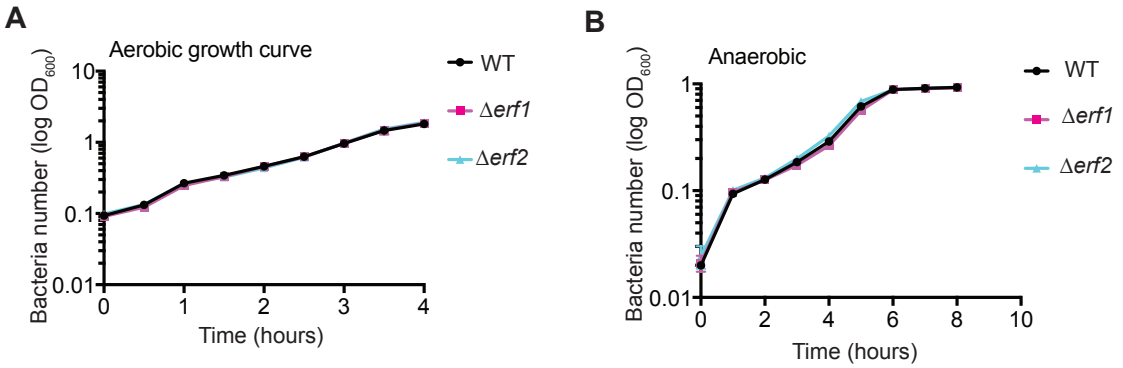


Figure 4.10 Erf1 and Erf2 do not affect EHEC growth. WT 86-24, Δ*erf1*, and Δ*erf2* were grown (A) aerobically and (B) anaerobically in DMEM with 10 mM EA. Bacterial growth was measured by $O.D._{600}$ reading and results are displayed on a logarithmic scale.

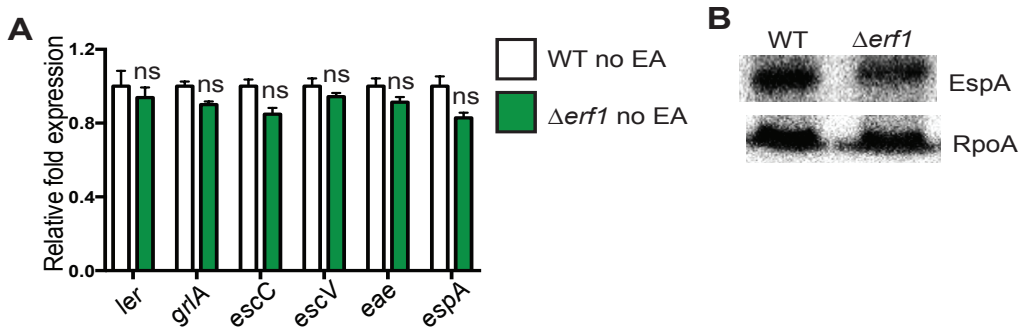


Figure 4.11 Erf1 does not influence virulence gene expression in the absence of EA. (A) qRT-PCR of *ler, grlA, escC, escV, eae,* and *espA* in WT 86-24 and the Δ*erf1* strains in the absence of EA. (B) Representative Western blot of the LEE-encoded EspA protein in WT and the Δ*erf1* strain. RpoA is shown as a loading control. ns = not significant.

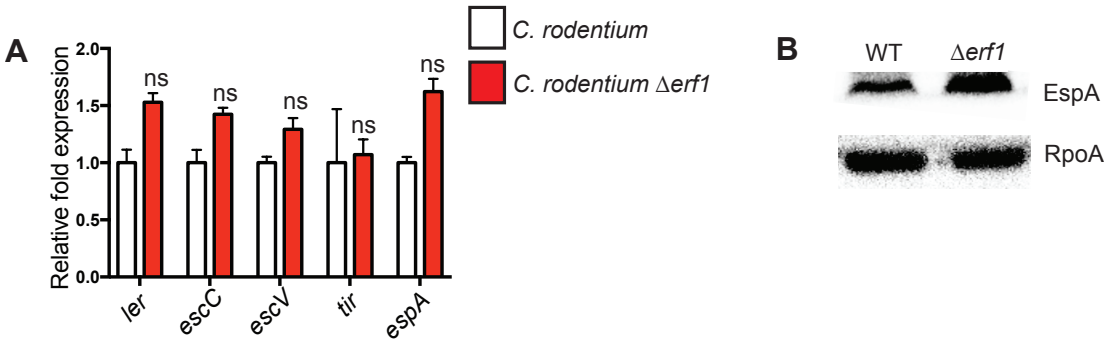


Figure 4.12 Erf1 does not regulate the LEE in *C. rodentium*. (A) qRT-PCR of *ler, escC, escV, tir,* and *espA* in WT *C. rodentium* (DBS100) and the Δ*erf1* strains. (B) Representative Western blot of the LEE-encoded EspA protein in WT *C. rodentium* and the Δ*erf1* strain. RpoA is shown as a loading control. ns = not significant.

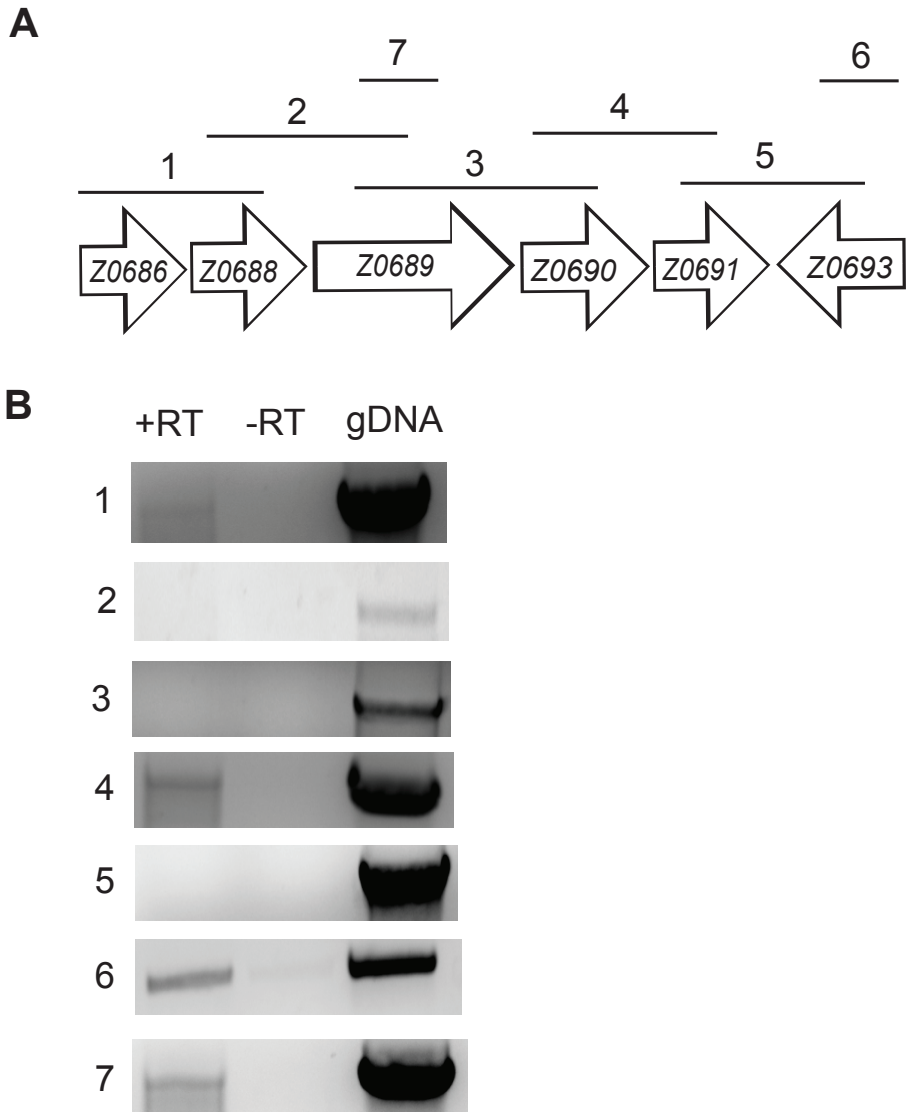


Figure 4.13 *loc3* expression. (A) Schematic representation of *loc3* (*Z0686-Z0693, sfm*) genetic locus in EHEC. (B) RT-PCR of *loc3*. Genomic DNA (gDNA) was used as a positive control and a reaction without RT was used as a negative control.

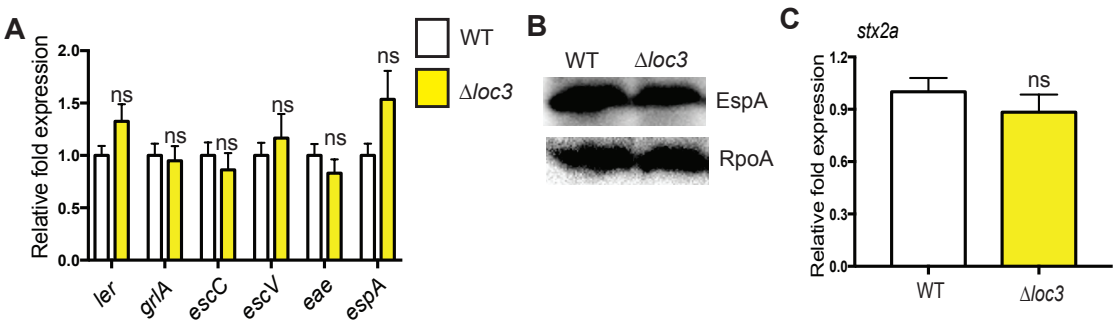


Figure 4.14 Loc3 does not influence virulence gene expression. (A) qRT-PCR of *ler, grlA, escC, escV, eae,* and *espA* in WT 86-24 and the Δ*loc3* strains. (B) Representative Western blot of the LEE-encoded EspA protein in WT and the Δ*loc3* strain. RpoA is shown as a loading control. (C) qRT-PCR of *stx2a* in WT 86-24 and the Δ*loc3* strains. ns = not significant.

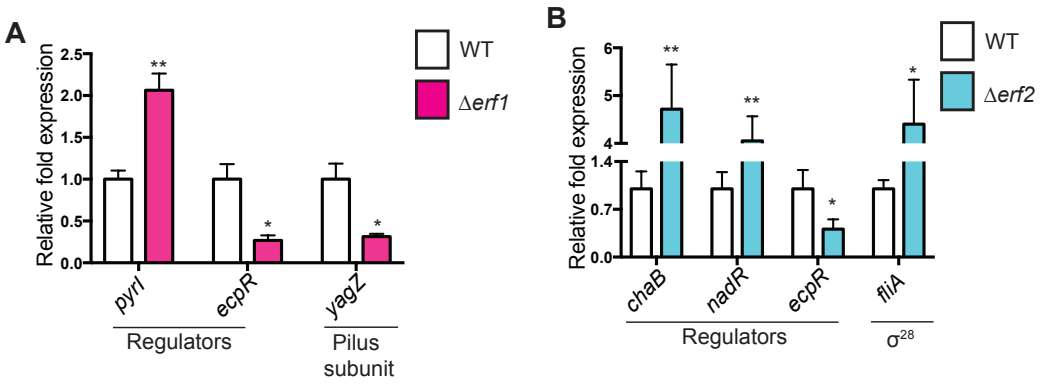


Figure 4.15 Expression of select genes from the Δ*erf1* and Δ*erf2* microarrays. (A) qRT-PCR of *pyrI, ecpR,* and *yagZ* in WT 86-24 and the Δ*erf1* strains. (B) qRT-PCR of *chaB, nadR, ecpR,* and *fliA* (σ^{28}) in WT 86-24 and the Δ*erf2* strains. *, P≤ 0.05; **, P≤ 0.005.

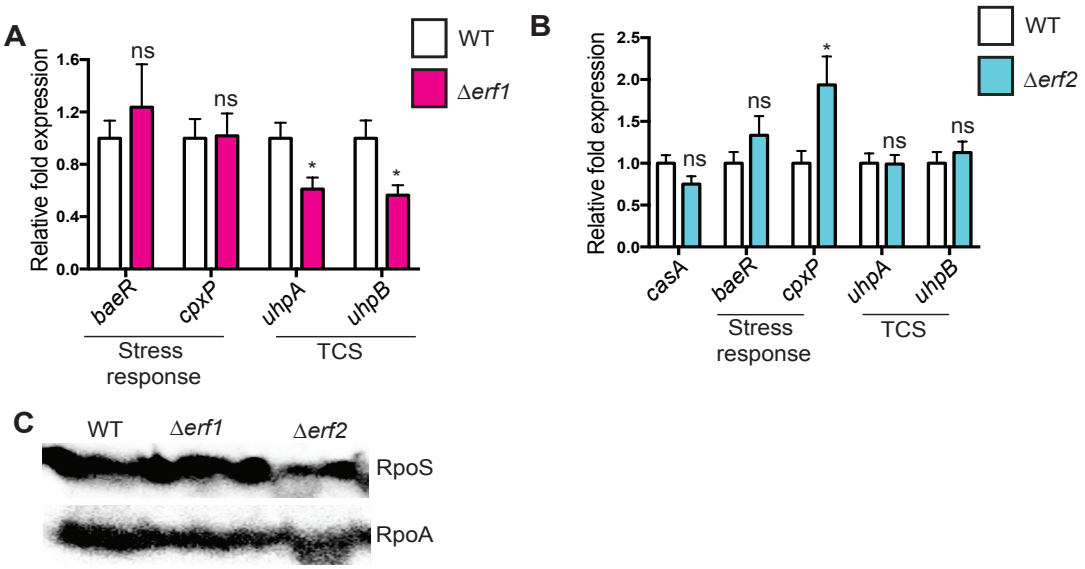


Figure 4.16 Expression of potential regulatory intermediates for Erf1 and Erf2 regulation of the LEE. (A) qRT-PCR of *baeR, cpxP, uhpA,* and *uhpB* in WT 86-24 and the Δ*erf1* strains. (B) qRT-PCR of *casA, baeR, cpxP, uhpA,* and *uhpB* in WT 86-24 and the Δ*erf2* strains. (C) Representative Western blot of RpoS (σ^{38}) in WT and the Δ*erf1* strain. RpoA is shown as a loading control. (D) Quantification of RpoS expression from (C). *, P≤ 0.05; ns = not significant.

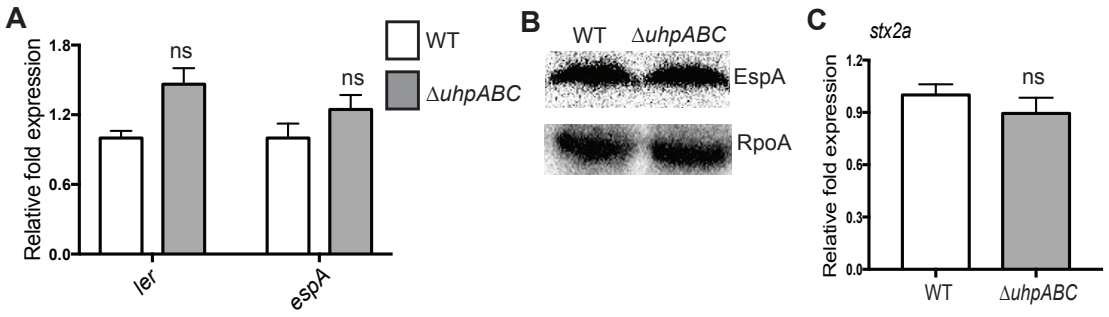


Figure 4.17 UhpABC does not influence virulence gene expression. (A) qRT-PCR of *ler* and *espA* in WT 86-24 and the Δ*uhpABC* strains. (B) Representative Western blot of the LEE-encoded EspA protein in WT and the Δ*uhpABC* strain. RpoA is shown as a loading control. (C) qRT-PCR of *stx2a* in WT 86-24 and the Δ*uhpABC* strains. ns = not significant.

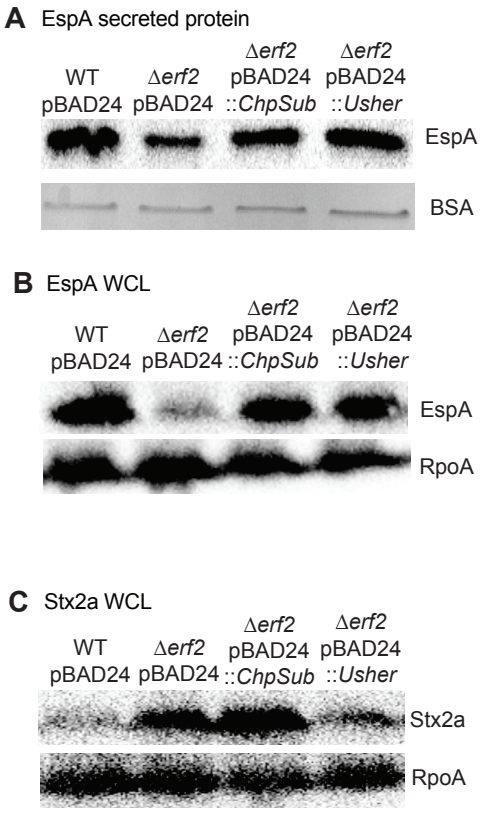


Figure 4.18 The entire Erf2 fimbrial locus is NOT necessary to virulence expression. (A) Representative Western blot of the secreted EspA protein in WT transformed with an empty vector control, the Δ*erf1* strain transformed with an empty vector control, the Δ*erf1* strain transformed with the chaperone and major subunit (*ChpSub*, *yadN* and *ecpD*, *Z0151-Z0152),* and the Δ*erf1* strain transformed with the usher (*Usher*, *htrE*, *Z0150)*. BSA is shown as a loading control. (B) Representative Western blot of WCL EspA protein. RpoA is shown as a loading control. (C) Representative Western blot of WCL Stx2a protein. RpoA is shown as a loading control.

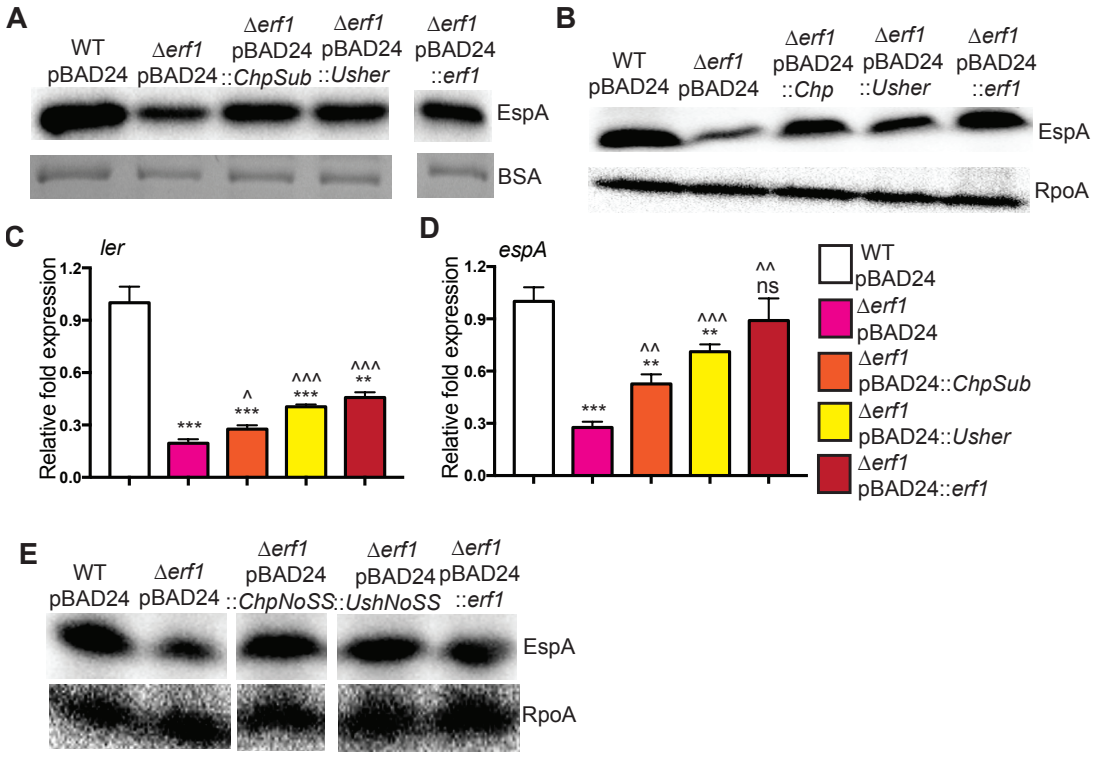


Figure 4.19 The entire Erf1 fimbrial locus is NOT necessary for expression of the LEE. (A) Representative Western blot of the secreted EspA protein in WT transformed with an empty vector control, the Δ*erf1* strain transformed with an empty vector control, the Δ*erf1* strain transformed with the chaperone and major subunit (*ChpSub*, *yehDC*, *Z3278-Z3279),* and the Δ*erf1* strain transformed with the usher (*Usher*, *yehB*, *Z3277)*. BSA is shown as a loading control. (B) Representative Western blot of WCL EspA protein in WT transformed with an empty vector control, the Δ*erf1* strain transformed with an empty vector control, the Δ*erf1* strain transformed with the chaperone (*Chp*, *yehC*, *Z3278),* and the Δ*erf1* strain transformed with the usher (*Usher*, *yehB*, *Z3277)*. RpoA is shown as a loading control. (C) qRT-PCR of *ler* and (D) *espA* in WT 86-24 transformed with an empty

vector control, the Δ*erf1* strain transformed with an empty vector control, the Δ*erf1* strain transformed with the chaperone and major subunit (*ChpSub*, *yehDC*, *Z3278-Z3279),* and the Δ*erf1* strain transformed with the usher (*Usher*, *yehB*, *Z3277)*. (E) Representative Western blot of EspA protein in WT transformed with an empty vector control, the Δ*erf1* strain transformed with an empty vector control, the Δ*erf1* strain transformed with the chaperone without the putative signal sequence (*ChpNoSS*, *yehC*, *Z3278),* and the Δ*erf1* strain transformed with the usher without the putative signal sequence (*Usher*, *yehB*, *Z3277)*. Statistical significance shown relative to WT 86-24 as: **, P≤ 0.005; ***, P≤ 0.0005; ns = not significant. Statistical significance shown relative to Δ*erf1* as: ^, P≤ 0.05; ^^, P≤ 0.005; ^^^, P≤ 0.0005.

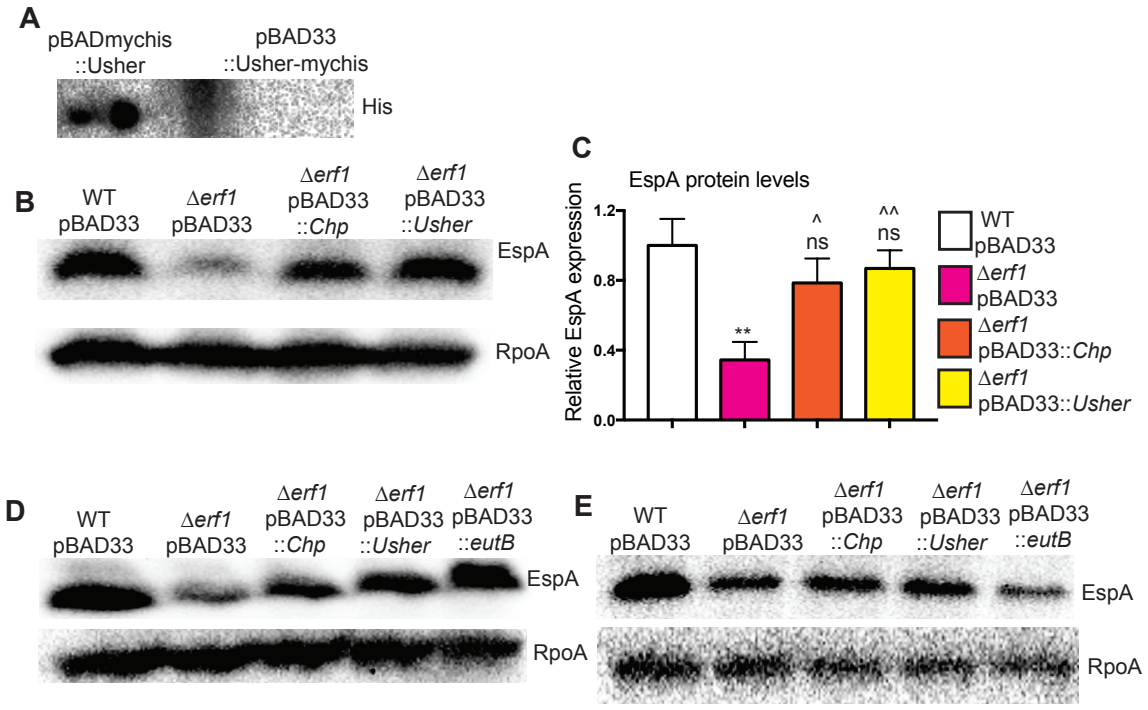


Figure 4.20 Transcription of certain genes from *erf1* MAY be sufficient to regulate expression of the LEE. (A) Western blot of the Usher::mychis (*yehB*, *Z3277)* from the pBADmychis vector and the pBAD33 vector. (B) Representative Western blot of EspA protein in WT transformed with an empty vector control, the Δ*erf1* strain transformed with an empty vector control, the Δ*erf1* strain transformed with the chaperone (*Chp*, *yehC*, *Z3278),* and the Δ*erf1* strain transformed with the usher (*Usher*, *yehB*, *Z3277)*. RpoA is shown as a loading control. (C) Quantification of EspA expression from (B). (D and E) Two representative Western blots of EspA protein in WT transformed with an empty vector control, the Δ*erf1* strain transformed with an empty vector control, the Δ*erf1* strain transformed with the chaperone (*Chp*, *yehC*, *Z3278),* the Δ*erf1* strain transformed with the usher (*Usher*,

yehB, *Z3706),* and the Δ*erf1* strain transformed with a random DNA sequence (*eutB*, *Z3277)*. Statistical significance shown relative to WT 86-24 as: **, P≤ 0.005; ns = not significant. Statistical significance shown relative to Δ*erf1* as: ^, P≤ 0.05; ^^, P≤ 0.005.

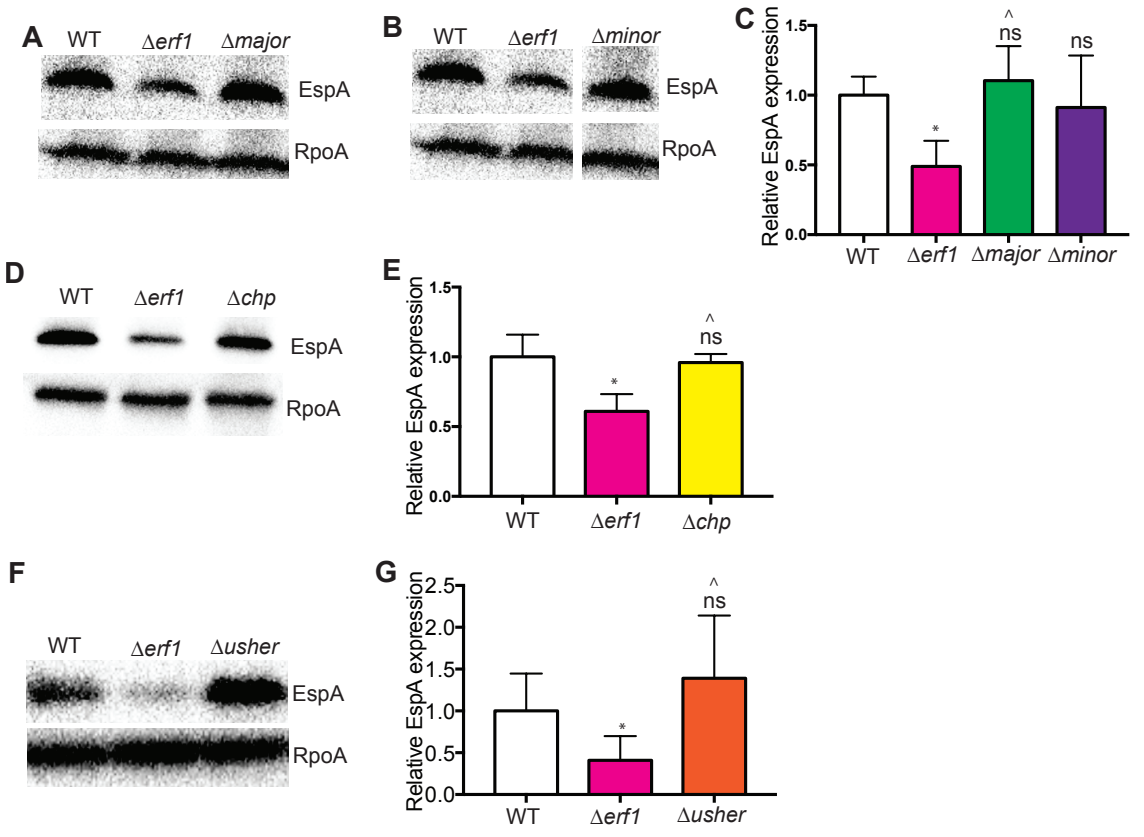


Figure 4.21 The entire Erf1 fimbrial locus is NOT necessary to expression of the LEE. (A) Representative Western blot of the LEE-encoded EspA protein in WT 86-24, the *Δerf1* strain, and the major subunit deletion strain (*Δmajor, yehD, Z3279*). (B) Western blot of EspA in WT 86-24, the *Δerf1* strain, and the minor subunit deletion strain (*Δminor, Z3276*). (C) Quantification of EspA protein levels from (A) and (B). (D) Western blot of EspA in WT 86-24, the *Δerf1* strain, and the chaperone deletion strain (*Δchp, Z3278*). (E) Quantification of EspA protein levels from (D). For all Western blots, RpoA is shown as a loading control. (F) Western blot of EspA in WT 86-24, the *Δerf1* strain, and the usher deletion strain (*Δusher, Z3277*). (G) Quantification of EspA protein levels from (F).

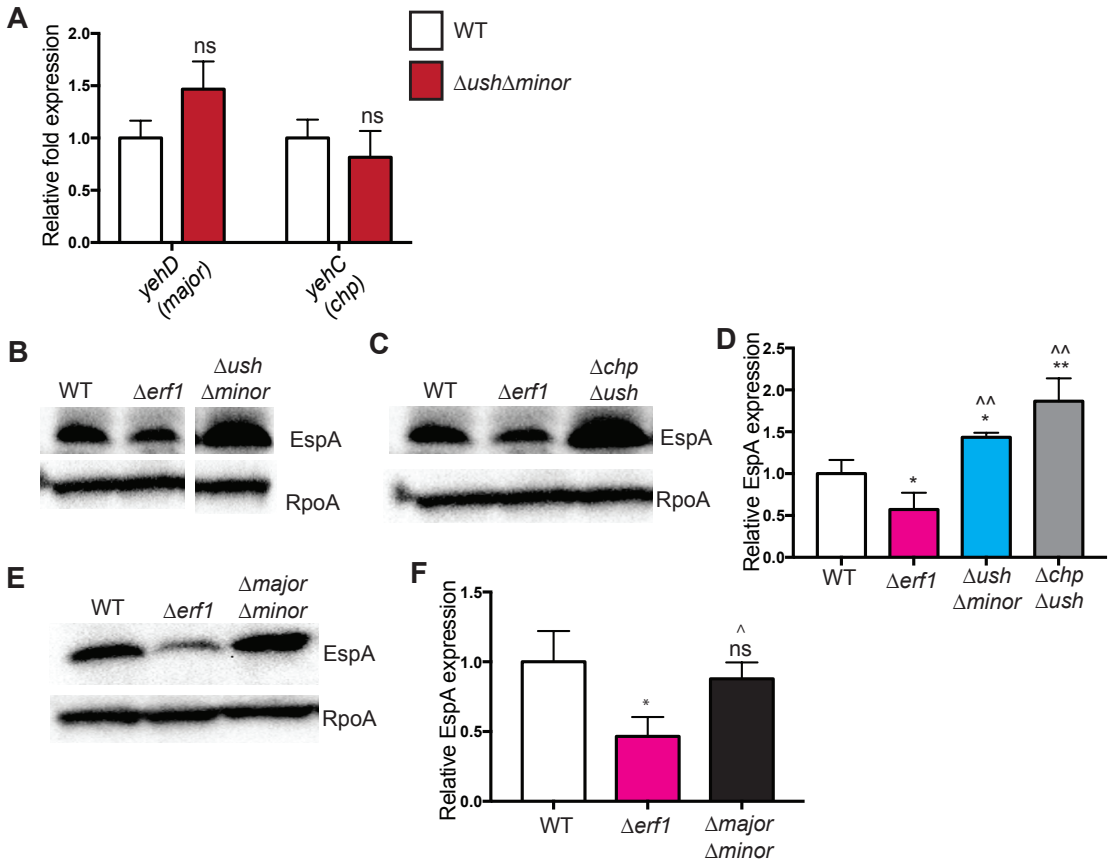


Figure 4.22 Expression of the Erf1 major and minor subunit or of the chaperone and usher is sufficient to regulate expression of the LEE. (A) qRT-PCR of *yehD* (major subunit) and *yehC* (chaperone) in WT and the *ΔushΔminor* (usher and minor subunit, *yehB* and *Z3276*). (B) and (C) Western blot of EspA in WT 86-24, the *Δerf1* strain, and the usher and minor subunit deletion strain (*ΔushΔminor, yehB* and *Z3276, Z3276-Z3278*) or the usher and minor subunit deletion strain (*ΔchpΔush, yehCB, Z3277-Z3278*), respectively. (D) Quantification of EspA protein levels from (B) and (C). (E) Western blot of EspA in WT 86-24, the *Δerf1* strain, and the major subunit and minor subunit deletion strain (*ΔmajorΔminor, yehD* and *Z3276, Z3276* and *Z3279*). (F) (D) Quantification of EspA protein levels from (E). RpoA is shown as a loading control.

Table 4.1 Size of deletions in the Erf1 fimbrial mutants chart

Deletion strain name	**Gene being deleted**	**Deletion size**	**Remaining of the deletion gene upstream**	**Remaining of the deletion gene downstream**	**5' UTR present?**
Δerf1	*yehDCB Z3276*	4777 bp of *erf1*	54 bp of *yehD*	15 bp of *Z3276*	Yes
Δmajor	*yehD*	482 of *yehD*	48 bp of *yehD*	13 of *yehD*	Yes
Δminor	*Z3276*	973 bp of *Z3276*	47 bp of *Z3276*	15 bp of *Z3276*	Yes
Δchp	*yehC*	672 of *yehC*	28 bp of *yehC*	23 bp of *yehC*	Yes
Δusher	*yehB*	2140 bp of *yehB*	185 bp of *yehB*	156 bp of *yehB*	Yes
Δmajor Δminor	*yehD, Z3276*	478 bp of *yehD* 973 bp of *Z3276*	52 bp of *yehD* 47 bp of *Z3276*	13 bp of *yehD* 15 bp of Z3276	Yes
Δchp Δush	*yehCB*	3220 bp of *yehCB*	None. (missing 10 bp of the 39 bp intergenic	9 bp of *yehB*	Yes

			region of *yehDC*)		
Δush Δminor	*yehB, Z3276*	3474 bp of *yehB/Z3276*	41 bp of *yehB*	11 bp of *Z3276*	Yes

Chapter Five: Conclusions and Future Directions

Summary

EA regulates the expression of virulence traits including the LEE, Shiga toxin, and fimbriae [109, 166]; however, biochemical evidence for these interactions has been lacking. We demonstrated that the EA-dependent regulatory protein EutR, directly binds to the *ler* promoter to activate transcription of the LEE. Furthermore, we determined that EA does not regulate the expression of virulence traits in enteropathogenic *E. coli*. Additionally, we characterized the function of EtrB, a protein regulated by QseA that is not involved in EA signaling, as a regulator of virulence gene expression. EtrB activates the expression of 46 genes including the LEE, through direct regulation of *ler* and non-LEE encoded effectors. EtrB also functions to repress the expression of 70 genes including fimbrial genes and genes located in the ETT2. These findings indicate that EtrB plays a more global role in EHEC gene regulation including factors that are important for EHEC host adaptation and pathogenesis.

EA induces the expression of 15 of the 16 encoded fimbrial loci [166]. Two EHEC fimbrial loci, Erf1 and Erf2, influence AE lesion formation and Shiga toxin production (Gonyar, unpublished); however, the mechanism underlying fimbrial regulation of virulence traits in EHEC was lacking. We demonstrated that Erf1 and Erf2 contribute to colonization of the murine GI tract; however, we did not observe any adherence defect in the *erf1* and *erf2* mutant strains. We hypothesize that the decreased fitness of the *Δerf1* and *Δerf2* strains is not caused by a lack of Erf1- or Erf2-mediated fimbrial adherence but is due to differential regulation of LEE-encoded T3SS, secreted effectors, intimin, or other fimbrial adhesions that influence

EHEC colonization of the murine GI tract. We also demonstrated that regulation of the LEE and of Shiga toxin does not require formation of an Erf1 or Erf2 fimbrial structure on the surface. These data indicate that components of the *erf1* and *erf2* fimbrial loci are sufficient to regulate virulence gene expression. Furthermore, these data redefine the function of fimbrial loci as more than just adhesins and suggest a novel mechanism for fimbrial regulation of virulence traits. Overall these findings in EHEC expand our understanding of signaling molecules that contribute to the regulation of virulence traits in EHEC (Figure 5.1).

Coordination of EHEC metabolism and virulence

Nutrient utilization and signaling are important cellular processes for pathogens to establish infection and cause disease [370]. Our work described the first mechanistic studies of EutR gene regulation and determined a novel role for EutR in EHEC pathogenesis [110]. The regulation of virulence traits through EA is independent of EA metabolism [109]. However, it is unclear how EA coordinates both metabolism and virulence regulation to enhance EHEC pathogenesis. It is likely that EHEC exploits EA as a signal to coordinate metabolism and virulence, thereby enhancing EHEC colonization and pathogenesis, similar to the GI pathogen *Salmonella* Typhimurium [228]. Additionally, EHEC utilizes other host-derived metabolites such as D-serine, fucose, biotin, and glucose [114, 121, 286, 371-373] to aid in metabolic competition and virulence gene expression. We hypothesize that EA metabolism may be important for EHEC nutrient competition with the commensal microbiota in the lumen while EA regulation of virulence is important for EHEC

colonization of the epithelium. To test this, we could use an infant rabbit model to study EHEC infection [145, 374]. We would assess colonic colonization and AE lesion formation in competition experiments between WT/*ΔeutR,* WT/*ΔeutB,* *ΔeutB*/*ΔeutR* (the *ΔeutR* strain cannot sense EA and the *ΔeutB* stain cannot catabolize EA). If EA metabolism is important for EHEC competition with the microbiota, we expect that WT would outcompete *ΔeutR* and *ΔeutB* in both the WT/*ΔeutR* and WT/*ΔeutB* competitions. We would further expect that the *ΔeutR* strain would be reduced in AE lesion formation and association with the epithelium in comparison to the *ΔeutB* strain in the *ΔeutB*/*ΔeutR* competition, indicating that EA regulation of virulence, not metabolism, is important for EHEC colonization at the epithelium. To differentiate between *ΔeutB* and *ΔeutR* strains in the GI tract in a competition experiment, we could create a constitutively GFP-expressing *ΔeutB* strain and a constitutively RFP-expressing *ΔeutR* strain.

We demonstrated that EutR directly binds to target DNA sequences to regulate expression. However, the binding kinetics of EutR are currently unknown. Studying the binding affinity of EutR to target promoters involved in EA metabolism and EHEC virulence may further reveal how EA metabolism and virulence in EHEC are coordinated [375-377]. The concentration of EA in the human GI tract is currently unknown. A study of the bovine intestinal contents measured the concentration of free EA at 2 mM [224]. Determining the concentration of free EA present in the different colonic compartments of the human GI tract (lumen, mucus, epithelium), with the *in vitro* binding kinetics assay, would provide information as to how EA signaling modulates gene expression through EutR-dependent and

independent mechanisms. Differential binding of EutR to genes involved in metabolism or virulence may coordinate virulence and metabolic gene expression in EHEC. Together, these studies would further our understanding of the link between nutrient metabolism and virulence regulation.

Our work identified the role of the transcriptional factor EtrB in the regulation of EHEC virulence traits, including AE lesion formation, fimbriae, and metabolism. The effect of EtrB regulation on metabolism and the role this plays in EHEC pathogenesis is currently unclear. EtrB is encoded within the ETT2 pathogenicity island. The ETT2 is present in a majority of *E. coli* strains and in all strains except for enteroaggregative *E. coli* O42, the ETT2-encoded T3SS is incomplete due to frameshift mutations in structural genes [206]. The ETT2 is located on the same chromosomal position, the intergenic region of *yqeG-glyU,* in all strains suggesting that ETT2 was acquired in an ancestral *E. coli* strain and has become degenerate [206]. The presence of ETT2 in commensal strains suggests that it was likely involved in host colonization in a symbiotic relationship or in survival in the external environment [378-380]. We determined that EtrB modulates the expression genes involved in threonine, serine, and tryptophan metabolism and transport, maltose transport, and allantoin utilization [119]; however, all of these products are utilized by commensal *E. coli* [381-386]. Therefore, we hypothesize that EtrB regulation of metabolism is a shared trait of EtrB in *E. coli* species [387-k390]. We could test this hypothesis by determining the impact of EtrB on metabolism in other *E. coli* strains such as MG1655, a commensal *E. coli* strain that contains a non-functional ETT2-encoded T3SS, and O42, a strain of the pathogen

enteroaggregative *E. coli* strain that contains an ETT2-encoded T3SS that is predicted to be functional [206].

The effect of EtrB on metabolism could be due to indirect targeting of another pathway by EtrB. This could be tested by performing CHiP-seq to assess global binding targets of EtrB. If EtrB is directly regulating metabolism, we could mutate the binding sites of EtrB in the promoter regions to assess how EtrB-regulation of that metabolic pathway affects EHEC growth. If EtrB regulation of metabolism is unimportant for EHEC pathogenesis as we predicted, then we expect that mutation of the EtrB binding sites involved in metabolism will have no effect on EHEC fitness in the GI tract.

Overall, these studies will further our understanding of the link between metabolism and virulence regulation in pathogens. Coordination of metabolism and virulence in response to the nutrient environment is critical for pathogen niche recognition. Furthering our understanding of host-pathogen interactions could lead to novel therapeutic strategies that would selectively target specific pathogens to affect both growth and virulence. One strategy is to target pathways involved in the expression of virulence traits [391, 392]. For example, we could develop a small molecule to selectively inhibit EutR preventing the activation of virulence gene expression. A similar molecule could be developed in regards to EtrB; however, we would need to verify that EtrB is not an important regulator of metabolism in commensal *E. coli*.

Hierarchy and the spatio-temporal activation of regulators in EHEC

The expression of virulence traits is energetically expensive and should only occur at a suitable niche within the host. Pathogens have evolved complex pathways to coordinate virulence gene expression at precise locations [393]. Our work describes multiple mechanisms that EHEC utilizes to regulate virulence traits, focusing on regulation of the LEE and thus AE lesion formation (Figure 5.1). However, very little is known about the hierarchy of virulence gene regulation in EHEC. Previous studies have begun to characterize the transcriptional regulatory networks in *E. coli* by identifying the hierarchy underlying transcriptional regulation [376, 394, 395]. In *E. coli*, it is common for a group of genes to be involved in a complex regulatory cascade involving multiple transcription factors [396]. However, these studies have not investigated the hierarchy of transcription factors regulating virulence gene expression in EHEC.

To begin to assess the interplay between EutR, QseA, EtrB, and the Erf1 and Erf2 fimbrial loci in regards to regulation of the LEE, we could determine the global binding targets of EutR, QseA, and EtrB by CHiP-seq [397, 398]. This will define if there is a hierarchy of EutR, QseA, and EtrB in the regulatory cascade(s) of EHEC by providing an unbiased method of assessing if these transcription factors act upstream of other transcriptional regulators. We predict that when EHEC is grown anaerobically, we will observe EutR binding the *qseA* promoter region, QseA binding the *etrB* and *qseA* promoter region, and EtrB binding the *etrB* promoter region leading to a clearly defined signaling pathway and hierarchy of regulation.

Another possibility is that these regulatory proteins act antagonistically to each other by inhibiting binding to the target LEE promoter. For example, EutR binding to the *ler* promoter region could inhibit binding of EtrB. QseA binds both the proximal and distal *ler* promoters immediately upstream of the -35 promoter elements [46]. EutR binds upstream of the -35 proximal *ler* promoter region [119]. The QseA and EutR binding sites in the proximal *ler* promoter are separated by 34 basepairs and do not appear to overlap, suggesting that both EutR and QseA could potentially bind the *ler* promoter region. An increased understanding of how EutR, QseA, and EtrB promote gene expression would help determine if these regulatory proteins can act synergistically. We could perform *in vitro* transcription assays using purified EutR, QseA, and EtrB to determine if the presence of multiple regulator proteins increases transcription of *ler*.

It is also possible that these regulators are expressed under different conditions, in different spatial locations within the GI tract, and/or at different times during infection and do not form a signaling cascade. To investigate the spatial activation of various transcription factors, we can utilize animal models of EHEC infection [399, 400]. For assessment of *erf1* and *erf2* expression, we can use our previously described model for EHEC-murine infection. For EutR, QseA, and EtrB, we can use the previously described infant rabbit model of infection. The spatial activation of regulators can be determined by assessing reporter expression in cross-sections of the colon. We could create an EHEC expression strain that contains the promoter region of *eutR* fused to CFP, the promoter region of *qseA* fused to YFP, and the promoter region of *etrB* fused to RFP to determine if there is overlap in the

expression of these transcription factors. Previous studies have used CFP, YFP, and RFP reporters together to assess gene regulation with minimal excitation emission spectra overlap [401, 402].

Another method to assess the hierarchy of EutR, QseA, EtrB, and the Erf1 and Erf2 fimbrial loci in LEE regulation is to create multiple deletion strains to determine if these regulators act in the same regulatory pathway. For example, we could create a *ΔeutRΔetrB* strain and determine LEE expression by qRT-PCR and by measuring AE lesion formation during HeLa cell infection. If the *ΔeutRΔetrB* strain has decreased LEE expression compared to *ΔeutR* and *ΔetrB*, this suggests that EutR and EtrB regulate the LEE through independent pathways. By creating different deletion combinations of EutR, QseA, EtrB, and the Erf1 and Erf2 fimbrial loci, we can assess how these regulators interact with each other. In addition, we can further study potential feed forward loops that control the expression of virulence traits, a mechanism used to increase the activation of a target gene in response to stimulus [403, 404]. Investigating the phenotype of *ΔqseAΔetrB* would determine if QseA and EtrB act together in a feed forward loop, as hypothesized in Chapter 3, to amplify activation of the LEE. If QseA and EtrB are acting in a feed forward loop, we expect the *ΔqseAΔetrB* strain to have decreased LEE expression compared to the *ΔqseA* and *ΔetrB* single deletion strains. If there is no additive effect of the *ΔqseAΔetrB* deletion, this would indicate that QseA and EtrB are not in a feed forward loop and may indicate that they regulate the LEE at different times during EHEC infection.

One difficulty in using multiple deletion strains is that we may observe additive effects in the double mutant that are due to differential regulation of other

genes. For example, QseA is a global regulator of gene expression in EHEC [46, 214]. QseA and EtrB may function in the same LEE activation pathway but the *ΔqseAΔetrB* double mutant might display decreased LEE expression in comparison to the *ΔqseA* or *ΔetrB* mutant strains because of the influence of QseA on other LEE regulators. As an alternative approach, we could mutate the binding site in the *ler* promoter region for one regulator of interested, such as QseA, in both WT EHEC and a mutant background, such as *ΔetrB*. The WT EHEC with the mutated binding site should have decreased *LEE* expression, but no off target effects associated with the *ΔqseA* mutant strain. We can then assess *LEE* expression in the *ΔetrB* mutant strain with the mutated QseA binding sequence in the *ler* promoter, to determine if QseA and EtrB have linear or parallel effects on virulence gene expression. These experiments would define the potential hierarchy regulation of the transcription factors EutR, QseA, and EtrB.

Increasing our understanding of the complex mechanisms underlying transcriptional regulation will provide novel information regarding virulence signaling cascades. We can utilize our new understanding of transcriptional regulatory networks and the hierarchal control of virulence gene expression to create prediction models for regulators and how they may influence bacterial pathogenesis [405, 406]. This will aid in the development of novel therapeutic targets or vaccine development by narrowing the number the potential targets [389, 407].

One limitation of our work characterizing EHEC virulence regulation is the lack of data concerning the spatio-temporal activation of EutR, QseA, EtrB, and the

Erf1 and Erf2 fimbrial loci. Different regulators may be activated at different times during infection or in different locations within the GI tract, such as in the ascending colon compared to the transverse colon [408-412]. EHEC colonizes the colon, predominantly in the ascending and transverse colon as suggested by the predominance of hemorrhagic colitis pathology [147, 413]. The concentration and localization of EA in the human colon has not been measured, but is found at mM levels in the bovine intestinal contents [224]. Free EA is present in the GI tract from the turnover and subsequent breakdown of host enterocytes and the microbiota, as well as from the host diet [242, 243, 317, 414]. The concentration of EA in the lumen is high enough to support pathogen growth [224, 225]. We hypothesize that utilization of EA in the lumen provides a competitive growth advantage for EHEC and EA near the intestinal epithelium acts as a signaling molecule to activate virulence gene expression.

QseA is involved in the epinephrine/quorum sensing pathway to regulate the expression of virulence genes [46, 214]. We determined that *etrB* expression is directly regulated by QseA [119], suggesting that EtrB is involved in this signaling cascade. The temporal activation of virulence gene expression in response EA and epinephrine during EHEC infection is unknown. Epinephrine is present in the human GI tract, but the exact concentrations are unknown [415]. A study indicated that epithelial cells could secrete hormones such as epinephrine [416]. Another possibility is that epinephrine is released from the bloodstream during disruption of the intestinal epithelium in the onset of EHEC-associated hemorrhagic colitis [233, 417]. We hypothesize that during infection EHEC first senses free EA produced by

the intestinal epithelium resulting in expression of the LEE. Over the course of infection as the intestinal epithelium becomes more disrupted, epinephrine is released into the gastrointestinal tract leading to increased activation of the LEE. Using fluorescent reporters genes, we could monitor the expression of multiple virulence regulators over time to determine when each regulator is expressed and if certain regulators are expressed concurrently. We could use quantitative time-lapse microscopy paired with fluorescent reporters to observe the temporal activation of regulators in EHEC. This could be performed *in vitro* under a variety of conditions including pure culture growth or with EHEC infection of epithelial cells.

These studies would further define the regulatory networks underlying virulence in EHEC by assessing the spatio-temporal activation of virulence regulators. Understanding when pathogens encounter various molecules during infection and how this affects virulence would lead to a greater understanding of bacterial pathogenesis and the host environment. Additionally, this work could have broader implications for how pathogens utilize multiple environmental cues to colonize the host and cause disease.

Function of regulators in other pathogenic *E. coli* species

Pathogens have evolved complex signaling pathways to regulate the expression of virulence traits [418-421]. In many cases, these regulators have evolved from their previous function in a distant ancestor [422-426]. Currently, very little is known about the evolution of regulatory circuits in bacteria [426, 427]. Further understanding the evolution of regulators could lead to a better

understanding of virulence regulatory circuits. We can use this information to predict the function of homologous regulators in pathogenic and commensal species. This could also aid in the development of novel therapeutics by eliminating regulatory targets that have effects on the commensal microbiota.

EHEC has maintained the ability to utilize EA as a metabolite and has gained the ability to utilize EA as a signaling molecule to regulate virulence [109, 223, 224]. Our work demonstrates that the AE-lesion forming pathogen EPEC does not use EA as a signaling molecule to activate LEE expression, despite containing the putative EutR binding sequence in the EPEC *ler* promoter [110]. This lack of regulation is likely due to the absence of EutR activation in the presence of EA and B_{12}. The *eut* operon contains a large phage insertion between the *eutB* and *eutC* genes, which encode the ammonia lyase, making EPEC unable to utilize EA as a metabolite [110]. A BLAST comparison of the *eutR* sequence in EHEC and EPEC demonstrate that *eutR* has 99% identical with 10 mismatches in the *eutR* coding sequence (10/1053 mismatched nucleotides, no gaps), 5 of which are located in the DNA binding motif. At the protein level there are only 3 amino acids differences between EPEC and EHEC EutR and none of the changes are located in the DNA binding motif. There is a 98.6% similarity in the 1000 base pairs upstream of *eutR* between EHEC and EPEC (14/1000 mismatched base pairs, no gaps; 13 of the mutations are over 800 nucleotides immediately upstream of the EPEC *eutR* ATG start site). It is unclear if this phage insertion is the reason *eutR* expression in not induced or if it is because the *eutR* promoter region has changed in comparison to EHEC. We hypothesize that the lack of EutR regulation of the LEE in EPEC is due to an inability of EA to induce

expression of *eutR*. To test if EPEC-encoded EutR can induce virulence gene expression in response to EA, we could express EPEC EutR on an inducible plasmid and assess EPEC virulence in the presence and absence of EA and B_{12}. We expect that if the lack of EA regulated virulence gene expression is due to an absence of *eutR* induction then induced expression of EPEC *eutR* on a plasmid should confer the ability of EPEC to respond to the presence of EA.

These data suggest that the role of EutR in metabolism and virulence is different in various *E. coli* lineages. Studies in uropathogenic *E. coli* and *Salmonella enterica* serovar Typhimurium suggest that EA metabolism and signaling may be a widespread mechanism utilized by select pathogens to respond to the host environment [225, 228, 428]. Future work could explore the function of EutR in other pathogenic *E. coli* species such as enteroaggregative *E. coli,* which utilizes different virulence traits to colonize the GI tract.

The evolution of a bacterial signaling pathway can involve modifications of regulatory proteins changing its target promoters, resulting in the ability to transcribe horizontally acquired genes, such as virulence genes, while retaining transcriptional control of ancestral genes [426, 429, 430]. It is unclear what the function of EtrB is in relation to the ETT2 pathogenicity island it is encoded in. EtrB is a homologue of the *Salmonella* SPI-2 regulator SsrB with a 32% protein similarity [206]. SsrB activates the expression of genes encoded within and outside of the SPI-2 pathogenicity island it is encoded in [52, 431], indicating that EtrB may function in a similar capacity to regulate the ETT2 as well as genes located outside the ETT2. The ETT2 in enteroaggregative *E. coli* strain O42 is predicted to encode a functional

secretion system, which provides a system to potentially study the function of ETT2 as a type III secretion system and assess the role of the transcription factor EtrB in relation to the intact ETT2 [206, 432].

To further assess the function of EtrB, we could study EtrB in other pathogenic *E. coli* species with a degenerate ETT2, such as in the Shiga toxin-producing *E. coli* strain O103:H25 [208, 433, 434]. Analysis of the function of EtrB in the non-LEE-encoding, Shiga toxin-producing *E. coli* O103:H25 would provide more information about the function of EtrB in other *E. coli* lineages and could provide insight into potentially conserved functions of EtrB, such as the regulation of metabolism. To further determine whether the function of EtrB is different in various *E. coli* species, we could study the function of EtrB in the laboratory strain MG1655. Comparing the global targets of EtrB in EHEC [119], *E. coli* O103:H25 (Shiga toxin-producing *E. coli*), and *E. coli* MG1655 (laboratory strain), which all contain a degenerate ETT2, and *E. coli* O42 (enteroaggregative *E. coli*), which contains a functional ETT2, could provide information as to the previous function of EtrB in relation to the ETT2 and how the function of EtrB has changed in different strains of *E. coli*. We could determine the EtrB binding sequence in EHEC, *E. coli* O103:H25, *E. coli* MG1655, and *E. coli* O42 to assess how EtrB and its binding site has changed in different *E. coli* species.

Our work has demonstrated that EHEC fimbriae regulate virulence traits including Shiga toxin and the LEE. Erf1 and Erf2 represent novel mechanisms in *E. coli* to regulate virulence gene expression and could represent a way to ensure hierarchical expression of pathogenic traits in EHEC. Fimbriae in other pathogens

such as *Pseudomonas aeruginosa* and *Neisseria meningitidis*, modulate gene expression; however, most studies have focused on how fimbriae-mediated mechanical stress associated with attachment modulates gene expression [324, 354-356]. Our work suggests that expression of certain fimbrial components can modulate gene expression. Many pathogens encode fimbriae, including other pathogenic *E. coli* strains [313, 343, 435, 436]. The regulation of bacterial gene expression by components of a fimbrial locus could be a conserved mechanism among bacterial pathogens to coordinate virulence. Future work could assess the function of fimbriae in other pathogenic *E. coli* species to determine regulation of bacterial gene expression by fimbrial components is a widespread mechanism that has been overlooked.

Mechanisms of fimbrial regulation of EHEC virulence traits

Our work begins to define the mechanism by which Erf1 and Erf2 regulate EHEC virulence traits. We have demonstrated that Erf1 and Erf2 do not play a role in fimbriae-mediated adhesion to epithelial cells in culture, but do influence colonization in a murine model of infection Furthermore, we have determined that expression of Erf1 and Erf2 as surface structures is not necessary to regulate the expression of virulence traits. Figure 5.2 and 5.3 postulate potential mechanisms regarding Erf1 and Erf2 regulation of the LEE and Shiga toxin.

Our first, non-exclusive hypothesis is that expression of Erf1 and Erf2 can still form fimbrial structures in the partial locus deletions, and these fimbrial structures regulates LEE expression. Previous studies have shown that the transport

and assembly machinery of different fimbrial loci can bind fimbrial subunits encoded in other fimbrial loci to form functional fimbriae [205, 437-440]. Therefore, in our deletion strains, Erf1 and Erf2 could potentially still be expressed on the surface and could still regulate virulence gene expression by Erf1 and Erf2 fimbriae-mediated binding (Figures 5.2A and 5.3C). Our gene and protein expression experiments were performed in the absence of epithelial cells and we did not observe any clumping or EHEC settling, which might indicate bacterial attachment. However, Erf1 and Erf2 could bind to ligands on EHEC that would then modulate virulence gene expression. To test this hypothesis, we can generate an antibody to the fimbrial major subunit which can be used to determine if Erf1 and Erf2 major subunits are being expressed on the surface of EHEC in a strain that does not express the fimbrial transport and assembly machinery (Erf1: *ΔchaperoneΔusher, ΔyehCΔyehB;* Erf2: *Δerf2* pBAD24::*MajorsubunitChaperone, yadN ecpD*). This antibody can be used to identify the alternate transport machinery interacting with the Erf1 and Erf2 major subunit.

Our data suggests another potentially redundant signaling event for Erf1 regulation of the LEE, as shown in Figure 5.2B. We hypothesize that the Erf1 chaperone and usher are exporting fimbrial subunits encoded in a different fimbrial locus to regulate expression of the LEE [205, 437-439]. We can test this hypothesis by tagging the Erf1 chaperone and using the tag to pulldown all proteins associated with the Erf1 chaperone in a strain lacking the Erf1 major and minor subunits (*ΔyehDΔZ3276; ΔmajorΔminor*) [441-443]. Both hypotheses (Figure 5.2A and B) would likely involve one or more regulatory intermediates to mediate LEE

expression. To identify regulatory intermediates, we could perform a pulldown with the usher and/or the chaperone, which would identify any direct binding targets. If no proteins are binding the chaperone and usher besides the fimbrial subunits, we can investigate total protein levels by tandem mass spectrometry. This will provide an unbiased approach to identify differentially expressed proteins as potential candidates for regulatory intermediates. To assess if identified proteins are regulator intermediates as opposed to downstream targets, we could delete the gene of interest in WT EHEC and in the *erf1* mutant background and assess virulence gene expression. If the gene of interest is a regulator intermediate, we expect to observe a decrease in virulence gene expression in both the single deletion strain and the deletion in the *erf1* mutant background when compared to WT. Additionally, we expect expression of virulence genes to be comparable in the single gene of interest deletion and the deletion of the gene of interest in the *erf1* mutant background.

It is unclear from our data if the various transport, assembly, and structural components of Erf1 and Erf2 need to be trafficked to the periplasm to affect LEE expression. As another possibility to explain how Erf1 and Erf2 regulate virulence gene expression is that transcription of the Erf1 major and/or minor subunits (Figure 5.2C), transcription of the chaperone and/or usher (Figure 5.2D), transcription of the Erf2 usher, or transcription of the Erf2 chaperone and/or major subunits (Figure 5.3D) could regulate LEE expression. Pathogens encode a large number of small RNAs (sRNA) [444, 445] that regulate virulence traits [446-450]. Additionally, some sRNAs can also encode proteins [451, 452] suggesting that

components of the *erf1* and *erf2* loci could encode both sRNAs and proteins. The Erf1 and Erf2 fimbrial loci could encode small RNAs that influence expression of the LEE and Shiga toxin. To test if the fimbrial mRNA is sufficient to regulate virulence, we could mutate the ribosomal binding site so that specific components of the *erf1* and *erf2* fimbrial loci are transcribed but not translated. This would eliminate potential variability caused by vector copy numbers and artificial induction. As an unbiased approach to identifying a potential RNA regulatory sequence in *erf1* and *erf2*, we could evaluate the RNA transcriptome [453]. Once a target RNA sequence is identified, we could use an aptamer tag to purify RNA-bound proteins from EHEC to identify regulatory intermediates that affect LEE expression [454-456].

It is possible that protein components of the Erf1 and Erf2 loci are mediating LEE expression (Figure 5.2C and D, Figure 5.3 B and D), as opposed to RNA. The literature is lacking in examples concerning the fimbrial usher or chaperone regulating traits; however, fimbriae-mediated signals can be transduced by proteins containing a periplasmic domain [324] suggesting that fimbrial components could function in a similar capacity. To test this, we could delete the signal sequence from the gene of interest, for example with the Erf1 chaperone (*yehC*) and assess the impact on virulence gene expression. If fimbrial components are sufficient to regulate the LEE, we could identify potential regulatory intermediates by crosslinking, co-immunoprecipitating with a tag on the fimbrial component of interest, then performing mass spectrometry. We can also create single expression strains by deleting every gene from the fimbrial locus except for our gene of interest.

This will identify which components of Erf1 and Erf2 are sufficient to influence virulence gene expression in EHEC.

We hypothesize that expression of the Erf2 usher (*htrE*) increases EHEC membrane stability, which leads to an increase in LEE expression (specifically LEE2/4) and a decrease in Shiga toxin production (Figure 5.3A) [457]. Alterations in the *E. coli* membrane can induce an SOS response [458]. The protein composition of the EHEC outer membrane is largely unknown [459]. The Erf2 usher could be an integral component of the EHEC membrane and could help maintain the outer membrane stability and integrity. The change in membrane composition in the *erf2* mutant strain could induce an SOS response, leading to increased Shiga toxin production. There are many bacterial outer membrane associated proteins that play an integral role in outer membrane stability [460-464]. To determine if the SOS response is being induced in the *erf2* mutant strain, we can measure *recA* gene expression, which is an accurate of the SOS response [465]. If we do see an induction of *recA* in the *erf2* mutant strain, we can perform further experiments to determine if the membrane composition is altered. To assess the protein composition of the EHEC outer membrane, we could perform mass spectrometry on the EHEC outer membrane, which would determine if the Erf2 usher is an integral outer membrane protein. The function of Erf2 in EHEC membrane stability can be tested by determining the sensitivity of WT EHEC, *Δerf2,* and the *Δerf2* pUsher (pBAD24::*htrE*) complement strain to vancomycin [466]. Changes in EHEC resistance to vancomycin would indicate differences in outer membrane properties and would indicate a role for the Erf2 usher in EHEC membrane homeostasis.

In summary, we anticipate that the experiments described above will provide novel insights into the mechanisms underlying fimbrial regulation of virulence traits in EHEC. Further understanding of these mechanisms will provide insight into how pathogens utilize fimbriae to exhibit hierarchical control over the expression virulence traits.

Implications of this study to the field

We have shown EutR directly modulates the expression of virulence genes including *eutS* and *ler*. Other studies have determined that EA is a critical metabolite and a signal for *Salmonella* Typhimurium disease progression [228]. Together, this suggests that EA signaling may be a conserved strategy used by a diverse group of pathogens to coordinate pathogen host adaptation and virulence during colonization and disease progression.

We identified and characterized the function of a novel regulatory protein, EtrB, encoded in the cryptic ETT2 locus. Notably, we demonstrated that EtrB influences the expression of EHEC virulence traits. EtrB directly activates expression of the LEE, activates the expression of non-LEE encoded effectors, and represses expression of ETT2-encoded genes, genes involved in metabolism, and the fimbrial locus 11. Our findings demonstrate mechanistically how ETT2-encoded regulators influence bacterial pathogenesis. The presence of EtrB in other pathogenic *E. coli* species indicates that EtrB could be a widespread regulator responsible for the coordination of factors important for host adaptation and virulence.

Our work further characterized the role of the two EA-regulated fimbriae, Erf1 and Erf2 to EHEC pathogenesis. Although we did not see a role for fimbrial-mediated adherence to Caco-2 cells *in vitro*, we determined that Erf1 and Erf2 are critical for colonization of the murine GI tract. Notably, this is the first evidence describing the contribution of a single fimbrial locus to EHEC colonization in an *in vivo* model. Overall, our findings demonstrate that EHEC fimbriae are important for EHEC pathogenesis.

Additionally, we have begun studying the mechanisms underlying Erf1 and Erf2 regulation of other virulence traits, mainly regulation of the LEE and Shiga toxin expression. We have shown that expression of the entire *erf1* or *erf2* fimbrial locus is not necessary to regulate the expression of virulence traits. Our data suggest that multiple signaling pathways exist in *erf1* and *erf2* to influence EHEC pathogenesis. These results reveal a novel mechanism by which fimbriae regulate virulence gene expression, independent of the previously characterized mechanisms involving the fimbrial locus 5' UTR or expression of fimbriae on the surface [324, 331, 333].

In summary, our data provide a better understanding of the complex regulatory circuits that pathogens employ to regulate virulence traits. The critical role for these transcription factors could be exploited to develop novel therapeutics targeted to various regulatory circuit. Further characterization of the spatio-temporal regulation of different signaling pathways to further understand when they are regulated and how their expression changes over the course of infection

could lead to a better understanding of mechanisms of pathogenesis and signals that pathogens encounter in the GI tract.

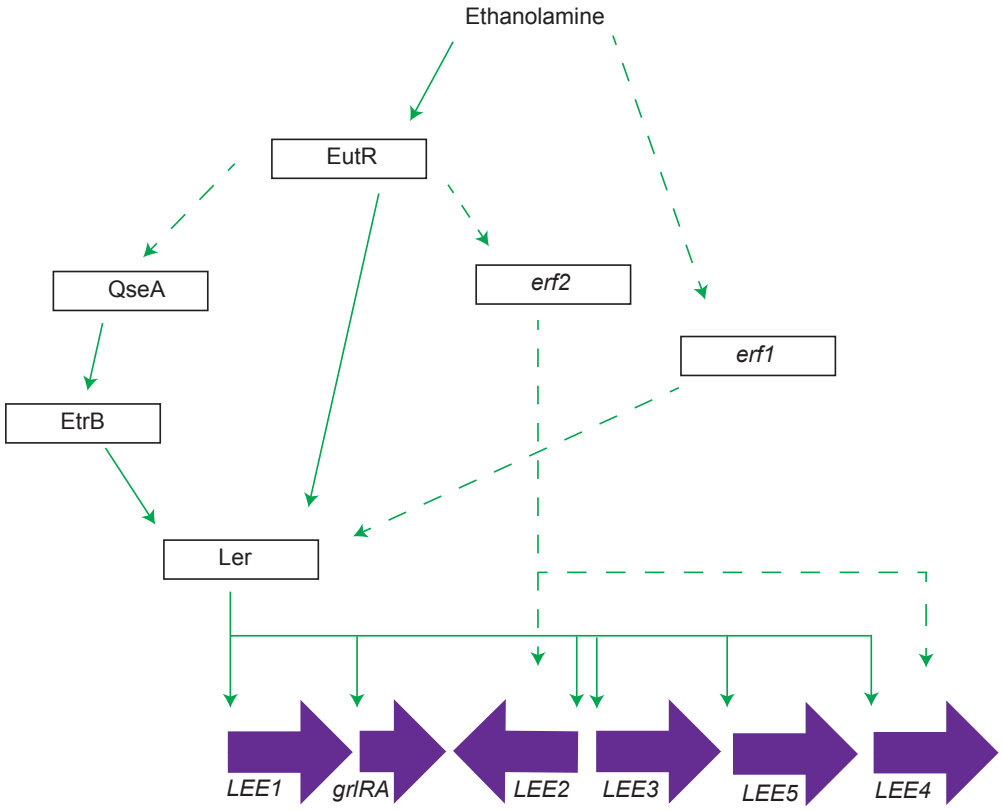


Figure 5.1 Model of total LEE regulatory circuit described in this work. Direct interactions are show with solid arrows, indirect interactions are shown with dashed arrows.

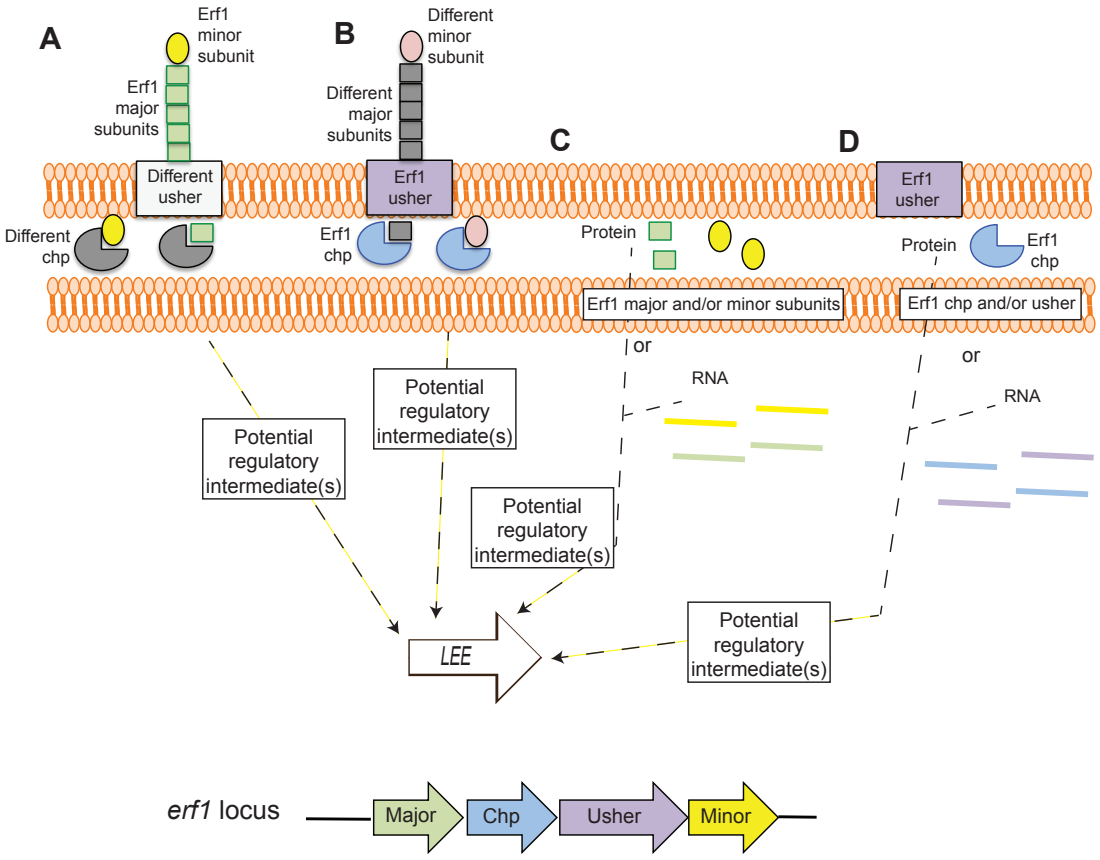


Figure 5.2 Model for possible mechanisms of Erf1 fimbrial regulation of the LEE. (A) A different chaperone and usher complex is exporting Erf1 major and minor subunits to the surface to form an Erf1 fimbrial structure. This structure would then signal through unknown regulatory intermediates to activate LEE expression. (B) The Erf1 chaperone and usher are exporting different fimbrial subunits, which activates LEE expression through unknown intermediates. (C) The Erf1 major and/or minor subunit protein or mRNA activates LEE expression. This pathway could involve other regulatory proteins to then activate the LEE. (D) Similar to (C), the Erf1 chaperone and/or usher protein or mRNA activates LEE expression, possibly through another regulatory protein. Dashed arrows highlight proposed signaling pathways.

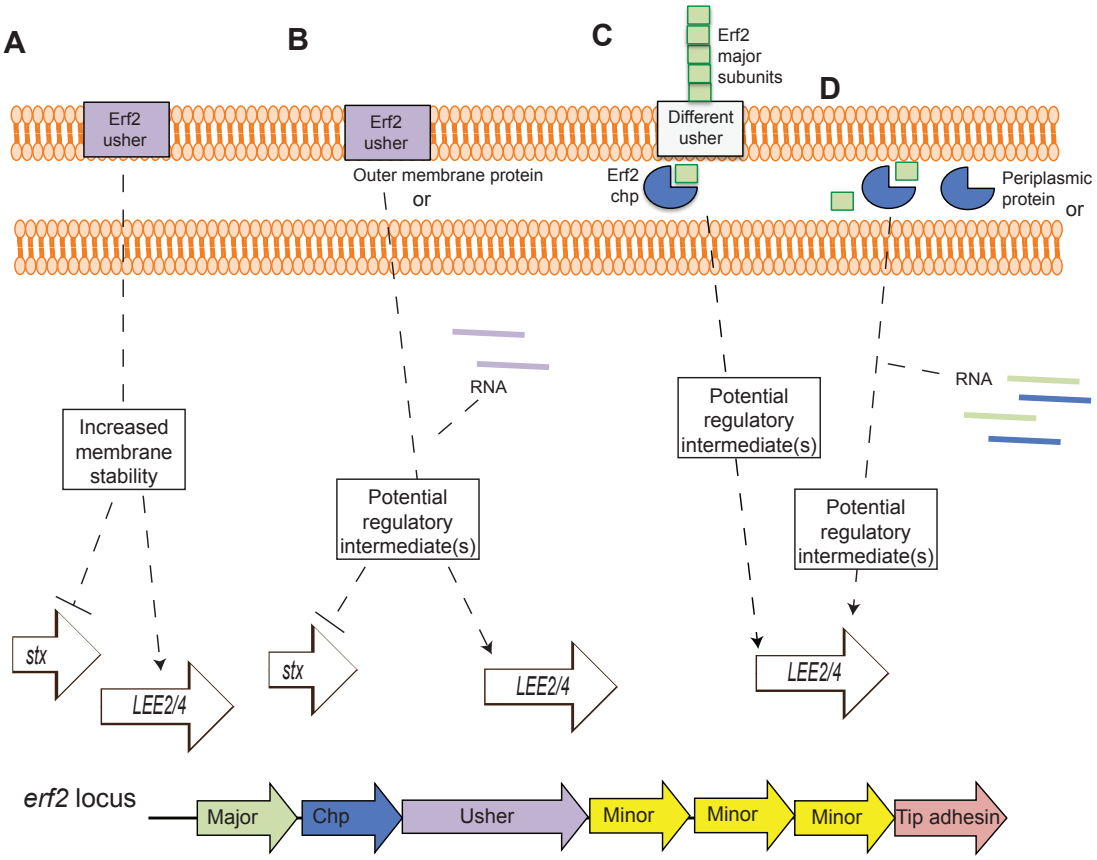


Figure 5.3 Model for possible mechanisms of Erf2 fimbrial regulation of the LEE and Shiga toxin. (A) The Erf2 usher in the outer membrane increases membrane stability, which represses Shiga toxin expression and activates expression of *LEE2/4*. (B) The Erf2 usher protein in the outer membrane or the usher mRNA repress Shiga toxin expression and activate *LEE2/4* expression, potentially through an unknown regulatory intermediate. (C) The Erf2 chaperone and major subunit interact with a different usher to form a partial Erf2 surface fimbriae composed of major subunits. This Erf2 structure interacts with other proteins to activate expression of *LEE2/4*. (D) The Erf2 chaperone and/or major subunit periplasmic proteins or mRNA activates *LEE2/4* expression, possibly

through another regulatory protein. Dashed arrows highlight proposed signaling pathways.

Appendices

Table A2.1 Strains and plasmids used in Chapter 2

Strain	Genotype Description	Reference or Source
86-24	Wild-type EHEC (serotype O157:H7)	[149]
MK37	86-24 *eutR* mutant	[109]
VS??	Wild-type EHEC with plasmid pVS23	[211]
E2348/69	Wild-type EPEC (serotype O127:H6)	[467]
BL21(DE3)	F- ompT hsdSB (rB - mB -) gal dcm (DE3)	Invitrogen
Plasmids	**Genotype description**	**Reference or Source**
pMAL-c5X	Cloning Vector	NEB
pMK53	EHEC 86-24 *eutR* in pMAL-c5X	This study
pVS23	Regulatory region of EHEC 86-24 *LEE1* in pBluescript	[211]

Table A2.2 Primers used in Chapter 2

Primer Name	Sequence	Primer use
ler_emsaF	ATGCAATGAGATCTATCTTA	EMSA and primer extension
ler_emsaR	AATATTTTAAGCTATTAGCG	EMSA and primer extension
eutS_emsaF	CATGCGTCGCATATGAAAGT	EMSA and primer extension
eutS_emsaR	GCCTGTGAAAAACTTTCGTG	EMSA and primer extension
amp_emsaF	GGAATTCGAAAGGGCCTCGTGATACGC	EMSA
amp_emsaR	CGGGATCCGGTGAGCAAAAACAGGAAGG	EMSA
EutRexp_F1	GGAATGTCCATATTGCATATC	eutR::MBP fusion
EutRexp_R1	TTACAACTCATAGCTGATGG	eutR::MBP fusion
eutS_RTrans_F1	ACGCATCATTCAGGAATTTG	RT-PCR
eutS_RTrans_R1	CAAATTCCTGAATGATGCCT	RT-PCR
eutS_RT_F	TTGGTCATTTCACAAAGCGT	qRT-PCR
eutS_RT_R	GCGCAGTAGAAGAAGCGTT	qRT-PCR
eutR_RT_F	TTGCCCCAGATGCCAGAA	qRT-PCR
eutR_RT_F	CGCAAAGCACAACGGTAAAAG	qRT-PCR
eutB_RT_F	CACCGGATTATTGCGGATGT	qRT-PCR
eutB_RT_R	GCGTGGCGGCAAAGC	qRT-PCR

rpoA_RT_F	GCGCTCATCTTCTTCCGAAT	qRT-PCR
rpoA_RT_R	CGCGGTCGTGGTTATGTG	qRT-PCR

Table A3.1 Strains and plasmids used in Chapter 3

Strain	Genotype Description	Reference or Source
86-24	Wild-type EHEC (serotype O157:H7)	[149]
DL01	86-24 *etrB* mutant	Luzader, EtrB study
DL02	etrB empty pGEN-MCS	Luzader, EtrB study
DL03	DL01 with plasmid pDL01 (*etrB* complement)	Luzader, EtrB study
VS145	86-24 *qseA* mutant	[214]
VS151	VS145 with plasmid pVS150 (*qseA* complement)	[214]
MK37	86-24 *eutR* mutant	[109]
BL21(DE3)	F- ompT hsdSB (rB - mB -) gal dcm (DE3)	Invitrogen

Plasmids	Genotype description	Reference or Source
pKD3	pANTSλ derivative containing FRT-flanked kanamycin resistance	[287]
pKD46	λ red recombinase expression plasmid	[287]
pCP20	TS replication and thermal induction of FLP synthesis	[287]

pGEN-MCS	Cloning Vector	[288]
pMAL-c5x	Cloning Vector	NEB
pVS150	EHEC 86-24 *qseA* in pACYC177	[214]
pMK08	EHEC 86-24 *qseA* in pET28	[46]
pDL01	EHEC 86-24 *etrB* in pGEN	Luzader, EtrB study
pDL02	EHEC 86-24 *etrB* in pMAL-C5x	Luzader, EtrB study
pDL03	EHEC 86-24 *etrB* promoter in pGEN-luxCDABE	Luzader, EtrB study

Table A3.2 Primers used in Chapter 3

Primer Name	Sequence	Primer use
etrB_GenComp_F	AAGCTTGTATTCTTTGGATTTTGCTTA	etrB complement
etrB_GenComp_R	CCATGGACTAGGCTTAATGAACTAGA	etrB complement
etrB_MalComp_F	CCTGCAGGTATAGTGCACACACCCATAC	etrB::MBP fusion
etrB_MalComp_R	CCATGGGAATGATGGGGGCCGAACTC	etrB::MBP fusion
etrB_LR_F	TATTTTAGGAGAATTTGCAGGTGGAATG ATGGGGGCCGAACTCGTAAAATTGTGTA GGCTGGAGCTGCTTC	etrB mutant
etrB_LR_R	CAAAATGAGCCTAAAGCCTCTTTTTTTT ATATAGTGCACACACCCATACGTCATAT GAATATCCTCCTTAG	etrB mutant
etrB_RT_F1	GGGCCGAACTCGTAAAATGG	qRT-PCR
etrB_RT_R1	ATACGCATCCTTTCGCACCT	qRT-PCR
ler_RT_F1	CGACCAGGTCTGCCC	qRT-PCR
ler_RT_R1	GCGCGGAACTCATC	qRT-PCR
grlA_RT_F1	CCGGTTGTTCCAGGACTTTC	qRT-PCR
grlA_RT_R1	TAAGCGCCTTGAGATTTTCATTT	qRT-PCR
escC_RT_F1	GCGTAAACTGGTCCGGTACGT	qRT-PCR
escC_RT_R1	TGCGGGTAGAGCTTTAAAGGCAAT	qRT-PCR
escV_RT_F1	TCGCCCCGTCCATTGA	qRT-PCR
escV_RT_R1	CGCTCCCGAGTGCAAAA	qRT-PCR

espA_RT_F1	TCAGAATCGCAGCCTGAAAA	qRT-PCR
espA_RT_R1	CGAAGGATGAGGTGGTTAAGCT	qRT-PCR
eae_RT_F1	GCTGGCCCTTGGTTTGATCA	qRT-PCR
eae_RT_R1	GCGGAGATGACTTCAGCACTT	qRT-PCR
rpoA_RT_F1	GCGCTCATCTTCTTCCGAAT	qRT-PCR
rpoA_RT_R1	CGCGGTCGTGGTTATCTG	qRT-PCR
ygeI_RT_F1	TAGCGAATGCAACGGGTGAT	qRT-PCR
ygeI_RT_R1	GACGCCATCCATGTTGAAACT	qRT-PCR
yqeK_RT_F1	ATGGACATTGAGTTTTCGCAGA	qRT-PCR
yqeK_RT_R1	CCCATGATGTTGTTTGCGTGA	qRT-PCR
ryeA_RT_F1	AGATGACGACGCCAGGTTTT	qRT-PCR
ryeA_RT_R1	ACCAGAACGGGCGGTTTTTA	qRT-PCR
tnaA_RT_F1	TGTACACCGAGTGCAGAACC	qRT-PCR
tnaA_RT_R1	CCGTCATACAGACCTACGGC	qRT-PCR
eivF_RT_F1	TTGTTTGCTGATGCCTTGCC	qRT-PCR
eivF_RT_R1	CGCTGCTCAGATAAGTGGCT	qRT-PCR
etrA_RT_F1	TGCAAGTCTTTTCCAGTGATGTC	qRT-PCR
etrA_RT_R1	CCAACGCAACTAAATCGCTGT	qRT-PCR
malK_RT_F1	CGCAATCGATCAAGTGCAGG	qRT-PCR
malK_RT_R1	GTCAGCGATATCACTCGGCA	qRT-PCR
nleA_RT_F1	TGTTGAAGGCTGGAAGTTTGTTT	qRT-PCR
nleA_RT_R1	CCGCTACAGGGCGATATGTT	qRT-PCR
Z4498_RT_F1	CTTGGCAAAAGTGGGCTCTT	qRT-PCR

Z4498_RT_R1	CCGCATCGTCAATACGGATA	qRT-PCR
stx2a _RT_F1	ACCCCACCGGGCAGTT	qRT-PCR
stx2a _RT_R1	GGTCAAAACGCGCCTGATA	qRT-PCR
etrB_prom_F1	TATTATTTCATCAATGTATTCTTT	EMSA
etrB_prom_R1	CGATTTAACCCATTTTACGA	EMSA
kan_EMSA_F1	CCGGAATTGCCAGCTGGGGCG	EMSA
kan_EMSA_R1	TCTTGTTCAATCATGCGAAACGATCC	EMSA
ler_emsaF	ATGCAATGAGATCTATCTTA	EMSA
ler_emsaR	AATATTTTAAGCTATTAGCG	EMSA
amp_emsaF	GGAATTCGAAAGGGCCTCGTGATACGC	EMSA
amp_emsaR	CGGGATCCGGTGAGCAAAAACAGGAAGG	EMSA
Z4175_R	GATAGCATAGGGAAGAACAG	RT-PCR
Z4177_F	TCACTGGCTCAGGTTTAATG	RT-PCR
Z4178_F	GGAATGTCCATATTGCATATC	RT-PCR
Z4176_F	TTACAACTCATAGCTGATGG	RT-PCR
Z4176_R	TTCCATACGCATCCTTTCGC	RT-PCR
etrB_PE_R	CGACTGTTTTCCTGCTTAAC	RT-PCR

Table A4.1 Strains and plasmids used in Chapter 4

Strain	Genotype Description	Reference or Source
86-24	Wild-type EHEC (serotype O157:H7)	[149]
LG01	86-24 *erf1* (*Z3276-Z3279, yeh*) mutant	This study
LG02	86-24 *erf2* (*Z0146-Z0152, yad*) mutant	This study
BL21(DE3)	F- ompT hsdSB (rB - mB -) gal dcm (DE3)	Invitrogen
LG03	*erf1* empty pGEN-MCS	This study
LG04	*erf1* complemented pLG01	This study
LG05	*erf2* empty pGEN-MCS	This study
LG06	*erf2* complemented pLG02	This study
DL07	86-24 *Z3279* mutant (major subunit)	This study
DL08	86-24 *Z3278* mutant (Chaperone)	This study
DL09	86-24 *Z3277 Z3276* mutant (usher and minor subunit)	This study
DL10	86-24 *Z3276* mutant (minor subunit)	This study
DL11	86-24 *Z3278 Z3277* mutant (Chaperone and usher)	This study
DL12	86-24 *Z3277* mutant (usher)	This study
DL13	*erf1* empty pBAD24	This study
DL14	*erf1* complemented pDL04	This study

DL15	*erf1* complemented pDL05	This study
DL16	*erf1* complemented pDL06	This study
DL17	*erf1* complemented pDL07	This study
DL18	*erf1* complemented pDL08	This study
DL19	*erf1* complemented pDL09	This study
DL20	*erf1* empty pBAD33	This study
DL21	*erf1* complemented pDL10	This study
DL22	*erf1* complemented pDL11	This study
DL23	*erf1* complemented pDL12	This study
DL24	*erf1* complemented pDL13	This study
DL25	86-24 locus 3 (*Z0686-Z0693, sfm*) mutant	This study

Plasmids	Genotype description	Reference or Source
pKD3	pANTSλ derivative containing FRT-flanked kanamycin resistance	[287]
pKD4	pANTSλ derivative containing FRT-flanked kanamycin resistance pKD46 λ red recombinase expression	[287]
pKD46	λ red recombinase expression plasmid	[287]
pCP20	TS replication and thermal induction of FLP synthesis	[287]
pGEN-MCS	Cloning Vector	[288]

pLG01	*erf1* in pGEN-MCS	This study
pLG02	*erf2* in pGEN-MCS	This study
pBADmycHis-A	Cloning Vector	Invitrogen
pBAD24	Cloning Vector	[318]
pBAD33	Cloning Vector	[318]
pDL04	*Z3279-Z3278* in pBAD24 (Major subunit and chaperone)	This study
pDL05	*Z3278* in pBAD24 (Chaperone)	This study
pDL06	*Z3278* without the first 138 base pairs of the annotated gene in pBAD24 (Chaperone No Signal sequence)	This study
pDL07	*Z3277* in pBAD24 (Usher)	This study
pDL08	*Z3277* without the first 110 base pairs of the annotated gene in pBAD24 (usher No Signal sequence)	This study
pDL09	*erf1* in pBAD24	This study
pDL10	*Z3279-Z3278* in pBAD33 (Major subunit and chaperone)	This study
pDL11	*Z3278* in pBAD33 (Chaperone)	This study
pDL12	*Z3277* in pBAD33 (Usher)	This study
pDL13	*Z3277*-mychis in pBAD33 (Usher:mychis)	This study
pDL14	*Z3278* in pBADmychis (Chaperone)	This study

pDL15	*Z3277* in pBADmychis (Usher)	This study
pMK52	*eutB* in pBAD33	[109]
pMK53	EHEC 86-24 *eutR* in pMAL-c5X	[110]

Table A4.2 Primers used in Chapter 4

Primer Name	Sequence	Primer use
erf1_LRF	ATCATGAAACATTCAATTATTGCTGTCGCTGTCTTA TCTTCTGTATTTATGTGTAGGCTGGAGCTGCTTC	Create Erf1 locus deletion strain and *Z3279* mutant (major subunit)
erf1_LRR	TTCTTATTATTAGCCACTTGCTCATCTTGCTTGTTA TTAATCGTATTTCACATATGAATATCCTCCTTAG	Create Erf1 locus deletion strain
erf2_LRF	AGGATGCATGTAATGAAAAAAGCACTTCTCGCAGC CGCTCTGGTTATGGCTT GTGTAGGCTGGAGCTGCTTC	Create Erf2 locus deletion strain
erf2_LRR	CGTTTTACTTATTCGTAGGTAAAGGAGAAGGTCGC GTTACCTGAAAATGTTCC AACACCATATGAATATCCTCCTTAG	Create Erf2 locus deletion strain
yehD delR	TTTAACTTACTGAAAAAACAAGATGATTATTTTAA ATATTTAATCCTGGGCATATGAATATCCTCCTTAG	86-24 *Z3279* mutant (major subunit)
yehC_LR_F1	TCATCTTGTTTTTTCAGTAAGTTAAAATGGCCGCCA TATTCATGGCGGCCTGTGTAGGCTGGAGCTGCTTC	86-24 *Z3278* mutant

		(Chaperone) and *Z3278 Z3277* mutant (chaperone, usher)
yehC_LR_R1	TCATTCTCAACATAGGCAGCTCCTGCAATTAAATTT TGTCACTAATATAGTCATATGAATATCCTCCTTAG	86-24 *Z3278* mutant (Chaperone)
yehB_LR_F1	AGGAGCTGCCTATGTTGAGAATGACCCCGCTTGCAT CAGCAATAGTCGCGTTGTGTAGGCTGGAGCTGCTTC	86-24 *Z3277 Z3276* mutant (usher, minor subunit)
yehB_LR_R1	ATTAGTAATATTCTATTTCTGAACATCATATTTCAC CTCTCACCGACAGATCATATGAATATCCTCCTTAG	86-24 *Z3277 Z3276* mutant (usher, minor subunit) and *Z3278 Z3277* mutant (chaperone, usher)
yehB_int_LR _F1	TCTTGATGATAATCAGCCTTTACCTGGGCAGTA CGACATTGATATTTATGTGTGTAGGCTGGAGCT	86-24 *Z3277* mutant (usher)

	GCTTC	
yehB_int_LR	TCATTGGTGCGAATAAATATCTGACTGCCCTGGCCGAC	86-24 *Z3277*
_R1	AACACCAATGTTTCATATGAATATCCTCCTTAG	mutant (usher)
Z3276 delF	AATATGATGTTCAGAAATAGAATATTACTAATATT	86-24 *Z3276*
	TATATTGTGGGCTAAGTGTAGGCTGGAGCTGCTTC	mutant (minor
		subunit)
Z3276 delR	CAGACACCTTATTATTTCTTATTATTAGCCACTTGC	86-24 *Z3276*
	TCATCTTGCTTGTTATTAATCGTATTCATATGAATA	mutant (minor
	TCCTCCTTAG	subunit) and
		Z3277 Z3276
		mutant (usher,
		minor subunit)
loc3_EHEC_L	GGTCGAAGGGGATGCGCCTATTTTGTCAGAAGCGG	locus 3 deletion
R_F	GGCGCGCTGTCAGGTGTGTAGGCTGGAGCTGCTTC	
loc3_EHEC_L	GCGGCCCACGACTTAGAAGGTCGTTGCTCTATCCAA	locus 3 deletion
R_R1	CTGAGCTAAGGGCGTCATATGAATATCCTCCTTAG	
Erf1_CF	CTAGGAATTCTTGCCAACACCGTTTTAAGCAT	pGEN:Erf1
		complement
Erf1_CR	CTAGCCTAGGGATCAGCGACACCGACGGTA	pGEN:Erf1
		complement
Erf2_CF	CTAGGAATTCAGCACTTCATGCAAATAGATTAGGC	pGEN:Erf2
		complement

Erf2_CR	CTAGCCTAGGAGCAGAACTCTGGTGCGATG	pGEN:Erf2 complement
erf1_pBADcomp_F1	GGTACCGAGATACAGACTCTTAACAA	pBAD24:Erf1 complement
erf1_pBADcomp_R1	AAGCTTTTATTATTAGCCACTTGCTC	pBAD24:Erf1 complement
yehD comp F	CTAGTCTAGAATGAAACATTCAATTATTG	*Z3279-Z3278* in pBAD24 (Major subunit, chaperone)
yehC comp Rv	CTAGAAGCTTTTAAATTTTGTCACTAATAT	*Z3279-Z3278* in pBAD24 (Major subunit, chaperone)
erf1_ChpTag_F2	CCATGGGTTAAAATGGCCGCCATATT	*Z3278* in pBAD24 (Chaperone)
erf1_ChpNoSigSeq_F1	CCATGGTTAATCATCTCTTCTATGAT	*Z3278* without the first 138 base pairs of the annotated gene in pBAD24 (Chaperone No

		Signal sequence)
erf1_ChpNoSigBAD24_R1	AAGCTTCAGCTCCTGCAATTAAATTT	*Z3278* in pBAD24 (Chaperone) AND Z3278 without the first 138 base pairs of the annotated gene in pBAD24 (Chaperone No Signal sequence)
yehB comp Fd	CTAGTCTAGAATGTTGAGAATGACCCCGCT	*Z3277* in pBAD24 (Usher)
yehB comp Rv	CTAGAAGCTTTCACCGACAGATATAATTTTTAC	*Z3277* in pBAD24 (Usher)
erf1_UshNoSigSeq_F1	CCATGGGCAGAAGAAACCTTTGACAC	*Z3277* without the first 110 base pairs of

		the annotated gene in pBAD24 (usher No Signal sequence)
erf1_UshBAD24_R1	AAGCTTTTCACCTCTCACCGACAGAT	*Z3277* without the first 110 base pairs of the annotated gene in pBAD24 (usher No Signal sequence)
yedD_pB33_F1	GGTACCATGAAACATTCAATTATTGC	*Z3277* in pBAD33 (Usher)
yehC_pB33_R1	AAGCTTTTAAATTTTGTCACTAATAT	*Z3277* in pBAD33 (Usher)
yehB_pB33_F1	GGTACCATGTTGAGAATGACCCCGCT	*Z3277* in pBAD33 (Usher)
yehB_pB33_	AAGCTTTCACCGACAGATATAATTTT	*Z3277* in

R1		pBAD33 (Usher)
Ush_erf1_His tag_pB33_F1	GGTACCTGGATGTTGAGAATGACCCC	*Z3277*-mychis in pBAD33 (Usher:mychis)
pBmh_His_R 3	AAGCTTTGGAGACCGTTTAAACTCAATGATG	*Z3277*-mychis in pBAD33 (Usher:mychis)
erf1_UshTag _F1	CCATGGATGTTGAGAATGACCCCGCT	*Z3277* in pBADmychis (Usher)
erf1_UshTag _R1	AAGCTTCCGACAGATATAATTTTTAC	*Z3277* in pBADmychis (Usher)
yehD_prom_ F1	AGTGGTAGCTGTGGGTAATA	Primer extension primer for erf1
yehD_cDNA_ R1	AATGTACATGGTGATTCTGC	Primer extension primer for erf1
ler_emsaF	ATGCAATGAGATCTATCTTA	*ler* EMSA
ler_emsaR	AATATTTTAAGCTATTAGCG	*ler* EMSA
yadN_emsaF	CAGTACTACAACCTGCCATC	*erf2* EMSA

yadN_emsaR	GATATGACCACCATCTACA	*erf2* EMSA
amp_emsaF	GGAATTCGAAAGGGCCTCGTGATACGC	*kan* EMSA
amp_emsaR	CGGGATCCGGTGAGCAAAAACAGGAAGG	*kan* EMSA
ler_RT_F1	CGACCAGGTCTGCCC	qRT-PCR
ler_RT_R1	GCGCGGAACTCATC	qRT-PCR
grlA_RT_F1	CCGGTTGTTCCAGGACTTTC	qRT-PCR
grlA_RT_R1	TAAGCGCCTTGAGATTTTCATTT	qRT-PCR
escC_RT_F1	GCGTAAACTGGTCCGGTACGT	qRT-PCR
escC_RT_R1	TGCGGGTAGAGCTTTAAAGGCAAT	qRT-PCR
escV_RT_F1	TCGCCCCGTCCATTGA	qRT-PCR
escV_RT_R1	CGCTCCCGAGTGCAAAA	qRT-PCR
espA_RT_F1	TCAGAATCGCAGCCTGAAAA	qRT-PCR
espA_RT_R1	CGAAGGATGAGGTGGTTAAGCT	qRT-PCR
eae_RT_F1	GCTGGCCCTTGGTTTGATCA	qRT-PCR
eae_RT_R1	GCGGAGATGACTTCAGCACTT	qRT-PCR
rpoA_RT_F1	GCGCTCATCTTCTTCCGAAT	qRT-PCR
rpoA_RT_R1	CGCGGTCGTGGTTATCTG	qRT-PCR
stx2a _RT_F1	ACCCCACCGGGCAGTT	qRT-PCR
stx2a _RT_R1	GGTCAAAACGCGCCTGATA	qRT-PCR
Z3279 RT F	TGGCGGTGATTCAGTCAGTATT	*erf1* RT and qRT

Z3279 RT R	ACCAACCGCGCCATCATA	*erf1* RT and qRT
Z3278 RT F	CCACCGAATAGCCCAGAAGA	*erf1* RT and qRT
Z3278 RT R	TACCGGAGCAATACCAGCAG	*erf1* RT and qRT
Z3277 RT F	ATTGCTCGGCATTGAAGCAC	*erf1* RT and qRT
Z3277 RT R	CAATGTCGTACTGCCCAGGT	*erf1* RT and qRT
Z3276 RT F	GCGGGGGATACATTTACGCT	*erf1* RT and qRT
Z3276 RT R	TCCTTACTGTTCGCCGCAAT	*erf1* RT and qRT
CR: ler_RT_F1	GGAAACTAATTCGCCCACAACA	*C. rodentium* qRT-PCR
CR: ler_RT_R1	AGCCGCTTTGCTTCTTGCT	*C. rodentium* qRT-PCR
CR: escC_RT_F1	CCGCGACCAAAATGTTGTC	*C. rodentium* qRT-PCR
CR: escC_RT_R1	CCATTACTTGCCATTGTCTTTAGGA	*C. rodentium* qRT-PCR
CR: escV_RT_F1	GCTTGAGGCAAAAGTTCTTCGT	*C. rodentium* qRT-PCR
CR: escV_RT_R1	CGCCCCGTCCATTGAG	*C. rodentium* qRT-PCR
CR: espA_RT_F1	GCGCCGGTGATTTGCA	*C. rodentium* qRT-PCR
CR: espA_RT_R1	CAAATTATTCGCTTTAGCCGAAA	*C. rodentium* qRT-PCR

CR: tir_RT_F1	AGGGCTTCCTGAACATCCATT	*C. rodentium* qRT-PCR
CR: tir_RT_R1	CAAATCCCCCATGCAAACAT	*C. rodentium* qRT-PCR
CR:rpoA_F1	CGTACCGACCTGGACAAGCT	*C. rodentium* qRT-PCR
CR: rpoA_R1	AATCGCCTCTTCAGGATCGA	*C. rodentium* qRT-PCR
pyrI_RT_F1	TGCCCGAACAGCAACTGTAT	Microarray confirmation primers
pyrI_RT_R1	GCGATATCATTGGCGCGTTT	Microarray confirmation primers
ecpR_RT_F1	ATTGCGGCCAGAAAGTCAGA	Microarray confirmation primers
ecpR_RT_R1	GCGTATACCACGCCCCTAAT	Microarray confirmation primers
yagZ_RT_F	CGTGGCTATCGAGGGTGACT	Microarray confirmation primers

yagZ_RT_R	CTGGGTTAATGTGTTGGTGATAAGA	Microarray confirmation primers
chaB_RT_F1	TTCAACAGCGCATGGGATCA	Microarray confirmation primers
chaB_RT_R1	CCACTTTATGCGCGGTTTCT	Microarray confirmation primers
nadR_RT_F1	TAGCTGATGCCAGCGGTATG	Microarray confirmation primers
nadR_RT_R1	TCCGGTATGCAGTGGGTAGA	Microarray confirmation primers
fliA_RT_F1	GAACGCTATGACGCCCTACA	Microarray confirmation primers
fliA_RT_R1	TCCAGTTGCCCTATTGCCTG	Microarray confirmation primers
BaeR_RT_F2	TCGTCTGCTGAAAACGCTCT	Potential regulatory

		intermediates
BaeR_RT_R2	ATGTGGCTGTCGATGGTACG	Potential regulatory intermediates
cpxP_RT_F1	AGCCATATGTTCGACGGCAT	Potential regulatory intermediates
cpxP_RT_R1	TGCATTGTCTCCAGTTCGCT	Potential regulatory intermediates
uhpA_RT_F1	ACTGGGCTTGTCACCGAAAA	Potential regulatory intermediates
uhpA_RT_R1	CAGCCATCAAACATACGGCG	Potential regulatory intermediates
uhpB_RT_F	CCTGTGGAGTATCAGCCTGC	Potential regulatory intermediates
uhpB_RT_R	AGCAGCACCGGCCAGTATC	Potential regulatory intermediates
casA_EHEC_	ATTAAGCGTGGCGTGACTGA	Potential

RT_F		regulatory intermediates
casA_EHEC_ RT_R	CAAGGGGGCTTGTTGATTGC	Potential regulatory intermediates
sfmA Fd	CGCTACGGGTAGTTATGCTG	*loc3* RT
sfmC_EHEC_ R	TCAGCTTTCCCTGCACTCCT	*loc3* RT
sfmC Fd	TTCGGCCAGCGTCCATGCTG	*loc3* RT
sfmD_R2	ACCTGGTTAAAACAGGGCAT	*loc3* RT
sfmD_EHEC_ F1	CCTGGTGTCTGGCCGGAATT	*loc3* RT
sfmH_R1	CGGTAATACTGATAGGCCAG	*loc3* RT
sfmH Fd	AAACATATCC TGGGCTACTG	*loc3* RT
sfmF Rv	CGCCACACCTGGCCGAACAC	*loc3* RT
sfmF_F1	TAGCTGTTTCTGCGGGTTAC	*loc3* RT
fimZ_EHEC_ F	GCACAGTGACCGTTGAACTA	*loc3* RT
fimZ Rv	CCGCTCCTATGAGACTGGTA	*loc3* RT

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