Ethanolamine-regulated fimbriae in enterohemorrhagic *Escherichia coli* O157:H7 pathogenesis

Laura Alice Gonyar

Holden, ME

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List of abbreviations

AE lesions- attaching and effacing lesions

CFU- colony forming units

CI- competitive index

CU- chaperone-usher

DMEM- Dulbecco's modified Eagle's medium

DPI- days post infection

EA- ethanolamine

EHEC- Enterohemorrhagic E. coli

GI- gastrointestinal

H- hours

H-NS- histone-like nucleoid-structuring protein

HUS- hemolytic uremic syndrome

LB- Luria broth

LEE- locus of enterocyte effacement

Log- logarithmic

PE- phosphatidylethanolamine

Stx- Shiga toxin

T3S- type III secretion

Tir- translocated intimin receptor

WT- wild type

Abstract

Ethanolamine (EA) is a signal for recognition of the host environment and regulation of virulence gene expression in enterohemorrhagic *Escherichia coli* O157:H7 (EHEC). Preliminary data suggested that EA increased early EHEC attachment to epithelial cells and also promoted a clustering adherence phenotype with other bacterial cells, which is classically mediated by fimbriae in bacteria. EHEC encodes sixteen fimbrial loci, and most were thought to be cryptic or incomplete. We hypothesize that EA promotes the expression of EHEC fimbriae and that these fimbriae are critical for EHEC colonization of the gastrointestinal (GI) tract. Expression of genes encoded in fifteen EHEC fimbrial loci was increased during growth with EA in comparison to growth without EA, and this increase in fimbrial transcription correlated with an increase in fimbrial structures present on the bacterial surface. We further characterized the expression of four EA-regulated fimbrial loci and found that all necessary subunits for fimbrial assembly are transcribed, suggesting that these four loci encode functional fimbriae. Together, these data demonstrate transcription of EHEC fimbriae, several for the first time, and suggest that they may be functional. To begin to elucidate the roles of EHEC fimbriae in colonization, we constructed deletion strains in two EA-regulated fimbriae, Erf1 and Erf2. Neither Erf1 nor Erf2 were necessary for early attachment to epithelial cells *in vitro*; however, these two EA-regulated fimbriae directed the expression of EHEC virulence determinants important for later stages in infection, including Shiga toxin and the locus of enterocyte effacement (LEE). Additionally,

Erf1 contributed significantly to EHEC colonization in a murine model of infection. Our findings demonstrate expression of EHEC fimbriae in response to the biologically relevant molecule EA, elucidate the contribution of fimbriae to EHEC early attachment to epithelial cells, and define a pivotal role for fimbriae in pathogenesis, beyond mediating attachment, as regulators of virulence gene expression.

Chapter One

Introduction

Fimbrial Adhesion in Bacteria

Adhesion is essential for bacterial persistence in any environment in order to resist clearance; a common mechanism that bacteria utilize to mediate attachment is the production of fimbriae [1]. Fimbriae are linear, unbranched proteinaceous structures that extend from the surface of the bacteria and are comprised of hundreds to thousands of polymerized fimbrial subunits [2]. Fimbriae can be expressed in a polar orientation or peritrichously, with hundreds of fimbriae distributed over the entirety of the bacterial cell [3]. In addition to differences in localization, the physical characteristics of these structures are diverse; fimbriae can be thin, fibrillar fibers (with a diameter of 2-5 nm) or thick, helically wound rods (with a diameter of approximately 7 nm) with a distinct, adhesive tip [4].

The chaperone-usher (CU) pathway is the largest and most conserved class of fimbriae in Gram-negative bacteria [5]. The two most extensively studied, prototypical CU fimbriae are type-1 fimbriae encoded by the *fim* operon and P fimbriae encoded by the *pap* operon, both produced by uropathogenic *Escherichia coli* [6]. Type 1 fimbriae form rod-like fibers that bind mannose [7]. During CU assembly, fimbrial subunits enter the bacterial periplasm through the Sec pathway and then interact with a periplasmic chaperone to prevent aggregation and degradation [8]. The chaperone completes the fold of the subunit through donor strand complementation [8]. The complex can then interact with the usher, which acts as an assembly platform in the bacterial outer membrane [8]. The usher

facilitates uncapping and polymerization of subunits into the fimbrial structure [8]. The required subunits for fimbrial assembly are a major subunit, a chaperone, and an usher, but some fimbrial loci also encode other components, such as tip adhesins and regulators. In addition to a chaperone and an usher, type 1 fimbriae encode a major subunit that makes up the helical rod structure, two adaptor proteins and a tip adhesin that make up the tip fimbrillum, two site-specific recombinases involved in regulation of the *fim* operon through phase variation, as well as a protein of unknown function that is required for fimbrial assembly [9, 10].

Although CU fimbriae make up the largest and most diverse class of bacterial fimbriae, fimbriae can also be assembled through alternate pathways. Type IV pili are morphologically thin, flexible fibers that tend to aggregate into bundles. They share many of the typical functions of chaperone-usher pathway fimbriae, including host cell adhesion and biofilm formation [11]. Type IV pili can also perform the unique functions of DNA uptake, twitching motility, and phage transduction [11]. Assembly of type IV pili is less understood than the assembly of fimbriae through the chaperone-usher pathway. Structural subunits are synthesized as precursors (prepilins), transported across the inner membrane through the Sec pathway, and then cleaved by a specific inner-membrane prepilin peptidase [12]. Other required components include an ATPase to power assembly, an integral inner-membrane protein to recruit the ATPase, and an integral outer membrane secretin for export of pili to the bacterial surface [13]. In order to participate in twitching motility, the pili must be able to retract and generate force, and this requires an additional retraction

ATPase [14]. However, not all bacterial species that express type IV pili encode a retraction ATPase and exhibit twitching motility [11].

Curli are proteinaceous, coiled fibers expressed on enteric bacteria [15]. Curli fibers are biochemically and structurally similar to eukaryotic amyloid fibers implicated in the neurodegenerative Alzheimer's and Parkinson's diseases [15, 16]. Curli fimbriae contribute to biofilm formation, adhesion to host cells as well as surfaces, aggregation with other bacterial cells, and the induction of the host immune response [17-20]. Curli fibers are assembled through the nucleation precipitation pathway [21]. The curlin major subunit (CsgA) is secreted from the bacterial cell and requires a second secreted protein (CsgB) for polymerization [22]. In many systems, these two proteins do not need to be secreted from the same bacterial cell in order to create curli fibers [22]. Four accessory proteins are also required for curli assembly: a transcriptional regulator, an outer membrane lipoprotein involved in secretion of CsgAB, and two periplasmic proteins of unknown function [16].

In the context of infection, fimbrial structures bind to molecules expressed by host epithelial cells to promote attachment and determine host specificity [1]. Many pathogens encode a large repertoire of fimbriae, but whether they function redundantly or independently to promote attachment is unclear. Expressing multiple fimbriae could be advantageous in the host environment through the ability to bind to unique targets at the same body site, strengthening bacterial attachment [1]. Alternatively, individual fimbriae could mediate attachment in

diverse niches (example: adherence in the human host versus animal reservoirs) [23]. The maintenance of many fimbrial loci in pathogenic bacteria highlights their importance in mediating initial interactions and attachment to host tissue.

Escherichia coli

E. coli is a diverse group of Gram-negative bacteria, comprised of both commensals and pathogens [24, 25]. Several highly adapted groups of *E. coli* strains, referred to as pathotypes, cause a shared disease manifestation through a common collection of virulence factors. The eight well described *E. coli* pathotypes are enteropathogenic *E. coli*, enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli*, enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli*, diffusely adherent *E. coli*, uropathogenic *E. coli*, and meningitis-associated *E. coli*. The first six pathotypes cause diarrheal disease, and the following two cause urinary tract infections or meningitis and sepsis, respectively. Adhesion is an essential step in colonization of the human host and attachment mechanisms, as well as localization to a body compartment, are often a component of pathotype classification [25]. These pathotypes have emerged through the acquisition of factors that increase fitness and host damage (what we recognize as disease) at a specific niche within the human host. For example, EAEC and EHEC both colonize the colon but use different factors to adhere to host tissue and cause host damage. EHEC produces Shiga toxin, which contributes to both the local and systemic damage associated with infections.

EHEC adheres to host cells through an intimate, receptor-mediated attachment, which requires genes encoded within the locus of enterocyte effacement (LEE). EAEC adheres to host cells as well as other bacterial cells through the expression of fimbriae in a defining "stacked-brick" aggregation pattern [26]. EAEC causes host damage resulting in diarrhea through the activities of enterotoxins and cytotoxins [27]. An EAEC strain that had acquired Shiga toxin was implicated in a 2011 outbreak in Germany, indicating that these pathotypes are still constantly evolving [28].

Enterohemorrhagic *E. coli* O157:H7

EHEC O157:H7 is a foodborne pathogen responsible for outbreaks of bloody diarrhea and colitis worldwide [29]. The highest incidences of human disease have been reported in Canada, the United States, Japan, and Scotland [30, 31]. EHEC exists in the environment by asymptomatically colonizing ruminants, and cattle are the primary reservoir for human infection [32]. Most cattle are colonized at low levels but some members of the population release more infectious units (frequently more than 5 logs greater) and are termed super-shedders [33]. Less than 10% of cattle are super-shedders but provide about 96-99% of bacteria shed into the environment [33]. Humans become infected by consuming food products contaminated by the feces of infected animals [34]. The first outbreak of EHEC O157:H7 occurred in the United States in 1982, and the source was contaminated,

undercooked hamburgers at a fast-food restaurant chain [35]. While the first outbreaks involved undercooked beef, more cases are now associated with cross-contamination of juices, milk, produce, and ready-to-eat foods [34, 35]

The disease usually resolves within 10 days, but approximately 3-20% of infected individuals develop the life-threatening complication hemolytic uremic syndrome (HUS) [36, 37]. Young children and the elderly are at increased risk for developing HUS [38]. HUS presents clinically as acute renal failure, hemolytic anemia, and thrombocytopenia. HUS has a fatality rate between 3-5% with approximately half of survivors experiencing chronic renal sequelae [37]. Antibiotics are not recommended as treatment for EHEC infections because they correlate with an increased risk for development of HUS [36, 39, 40]. The lack of treatment options, low infectious dose (less than 100 cells), and threat of life-threatening complications make EHEC a significant public health concern [41].

Virulence factors

Shiga toxin (Stx) and the LEE are the two defining virulence traits for EHEC. EHEC also possesses other virulence factors (fimbriae and flagella, for example), but Stx and the LEE are essential in the classification of a strain as EHEC [25].

Stx

Stx was first identified and named in *Shigella dysenteriae* more than 100 years ago [42]. Stx causes HUS and also contributes to local damage in the GI tract that manifests as bloody diarrhea, hemorrhagic colitis, necrosis, and intestinal perforation [43]. Stx is an AB toxin, which is comprised of a pentamer of B subunits responsible for binding to target cells and a catalytic A subunit responsible for toxin activity [44, 45]. The major receptor for Stx is the glycolipid globotriaosylceramide (Gb3), and the kidneys and brain are susceptible to intoxication due to high levels of Gb3 expression on epithelial cells [46, 47]. Stx inhibits protein synthesis through RNA N-glycosidase activity that removes one adenine from adenosine in 28S ribosomal RNA, inhibiting binding of amino-acyl-tRNA to the 60S ribosomal subunit [48, 49]. The toxin induces apoptosis in target cells. Stx can also alter cytokine expression, inducing IL-8 expression from epithelial cells and IL-1, IL-6, and tumor necrosis factor from macrophages and human proximal tubule cells [50, 51].

The two main types of Stx produced by EHEC strains, Stx1 and Stx2, are approximately 55% homologous on the amino acid level [52]. Stx1 is highly similar to Stx produced by *Shigella*, and Stx2 is associated with increased disease severity [52-54]. EHEC strain EDL933 was isolated from the first outbreak in 1982 in Oregon and Michigan and encodes both *stx1* and *stx2* [26, 55, 56]. EHEC strain 86-24 was isolated from a later outbreak in 1986 and encodes only *stx2* [57]; however, 86-24 is thought to be more virulent than EDL933 with increased progression to HUS [58-61].

The *stx* (*stxA* and *stxB*) genes are encoded on chromosomally inserted bacteriophages [62]. Stx expression is activated during the phage lytic cycle, which is induced through the bacterial SOS response by DNA damage and regulated by RecA [63] (Figure 1.1). Initiation of the bacterial SOS response and activation of RecA leads to the degradation of *c*I, a key phage repressor [63]. Loss of *c*I repression leads to the expression of Stx as well as assembly of phage particles and release through bacterial cell lysis [64]. Antibiotic use is thought to trigger the bacterial SOS response, increasing toxin expression and progression to HUS [36, 39, 40].

LEE

The second major virulence trait is the LEE, which is a pathogenicity island encoding many of the genes required for type III secretion (T3S) and intimate attachment to intestinal epithelial cells through the formation of attaching and effacing (AE) lesions (Figure 1.2A). AE lesions are characterized by an effacement of the microvilli, an intimate receptor-mediated attachment of EHEC to host epithelial cells, and the formation of an actin-rich pedestal through manipulation of the host cell cytoskeleton. These effects are mediated by the transfer of bacterial effectors into the host cells through T3S. The T3S apparatus is multicomponent organelle made up by approximately 20 proteins. It is structurally composed of a series of protein rings spanning the inner and outer bacterial membrane and a needle-like

extension off the surface of the bacteria that can insert effectors directly into the cytoplasm of target cells (Figure 1.2B).

The bacterial receptor for intimate attachment is intimin, and EHEC translocates a cognate receptor (Tir, translocated intimin receptor) into target cells through type III secretion to facilitate binding. Both the intimin and Tir proteins form multimers, and binding of intimin to Tir leads to higher-order clustering, ultimately producing the signaling events that drive actin rearrangement and pedestal formation [65, 66] (Figure 1.2B). The non-LEE encoded EHEC effector $EspF_u$ interacts with Tir to recruit host cell proteins involved in actin reorganization, specifically the actin-nucleating factor Wiscott-Aldrich syndrome protein (N-WASP) and the insulin receptor tyrosine kinase substrate p53 (IRSp53) [67, 68]. This signaling cascade leads to the accumulation of actin, forming the characteristic pedestal-like structure at the site of bacterial attachment.

A complex regulatory network controls expression of the five operons (*LEE1-5*) encoded within the LEE as well as the regulators *grlRA* (Figure 1.3). The first gene product encoded in the LEE is Ler, which promotes expression of *LEE2-5* as well as genes encoded outside of the LEE [69]. The LEE is silenced by H-NS (histone-like nucleoid-structuring protein), and Ler functions to alleviate H-NS repression to allow expression [69, 70]. GrlA activates Ler to promote expression of the LEE, including *grlRA*, and GrlR negatively regulates GrlA activity post-translationally [71, 72]. GrlR is regulated post-translationally through protease degradation to relieve GrlA repression and activate Ler expression [73]. Many regulators encoded outside

the LEE respond to environmental conditions to regulate *LEE1/ler* expression. Expression of *ler* is activated by GrvA [74], QseCEF [75], QseA [76], IHF [77], PerC homologs (Pch) [78], ppGpp [79], Hfq [80], EutR [81], DsrA/RpoS [82], KdpE/Cra [83], and NsrR [84]. Additionally, *LEE1/ler* is negatively regulated by Hha [85], RpoS [73], FusKR [86], SdiA [87, 88], EtrA [89], GadX [84], and EivF [89]. Furthermore, the LEE is also regulated independently of Ler, allowing for expression of specific LEE operons; YhiEF represses expression of LEE2/4 [90]. The presence of this intricate web of regulators highlights the importance of controlled expression of the LEE.

EHEC fimbriae

Fimbriae are less well characterized EHEC virulence factors but are hypothesized to be essential for initial interactions with host cells. EHEC encodes 16 putative and characterized fimbrial loci [91]. Most EHEC fimbriae are assembled through the chaperone-usher pathway, but EHEC also encodes curli fimbriae and type IV pili (HCP) [5, 91]. Characterized fimbriae either lack robust roles in EHEC adherence in the host environment or have not been tested in an animal model, leaving the contribution of EHEC fimbriae to pathogenesis still undefined (Table 1.1).

The two most extensively studied fimbrial loci in EHEC are *lpf1* and *lpf2*, which encode homologs to the well-characterized long, polar fimbriae of *Salmonella*.

Lpf1 binds to fibronectin, laminin, and collagen IV [92] and is not encoded in the prototypical commensal strain *E. coli* K12 [93, 94]. Expression in non-fimbriated *E. coli* cells leads to production of short, peritrichous fimbriae and increases adherence to epithelial cell [93]. Deletion of *lpf1* in WT EHEC decreases adherence to epithelial cells and alters the adherence pattern, suggesting that Lpf1 is important for EHEC adherence [93, 95]. The *lpf1* locus is H-NS repressed, and Ler directly interacts with the promoter region to outcompete H-NS and relieve repression [96-98].

Lpf2 is also not encoded in *E. coli* K12 [94]. The adherence phenotype for Lpf2 is confusing; adherence is actually decreased when expressed in non-fimbriated *E. coli* [95]. However, mutants in the major fimbrial subunit in EHEC show a reduction in adherence to intestinal epithelial cells (Caco-2 cells) but not cervical epithelial cells (HeLa cells) [95]. Its regulation is not as well characterized as is that for *lpf1*, but *lpf2* expression may be induced in low iron conditions through the regulator Fur [99, 100].

Studies testing the abilities of single and double *lpf1/2* mutants to colonize animal hosts have shown that the effects of these two fimbriae may be redundant. Slight reductions in fecal shedding by the single mutants were observed in crossbred lambs, and there was no difference in colonization fitness between single mutants and the WT strain in the infant rabbit model [99, 101]. A more robust colonization defect was observed in the double *lpf1/2* mutant in both crossbred lambs and infant rabbits [99, 101].

EHEC also encodes curli fimbriae (csg, *loc7),* but mutants in curli production alone are not defective in adherence to epithelial cells [102]. In *Salmonella* and *E. coli* K12, curli fimbriae contribute to biofilm formation, which is not known to play a role in EHEC pathogenesis. Additionally, curli fimbriae expression is generally increased at 25°C in comparison to 37°C, which suggests that curli fimbriae may be important for EHEC persistence in the environment or on food products [15, 103]. Interestingly, a *lpf1/2* double mutant overexpresses curli fimbriae and exhibits increased adherence to colonic epithelial cells *in vitro* [101]. This study suggested that the regulatory networks controlling the expression of these three EHEC fimbriae may be connected and that curli fimbriae contribute to adherence in the host.

Genes encoded within *loc2* are upregulated in EHEC under acid stress, but expression of this fimbrial locus does not aid in acid resistance [104]. Increased adhesion in acid-stressed EHEC in comparison to unstressed EHEC relies on Loc2 [104]. *loc5* encodes peritrichous, flexible fine fibers that bind laminin but not collagen IV or fibronectin [105]. A strain lacking *loc5* shows reduced adherence to intestinal epithelial cells and explants [105], but nothing is known about the regulation of *loc5* expression or its role in EHEC colonization. The role of *loc8* in adherence has been briefly investigated and is unclear. Expression of the EHEC *loc8* in *E. coli* K12 increases adhesion to bovine intestinal epithelial cells, but expression in EHEC actually decreases adherence to bovine GI explant tissue [106]. Colonization of weaned cattle with a *loc8* mutant strain showed slightly reduced

shedding in comparison to WT EHEC that was not statistically significant [106]. This suggests that Loc8 is likely not responsible for tissue tropism but could still contribute to EHEC colonization. ECP, the *E. coli* common pilus that is found in both pathogenic and commensal *E. coli*, has been studied in EHEC, and there was a decrease in adherence to epithelial cells in an *ecp* mutant strain in comparison to WT [107]. *ecp* is positively regulated by an internal regulator (EcpR) as well as integration host factor (IHF) to relieve H-NS repression [108]. HCP, the hemorrhagic colitis pilus, has been shown to mediate adherence to human intestinal and bovine renal epithelial cells as well as to porcine and bovine gut explants [109]. HCP is a type IV pilus that forms bundles [109]. Together, these data suggest that many of the EHEC fimbriae are functional when expressed and may contribute to adhesion to host tissue, but the conditions that induce expression of most of these fimbriae in the host are unclear.

In order to investigate the expression of EHEC fimbriae, one study utilized promoter fusions and found that 11/15 major fimbrial genes were not expressed under the conditions tested, which included varying temperatures, growth phases, aeration, and media [91]. The four fimbrial loci that were expressed were *loc4*, *loc7* (curli), *loc8*, and *loc9* [91]. Of note, while expression of *loc8* was detected, transcription was most highly increased at low temperatures, suggesting that this fimbria may aid in attachment outside of the host environment [91]. No phase variation was detected [91]. Three fimbrial loci (*lpf1*, *lpf2*, and *loc5*) encode premature stop codons [91]. Previous studies have shown that *lpf1* and *lpf2* encode

functional fimbriae in EHEC, which suggests that mechanisms for complementation by other fimbrial loci may exist or that there could be sequence diversity in fimbrial loci amongst EHEC isolates. Only *loc8*, *loc10*, and *loc14* encode a typical adhesin gene with homology to FimH [91]. Other EHEC fimbrial loci could potentially contain adhesins that do not share homology with adhesins encoded within the *fim* and *pap* operons. This study concluded that EHEC possessed a very limited repertoire of expressed and functional fimbriae [91], but this assumption is premature due to a lack of understanding about which conditions promote expression of many of the EHEC fimbriae.

Ethanolamine

In addition to adherence, invading pathogens need to compete with the resident microbiota for nutrients to colonize the gastrointestinal tract. EHEC utilizes ethanolamine (EA, Figure 1.4A) as a non-competitive nitrogen source in the bovine intestinal tract [110]. EA is derived from phosphatidylethanolamine (PE), which is a component of both bacterial and mammalian membranes. PE is the most abundant phospholipid in microbial membranes and the second most abundant in plant and animal membranes [111, 112]. The concentration of EA in bovine intestinal content has been measured at 2.2 mM [110], likely through both the high rate of turnover of bacterial and host cells as well as contributions from the diet [113].

The presence of EA utilization operons correlates with the ability to cause food poisoning; essentially, this means that bacteria that cause food-borne disease are more likely to encode the genes required to utilize EA as a nutrient source [114, 115]. Examples of foodborne pathogens encoding EA utilization operons include *Clostridium*, *Salmonella*, *Shigella*, *Listeria*, and EHEC [114, 115].

In *Enterobacteriaceae*, the genes required for the utilization of EA are encoded in the *eut* operon, and expression of these genes is induced during growth in EA [116]. The *eut* operon encodes 17 genes involved in the degradation of EA [117]. The ethanolamine ammonia-lyase (EutBC) enzyme converts EA into acetylaldehyde and ammonia. Ammonia can be readily used as a nitrogen source, and then acetylaldehyde is further metabolized into acetyl-CoA and ethanol by an acetaldehyde dehydrogenase (EutE). Acetyl CoA enters the TCA cycle to generate carbon and energy. However, EHEC is unable to use EA as a carbon source. EutR is the transcriptional regulator of the *eut* operon [118] (Figure 1.4B).

In EHEC pathogenesis, EA serves as a signal to regulate EHEC virulence gene expression (Figure 1.4B). Expression of the LEE and Stx is increased when EHEC is grown in the presence of EA in comparison to its absence [81]. EA-dependent virulence gene expression is independent of EA metabolism, as an *eutB* mutant and WT EHEC respond equivalently to EA to activate virulence gene expression [81]. EutR, the transcriptional regulator of the *eut* genes, also regulates EHEC virulence genes [81, 119]. A subset of EA-responsive gene regulation occurs independently of EutR, indicating that there is at least one other EA sensor [81].

Additionally, EHEC binds to PE [120]. EHEC does not bind to phosphatidylserine or phosphatidylcholine, indicating that EHEC binding to PE is specific [120]. Incubation of epithelial cells with EHEC induces apoptosis and increases PE expression on the cell surface, and this effect is independent of toxin production [121]. It is clear that EA is a critical factor in EHEC pathogenesis, and we are just beginning to understand the multiple mechanisms in which EA influences the interactions between EHEC and the host.

Project rationale

Microarray analysis of EHEC grown in the presence and absence of EA identified that multiple fimbrial genes exhibited altered expression during growth in EA [81]. The hypothesis for this project is that EA is a critical signal in EHEC for fimbrial expression and that EA-regulated fimbriae promote colonization of the GI tract. We investigated how EA influences EHEC fimbrial expression and early adhesion as well as the contribution of two EA-regulated fimbriae to EHEC pathogenesis. Furthering our understanding of the role of both EA signaling and fimbriae in EHEC colonization is critical for the development of therapeutic interventions to prevent infection, which are critical because of limited treatment options for EHEC disease due to antibiotic use being contraindicated.

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

Locus	*In vitro* adherence	*In vivo* adherence	Reference
loc1	NT	NT	
loc2	+	NT	[104]
loc3	NT	NT	
loc4	NT	NT	
loc5	+	NT	[105]
loc6	NT	NT	
loc7/curli	+/-	NT	[101, 102]
loc8	+/-	-	[106]
loc9	NT	NT	
loc10	NT	NT	
loc11	NT	NT	
loc12/lpf1	+	-*	[93, 95, 99,

			101]
loc13/lpf2	+/-	-*	[95, 99, 101]
loc14	NT	NT	
ecp	+	NT	[106]
hcp	+	NT	[109]

\+ Fimbrial locus increases adherence to tissue culture cells *in vitro*

\- Fimbrial locus does not increase adherence under conditions tested

NT Not tested

* An *lpf1/2* double mutant shows reduced colonization compared to WT EHEC in both crossbred lambs and infant rabbits.

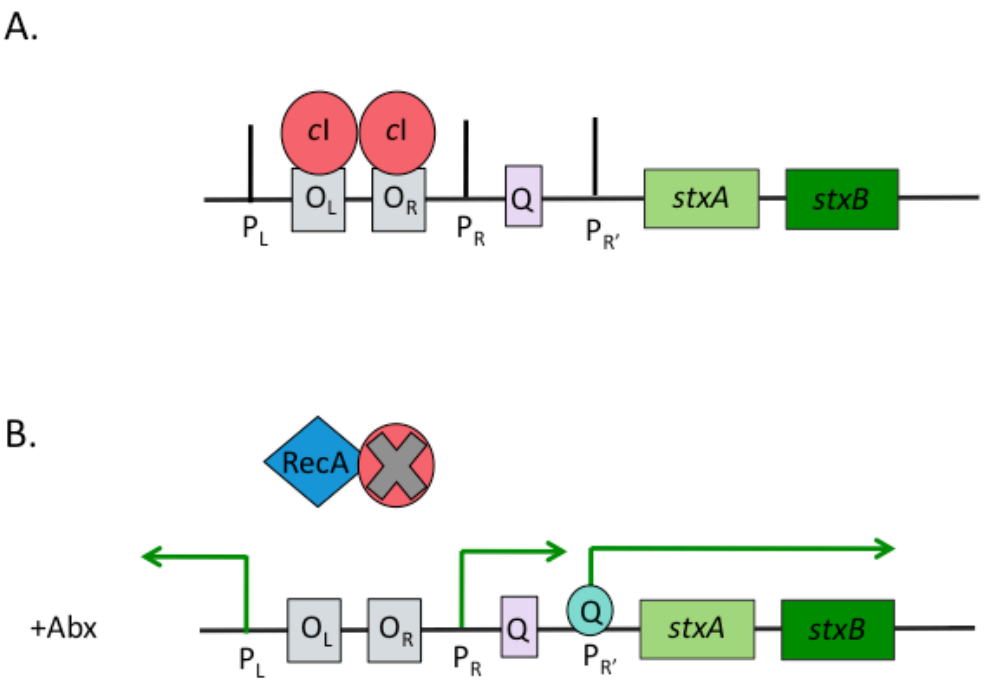

Figure 1.1 Shiga toxin (Stx) expression in EHEC. Stx is phage encoded and expressed under the $P_{R'}$ promoter with the phage late genes. A. Stx transcription is normally repressed by *c*I binding to left and right operator sites (O_L and O_R). B. Antibiotic treatment (Abx), specifically by antibiotics that induce DNA damage, can initiate the bacterial SOS response. When the bacterial SOS response is activated, RecA degrades *c*I and relieves repression of the P_L and P_R promoters, allowing for expression of the *stx* genes from $P_{R'}$. Protein Q (expressed under the P_R promoter) binds to the $P_{R'}$ promoter to activate *stx* transcription.

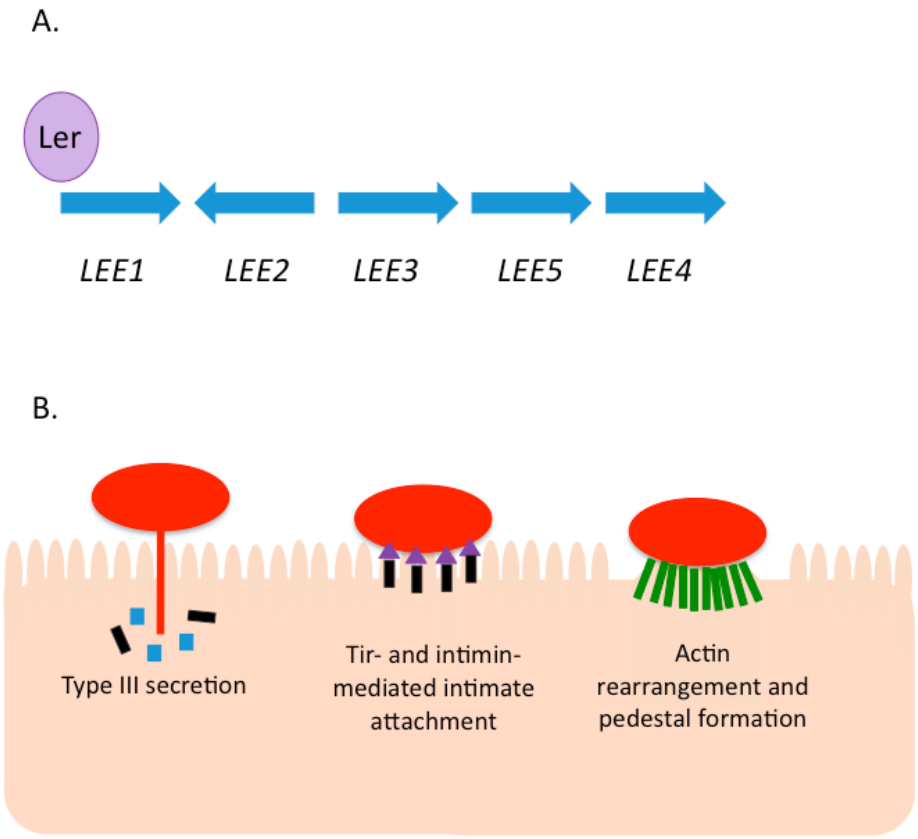

Figure 1.2 The locus of enterocyte effacement (LEE). A. A schematic of the five operons encoded within the LEE. Ler is the master regulator of the LEE and is encoded in *LEE1*. B. The LEE encodes the genes required for the formation of attaching and effacing (AE) lesions. EHEC translocates effector proteins into the host cell cytoplasm through the needle-like T3S apparatus. One of these effectors is the cognate receptor Tir (black rectangles) of the bacterial protein intimin (purple triangles). Intimin and Tir binding allows intimate attachment between the bacterial and host cells. Subsequent signaling events result in actin rearrangement and the formation of an actin-rich pedestal that cups the bacterial cell to the host cell.

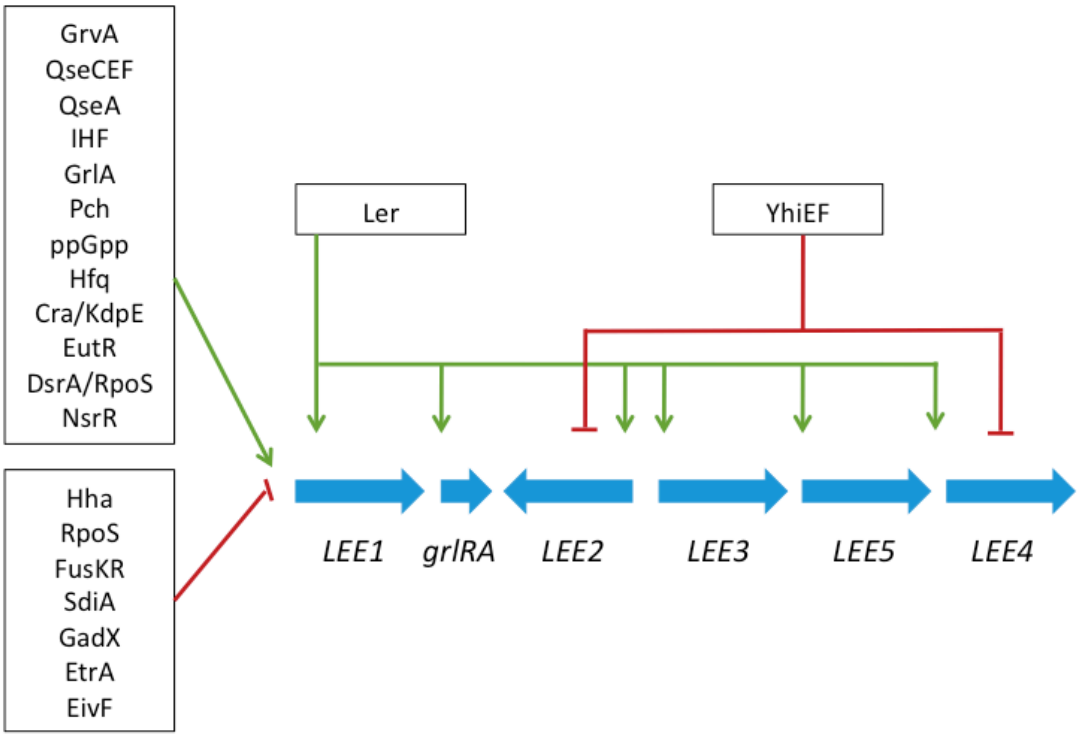

Figure 1.3 Regulation of the LEE. This schematic details known regulatory mechanisms controlling expression of the LEE. Positive regulation is indicated with green and negative regulation with red. Not all regulation is known to be direct.

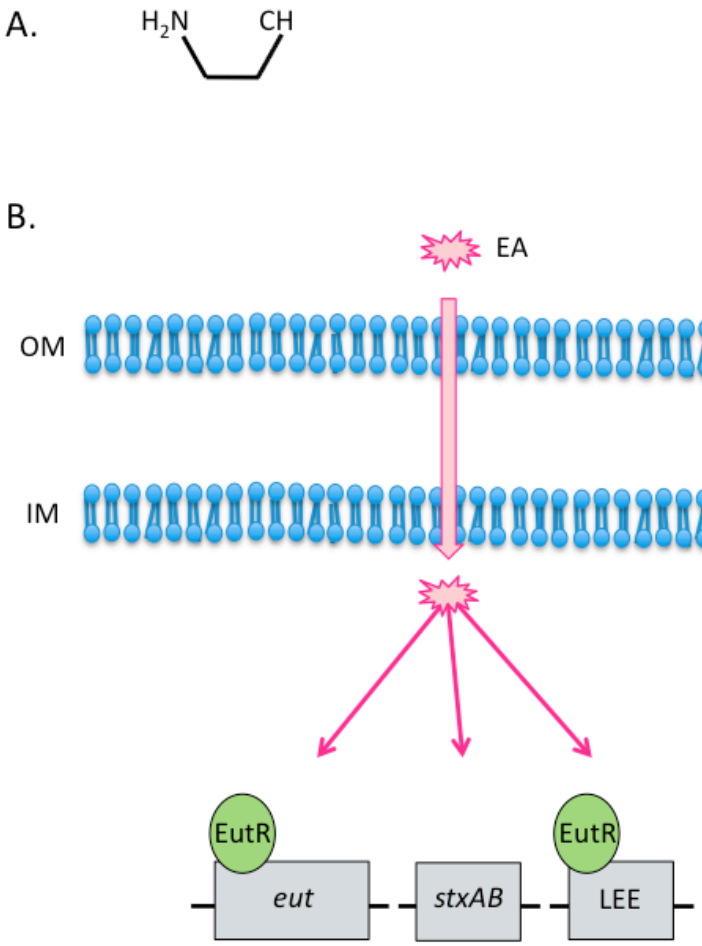

Figure 1.4 Ethanolamine (EA) signaling in EHEC. A. The structure of EA. B. Sensing of EA by EHEC leads to the upregulation of genes involved in the utilization of EA as a nutrient source through the transcriptional regulator of the *eut* operon, EutR. Additionally, EA signaling modulates virulence gene expression, including genes involved in Stx expression, T3S, and AE lesion formation. EutR is also involved in the regulation of virulence genes in EHEC in response to EA. OM, outer membrane. IM, inner membrane.

Chapter Two

Ethanolamine and choline promote expression of putative and characterized fimbriae in enterohemorrhagic *Escherichia coli* O157:H7

Adapted from "Ethanolamine and choline promote expression of putative and characterized fimbriae in enterohemorrhagic *Escherichia coli* O157:H7"

Laura A. Gonyar and Melissa M. Kendall

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Abstract

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 is an important food-borne pathogen responsible for disease outbreaks worldwide. In order to colonize the human gastrointestinal (GI) tract and cause disease, EHEC must be able to sense the host environment and promote expression of virulence genes essential for adherence. Ethanolamine (EA) is an important metabolite for EHEC in the GI tract, and EA is also a signal that EHEC uses to activate virulence traits. Here, we report that EA influences EHEC adherence to epithelial cells and fimbrial gene expression. Quantitative RT-PCR indicated that EA promoted transcription of genes encoded in characterized and putative fimbrial operons. Moreover, putative fimbrial structures were produced by EHEC cells grown with EA but not in medium lacking EA. Additionally, we defined two previously uncharacterized EA-regulated fimbrial operons, *loc10* and *loc11*. We also tested whether choline or serine, which are also components of cell membranes, activated fimbrial gene expression. In addition to EA, choline activated fimbrial gene expression in EHEC. These findings describe transcription of several putative fimbrial loci in EHEC for the first time. Importantly, the biologically relevant molecules EA and choline, that are abundant in the GI tract, promoted expression of these fimbriae.

Introduction

Bacterial pathogens interact directly with mammalian hosts by expressing adhesive structures, including fimbriae, on the outer surfaces of their cells. Fimbriae typically mediate the earliest stages of host colonization and are especially critical for enteric bacteria to successfully colonize the gastrointestinal (GI) tract in order to avoid displacement by the continuous flow of intestinal contents [1, 122]. Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 is a food-borne pathogen that causes bloody diarrhea and hemolytic uremic syndrome worldwide [123]. The first step in EHEC pathogenesis involves adherence to the intestinal epithelium and subsequent intimate attachment and formation of attaching and effacing (AE) lesions on epithelial cells [122]. The locus of enterocyte effacement (LEE) pathogenicity island encodes most of the genes required for AE lesion formation and intimate attachment to host cells [124].

However, factors encoded outside of the LEE may also play important roles in EHEC adherence to epithelial cells, especially during the initial stages of EHEC infection [125]. EHEC encodes 14-16 fimbrial loci [126, 127], suggesting that fimbriae are an important component of EHEC's virulence repertoire. However, very little is known about the roles of EHEC fimbriae in colonization of the mammalian GI tract or EHEC's tropism for the large intestine. This is due, at least in part, to the lack of understanding of environmental cues that promote expression of these fimbriae *in vitro* [91, 122, 128].

Ethanolamine (EA) is an important metabolite for EHEC in the GI tract [110] as well as a cue that EHEC uses to recognize the host environment and modulate expression of virulence traits, including EHEC's ability to adhere to epithelial cells *in vitro* [81]. EA is a major component of mammalian and bacterial cell membranes, and the turnover and exfoliation of intestinal cells is a key source of EA in the intestine [113]. Therefore, we hypothesized that EA could be a signal that EHEC uses to sense its proximity to epithelial cells and activate expression of fimbriae that contribute to EHEC colonization of the GI tract. The goal of this study was to determine the role of EA in promoting expression of EHEC fimbriae. Additionally, we examined whether other host- and bacterial-cell breakdown products promoted expression of fimbriae in EHEC. Our data indicate that biologically relevant concentrations of EA and choline, but not serine, significantly promote expression of EHEC fimbriae.

Materials and Methods

Strains and growth conditions

Wild type (WT) EHEC strain 86-24 [57] and the *ΔeutR* mutant strain MK37 [81] were used in this study. Cultures of 86-24 and MK37 were grown at 37 °C with shaking in LB (Invitrogen), Dulbecco's modified Eagle's medium (DMEM) (Invitrogen), or M9 minimal medium (WT only) [129] prepared without addition of a nitrogen source to the minimal salts. The nitrogen sources added to minimal

media were 2 mM EA hydrochloride, 5 mM choline, 5 mM serine, or 2 or 5 mM NH_4Cl (all from Sigma). For studies using DMEM, 10 mM EA or choline was added as indicated. Vitamin B12 (cyanocobalamin) was supplemented at 150 nM into the medium whenever EA was used as a nitrogen source. Streptomycin was added to overnight cultures of EHEC at a final concentration of 50 μg/ml.

RNA extraction and quantitative RT-PCR (qRT-PCR)

EHEC strain 86-24 was grown aerobically in LB overnight at 37˚C with shaking and then diluted 1:100 in M9 minimal media supplemented with the indicated nitrogen sources and grown at 37°C. RNA from three biological replicates of each condition was extracted at early logarithmic phase ($O.D._{600}$ of 0.2), mid- logarithmic phase ($O.D._{600}$ of 0.5), or late logarithmic phase ($O.D._{600}$ of 1.0). For qRT-PCR experiments from cells grown in DMEM, EHEC strains 86-24 and MK37 (Δ*eutR* strain) were incubated for 6 h statically in a 5% CO_2 atmosphere. RNA was extracted using the RiboPure bacteria kit (Ambion).

Primers used in real-time qRT-PCR assays were designed using Primer Express v1.5 (Applied Biosystems) and validated before use. Reaction mixtures were prepared as previously described [130]. qRT-PCR was performed using a one-step reaction in an ABI 7500-FAST sequence detection system (Applied Biosystems). All data were normalized to the levels of *rpoA* and analyzed using the comparative cycle threshold (CT) method [131]. The relative quantification method was used to

determine the expression level of target genes in various growth conditions. Statistical significance was determined by Student's *t* test, and a *p* value of ≤0.05 was considered significant. Specific genes from each fimbrial locus that was used in qRT-PCR are indicated in Table 2.1.

Reverse transcriptase PCR (RT-PCR)

RNA was isolated from EHEC grown to early-logarithmic growth phase in minimal medium with 2 mM or 10 mM EA. SuperScript II reverse transcriptase (Invitrogen) and random primers (Invitrogen) were used to synthesize cDNA. The resulting cDNA was used for regular PCR with gene specific primers (Table 2.1). A reaction without reverse transcriptase was used as a negative control, and genomic DNA was used as a positive control. PCR and gel electrophoresis were performed using standard methods.

Adhesion assay

Adhesion assays were performed as previously described [132]. Briefly, EHEC strain 86-24 was grown aerobically in LB overnight at 37 °C without shaking. HeLa cells were washed before infection and placed in low glucose DMEM without antibiotics. The bacterial overnight cultures were diluted to approximately 1.0 x 10^9 colony forming units (CFUs)/ ml to infect the HeLa cells. HeLa cells were incubated

with the bacteria for 3 and 6 h at 37 °C in 5% CO_2 in the presence and absence of 10 mM EA or 10 mM choline. The coverslips were then washed, fixed with methanol, and stained with Giemsa stain (Sigma) at room temperature. A replicate set of coverslips were washed, and subsequently, the HeLa cells were lysed with Triton-X 100 (Sigma), and bacterial cells were plated to determine CFU/ml. Adherence is presented as the percentage of bacteria that adhered to epithelial cells in the presence of EA or choline relative to bacteria adhered in DMEM alone (absence of EA or choline). Three individual experiments were averaged and a Student's *t* test was performed to determine statistical significance.

Transmission electron microscopy (TEM)

EHEC strain 86-24 was grown aerobically in LB overnight at 37 °C with shaking and then diluted 1:100 in M9 minimal media supplemented with either 10 mM EA or 20 mM NH_4; these cultures were then grown at 37 °C for 5 h under static conditions to best preserve fimbrial structures. The bacteria were washed in ultrapure water and then fixed overnight at 4 °C in 2.5% glutaraldehyde. After fixation, the bacteria were washed twice and diluted to an $O.D._{600}$ of approximately 0.9. The bacteria were allowed to adhere to formvar-coated copper grids (200 mesh) and excess liquid was wicked away with filter paper. The cells were then stained with 2% phosphotungstic acid and imaged on a JEOL 1230 TEM. To quantify the percentage of cells expressing fimbriae, a total of 45-65 cells per condition were

evaluated as either positive or negative for the presence of fimbrial structures.

Results

EA influences EHEC adherence to epithelial cells

EA plays an important role in promoting gene expression in EHEC [81]. A previous study demonstrated that EHEC binds to phosphotidylethanolamine (PE) of host cells [120], and another study recently showed that EA significantly increased EHEC attachment to epithelial cells [81]. To better understand the role of EA in EHEC adherence, we investigated whether EA influenced the adhesion profile of EHEC to epithelial cells. We grew EHEC with HeLa cells in the absence or presence of EA, and the cells were examined after 3 or 6 h of incubation. When EHEC was grown in the absence of EA, typical microcolony patterns of adherence to HeLa cells were observed. However, when EHEC was grown with EA, we observed that the cells appeared more clustered with EHEC adhering not only to the HeLa cells, but to the glass cover slips as well (Figure 2.1A). The arrangement of EHEC cells grown with EA resembles the stacked-brick pattern of adherence typically associated with enteroaggregative *E. coli* (EAEC) [133] as well as the adherence phenotype of a Δ*ler* (LEE-encoded regulator) strain of EHEC in which the Δ*ler* EHEC displayed increased adherence to epithelial cells as well as to glass [69]. Quantification of adhering bacteria (CFUs) showed that EA significantly increased the percentage of EHEC adhering to epithelial cells at 3 h post-infection (Figure 2.1B); however, there were

no significant differences in the percentage of adhering bacteria grown with or without EA at 6 h (Figure 2.1B). These data are consistent with our previous findings which showed that EA increases EHEC's ability to infect a higher percentage of epithelial cells [81] and also suggest that EA is important for the initial stages of EHEC adherence to epithelial cells.

EA promotes expression of fimbrial genes

Because fimbriae are involved in EAEC adherence [134] as well as the increased adherence of the Δ*ler* EHEC strain [69], we re-examined our microarray data [81] to determine whether EA promotes expression of fimbriae. The array data indicated that genes encoded within *loc2* (*yad*), *loc7* (*csg*/ curli), *loc10* (*stf*), *loc11* (*yra*), and *loc12* (*lpf1*) were significantly increased in EHEC cells grown with EA compared to EHEC cells grown in the absence of EA [81]. To confirm the microarray analysis and determine whether expression of additional fimbrial genes were modulated by EA, we performed qRT-PCR and measured transcription of one gene from each of the fimbrial loci (listed in Table 2.1) from EHEC cells grown in minimal medium with 2 mM NH_4 or 2 mM EA, which is a physiologically relevant concentration of EA [110]. Gene expression was examined in EHEC grown to early-, mid-, and late-logarithmic growth phases. These analyses provided interesting insights into EA-induced fimbrial gene expression. When EHEC was grown with EA, expression of genes encoded in 14 distinct fimbrial loci was significantly increased

compared to EHEC grown with NH_4 at both early- and mid- logarithmic growth phases (Figure 2.2 and Figure 2.3), and there were no significant differences at late-logarithmic phase (Figure 2.4). A possible explanation for the loss of significance differences at late-log may be a result of an increase in fimbrial gene expression in cells grown with NH_4 during this growth phase. To test this, we compared fimbrial gene expression (*loc7, 11*, and *12*) between cells grown with NH_4 to early- or late-logarithmic phase. The results indicated that there were no significant differences (Figure 2.5) in fimbrial gene expression in minimal medium with NH_4 at the varying growth phases. We also compared fimbrial gene expression (*loc7, 11*, and *12*) between cells grown with EA to early- or late- logarithmic phase. Fimbrial gene expression was significantly increased in cells grown to early- logarithmic phase compared to cells at late- logarithmic phase growth phase in minimal medium with EA (Figure 2.5). These results suggest that the depletion of EA in the culture medium leads to reduced fimbrial gene expression observed at late- logarithmic phase.

EA regulation of fimbrial genes is both EutR dependent and independent

Our recent findings suggested that EHEC encodes two EA sensors involved in virulence gene regulation [81]. EutR is a transcriptional regulator that activates expression of genes that allow for EA metabolism in response to EA and vitamin B12 [118, 119]. We showed that EutR binds EA and directly regulates *ler* that encodes

Ler [119], the master regulator of the LEE pathogenicity island [135-137]. To better understand EA-dependent fimbrial gene expression, we grew WT EHEC and the Δ*eutR* mutant strain MK37 anaerobically in DMEM for 6 h with or without the addition of EA and performed qRT-PCR on one gene from each of the fimbrial loci (indicated in Table 2.1). The pattern of gene expression in WT EHEC grown in DMEM with or without EA was similar to the gene expression profile of cells grown in minimal medium (Figure 2.6). Interestingly, we found that expression of only five fimbrial loci depended on EutR. EA-regulated fimbrial loci that were dependent upon EutR included *loc2*, *loc7*, *loc11*, *loc12*, and *ecp* (Figure 2.6). Expression of the remaining ten EA-regulated fimbrial loci was induced independently of EutR (Figure 2.6).

EA promotes expression of fimbrial structures

Our data demonstrate that EA influences fimbriae expression at the transcriptional level; however, in order to function in adherence, fimbriae must be expressed on the bacterial cell surface. Therefore, we performed transmission electron microscopy (TEM) of cells grown statically with NH_4 or EA for 5 h. EA-induced fimbriae displayed a short, spikey appearance and were evenly distributed on the outer surfaces of the cells; these structures were absent on cells grown with NH_4 (Figure 2.7). Additionally, significantly more cells produced fimbrial structures when grown with EA compared to NH_4 (Figure 2.7).

Transcriptional mapping of fimbrial loc10 and 11

To better characterize EA- induced fimbriae, we undertook operon mapping of *loc10* and *11*. These loci were chosen because we saw significant increases in expression of these loci in response to EA as part of our previous microarray analyses and because expression of these fimbriae has not been demonstrated previously for EHEC [91, 128]. Both loci encode putative fimbriae that belong to the chaperone-usher (CU) family of adhesins. Genes encoding fimbriae of the CU class are typically organized into operons that contain a minimum of a chaperone (that facilitates folding of fimbrial subunits in the periplasm), an usher (that acts as an assembly platform in the outer membrane of the bacterial cell), and a major fimbrial subunit [138]. Furthermore, CU fimbriae often encode additional minor fimbrial subunits, regulatory proteins and/or assembly proteins [138].

The putative fimbrial locus *loc10* contains seven open reading frames (ORFs) that are predicted to be organized in an operon (Figure 2.8A). To test this prediction, we defined the operon structure of *loc10* by RT-PCR analysis. Whole-cell RNA was isolated from EHEC strain 86-24 grown in minimal medium with EA as the nitrogen source, and cDNA was synthesized as a template. The RT-PCR analysis showed that all of the predicted minor fimbrial subunits are expressed; however, these minor subunits are transcribed as two separate transcriptional units, as reactions using primers that spanned *Z3595* to *Z3598* did not produce visible PCR

products (Figure 2.8B). In these initial RT-PCR analyses, the results demonstrated that the predicted major subunit (*Z3601*), usher (*Z3600*), and chaperone (*Z3599*) were not transcribed, as no products were obtained despite any primer combination that included these genes (including internal control primers) (Figure 2.8B). To investigate this further, we performed qRT-PCR on *Z3599- Z3601* and compared expression to *Z3597*. The results indicated that these genes were expressed relatively 5-fold lower than *Z3597* (Figure 2.8C); therefore, we repeated the operon mapping and increased the number of PCR cycles to 35 cycles (from 25). We were able to detect individual transcripts under these conditions for *Z3599, Z3600*, and *Z3601*, but no bands were detected using overlapping primers specific to *Z3599-Z3601*, indicating that these genes are not co-transcribed (Figure 2.8D). However, these data also revealed that *Z3599* is co-transcribed with *Z3597* and *Z3598* (Figure 2.8D).

The putative fimbrial cluster *loc11* also encodes seven ORFs including predicted transposase genes (*Z4503* and *Z4502)* (Figure 2.9A). The RT-PCR analysis showed that all seven genes within this locus are transcribed when EHEC is grown with EA and that all ORFs within this locus are transcriptionally coupled (Figure 2.9B), suggesting that this operon encodes a functional fimbria.

Roles of choline and serine in EHEC fimbrial gene expression

A significant source of lipids in the intestinal lumen derives from exfoliation of

intestinal cells [113]; therefore, we investigated whether choline and serine, the breakdown products of phosphotidylcholine (PC) and phosphotidylserine (PS), respectively, could also influence fimbrial gene expression in EHEC. In addition to PE, PS is an important phospholipid in both bacterial and mammalian cell membranes [139], whereas the lipid PC is the most abundant phospholipid in mammalian cell membranes [139]. With few exceptions, bacteria do not make PC [139]. In the initial assays, EHEC was grown in minimal medium with 2 mM NH_4, 2 mM choline, or 2 mM serine as the sole nitrogen source to early-log growth phase and fimbrial gene expression was measured by qRT-PCR. Our data indicated that choline significantly increased expression of all fimbrial genes examined (Figure 2.10) when grown in minimal medium. In contrast, our data revealed no significant differences in expression of any of the fimbrial genes examined when EHEC cells were grown with serine as compared to cells grown with NH_4 (Figure 2.11). The overall trends of fimbrial gene expression in cells grown with EA or choline were very similar. For example, the lowest fold change in expression was measured in the fimbrial locus *loc8* (*fim9*) in cells grown with EA or choline compared to cells grown with NH4, whereas the largest fold increase in expression was measured in the fimbrial locus *loc7* (*csg*), although the significances in gene expression did vary between these conditions.

To further investigate the role of choline in promoting fimbrial gene expression, we grew EHEC cells for 6 h anaerobically in DMEM with or without choline. Under these conditions, choline significantly increased expression of 13

fimbrial loci (Figure 12). Interestingly, we did not observe significant changes in gene expression in the EutR-regulated fimbrial *loc2*, *loc7* or *ecp*, although we did measure significant increases in expression of l*oc11* and *loc12* that are also regulated by EutR.

Choline increases adherence of EHEC to epithelial cells

To elucidate the role of choline on EHEC adherence to epithelial cells, we performed adhesion assays. When EHEC was grown with choline, we observed a clustered pattern of adherence (Figure 2.13A) that was similar to the adherence pattern of EHEC when grown with EA (Figure 2.1A). We also quantified the percentage of EHEC cells adhering to epithelial cells in the absence and presence of choline at 3 and 6 h post-infection and determined that significantly more EHEC cells adhered to epithelial cells when grown with choline compared to EHEC cells grown in the absence of choline at 3 h, whereas no significant differences were observed at 6 h (Figure 2.13B). Together, with the EA data, these results support a role for fimbriae during initial stages of infection.

Choline induces expression of fimbrial structures on EHEC cells

We performed TEM on cells grown with choline to determine if choline influenced the production of surface structures. Cells grown with choline produced

significantly more structures on the outer surface compared to cells grown in the absence of choline (Figure 2.14B). The choline-induced fimbriae appeared longer and less evenly distributed on the cell surface compared to the fimbrial structures produced in the presence of EA (Figure 2.14A); therefore, it is possible that EA and choline lead to the expression of different fimbrial proteins.

Discussion

Fimbriae are critical for the successful initiation and establishment of infection by many bacterial pathogens. Our findings demonstrate that EA and choline, host- and bacteria- derived molecules that are present in the GI tract, promote expression of characterized and putative fimbriae. These expression data are intriguing because, for many of the EHEC fimbrial loci, only limited data exist describing conditions that promote expression. Expression of the *lpf1* loci (*loc12*) in EHEC is influenced by pH, temperature, and growth phase [99]. Temperature and growth phase as well as iron depletion also influence transcription of *lpf2 (loc13)* [99]. Expression of *ecp* (*E. coli* common pilus/*yagZ*) is regulated by temperature, oxygen tension, growth medium [107] as well as under conditions inducing acid stress [140]. Transcription of *loc5* (*elf/E. coli* laminin fimbriae, *ycb*,) was observed using various culture media; however, the protein was only expressed when cells were grown in Minca minimal medium, which is similar to conditions that promote HCP expression on EHEC cells (encoded by *hcp* hemorrhagic colitis pilus, *ppd*) [105, 109].

Expression of *yadK* encoded in fimbrial *loc2* has been shown to be induced in EHEC cells undergoing acid stress [104]. Finally, expression of curli, encoded in *loc7* (*csg*), varies among EHEC strains and is influenced by environmental conditions including temperature, oxygen-, salt-, and nutrient-availability, and acid stress [140, 141].

Here, we show that EA contributes to expression of the *loc12* and *13* (*lpf1/2)*, *loc5* (*elf), loc16 (hcp), loc2 (yad)*, and *loc7* (*csg)* fimbrial loci. No significant differences in expression were observed in the *ecp* or *loc8* (F9) fimbrial loci in experiments using minimal media. We performed these analyses using cells grown at 37°C under an aerobic atmosphere, whereas the ECP fimbriae locus is most highly expressed at lower temperatures as well as under a CO_2 atmosphere [107]. However, when we grew EHEC in DMEM under a 5% CO_2 atmosphere (Figure 2.6), we measured a significant increase in *ecp* expression in cells grown with EA compared to without EA. These data further highlight oxygen availability as an important environmental cue for regulation of this locus. Expression of *loc8* (F9*)* has been shown to occur in rich media in stationary-phase cultures that were incubated at 28°C [106]. Therefore, it is not surprising that we did not observe high levels of expression with our conditions. *In vitro* expression of the remainder of these fimbrial loci have not previously been reported, thus our findings provide an initial framework for elucidating the contributions of these fimbriae to EHEC colonization of the host intestine.

We also investigated the role of EutR in regulation of fimbrial expression and

found that increased expression of *loc2*, *loc7* (*csg*), *loc12* (*lpf1*), *loc13* (*lpf2*) and *ecp* during growth in the presence of EA is EutR-dependent. It has been previously shown that transcription of *lpf1* (*loc12*) is regulated by H-NS and the LEE-encoded regulator Ler, with Ler displacing H-NS to promote *lpf1* (*loc12*) expression [97]. Moreover, transcription of *ecp* is also negatively regulated by H-NS [108]. In *E. coli* K-12, several fimbrial loci have also been reported to be repressed by H-NS [128]. Hence, additional mechanistic studies are necessary to determine if EutR-dependent fimbrial gene expression is mediated indirectly by EutR activation of Ler, by direct binding of EutR to these fimbrial promoters, and/or antagonizing H-NS to overcome repression of these loci.

Our data indicate that increased expression of ten of the EA-induced fimbrial loci during growth with EA is EutR-independent (Figure 2.6). In all cases (except *hcp*), expression values were similar in WT EHEC compared to the Δ*eutR* strain, and both the WT and Δ*eutR* strains had significant increases in fimbrial gene expression when grown with EA. Expression of *hcp* was significantly decreased in the Δ*eutR* strain compared to WT EHEC; however, the Δ*eutR* strain was able to respond to EA. These data suggest that EA-induced expression of *hcp* may be controlled through both EA regulatory pathways. These results are consistent with our previously published data showing that EA promotes EHEC adherence to epithelial cells, including adherence of the Δ*eutR* strain [81]. These findings further support a role for adhesins encoded outside the LEE in EHEC attachment to epithelial cells [122].

We mapped two EA-induced fimbrial loci, *loc10* and *loc11*, and showed that all

genes encoded within these two loci are expressed during growth with EA. Although *loc10* transcribes the predicted chaperone and usher subunits at lower levels than measured for the minor subunits, this operon may still encode a functional fimbriae. Alternatively, the minor subunits may function in adherence in conjunction with different transport and assembly machinery of distinct fimbriae [142]. Previous work has demonstrated that the transport and assembly machinery of separate fimbriae are able to bind and protect the heterologous subunits and incorporate these subunits into functional hybrid fimbriae [142-144]. Furthermore, the *lpf1* (*loc12*) and *elf* (*loc5*) of EHEC as well as the *stg* fimbrial operon of *Salmonella enterica* contain premature stop codons; however, these fimbriae play a role in adherence of these pathogens [93, 105, 145-147]. Moreover, production of Lpf1 and Elf fimbriae on the outer surface of EHEC has been demonstrated [93, 105]. Presumably, these loci express functional fimbriae using a combination of fimbrial systems [142]. Our data demonstrating expression of all genes encoded within *loc10* and *loc11* suggests that they could both encode functional fimbriae.

Additionally, we revealed an important role for choline in promoting expression of characterized and putative fimbriae. We previously showed that expression of *ler* and *stx2a* that encodes Shiga toxin did not depend on the EA catalytic enzymes; however, it is possible that EA and/or choline metabolism is a cue for EHEC to promote expression of fimbriae. Recently, the choline metabolic pathway was elucidated and the authors reported shared mechanistic similarities between EA and choline catabolism [148]. Although we observed distinct

phenotypes for EA- and choline-dependent fimbrial gene expression as well as different surface structures when EHEC was grown with EA compared to choline, additional studies are warranted to determine the interplay between these regulatory pathways. Altogether, these data further highlight the role of metabolites as cues for promoting expression of virulence traits that are involved in host colonization.

Table 2.1 Primers used in this study.

Primer Name	Primer Sequence	Notes
loc1Fd	CCAAGTATCGAAGTACTAGCATCAGAGT	qRT-PCR; *Z0024*
loc1Rv	CCCTGGTCTCATTCTGGTCAA	qRT-PCR; *Z0024*
loc2Fd	TCTGGGTGCTTGGTATGAAGTTAT	qRT-PCR; *Z0146*
loc2Rv	TGTGGTGCCGCAGATGAA	qRT-PCR; *Z0146*
loc3Fd	TCTCAAACCTGATGGGAATAGCTT	qRT-PCR; *Z0686*
loc3Rv	CGTGCAGAAAAATGAAGAACGT	qRT-PCR; *Z0686*
loc4Fd	CGCCCTGGCCATATCTCTAC	qRT-PCR; *Z0872*
loc4Rv	TTTAAAGTGGATTTCACCAGATCCT	qRT-PCR; *Z0872*
loc5Fd	GCAGATACCCGGTGGCATAT	qRT-PCR; *Z1290*
loc5Rv	TCCCTGGCCGCCTAAAC	qRT-PCR; *Z1290*
loc6Fd	ACGCCGTTTACGGTTTCTGT	qRT-PCR; *Z1538*
loc6Rv	GGTGGTGTTGATTGCCTGATC	qRT-PCR; *Z1538*
loc7Fd	GCGTTGTCAATGGATTGCAA	qRT-PCR; *csgD*

loc7Rv	TCAGGTAACTGGCAAGCTTTTG	qRT-PCR; *csgD*
loc8Fd	TGCCAGACAATTTCCAACGA	qRT-PCR; *Z2204*
loc8Rv	GTCCCTTTGCTGCATTCACTTA	qRT-PCR; *Z2204*
loc9Fd	TGGCGGTGATTCAGTCAGTATT	qRT-PCR; *Z3279*
loc9Rv	ACCAACCGCGCCATCATA	qRT-PCR; *Z3279*
loc10Fd	ACGGCCGAATCGTCAAAG	qRT-PCR; *Z3597*
loc10Rv	CGTGACCCTCAAATGGAAATG	qRT-PCR; *Z3597*
loc11Fd	GCGCTTATGCCTCCTCAGAA	qRT-PCR; *Z4501*
loc11Rv	CTGGTTCACCGTAACATCATCAA	qRT-PCR; *Z4501*
loc12Fd	TCTGTATTTGCTGCGGTTGGT	qRT-PCR; *Z4971*
loc12Rv	GGTATCGCAATCTTCCAGTTTGA	qRT-PCR; *Z4971*
loc13Fd	TGGTTTCGCCGCTAATGG	qRT-PCR; *Z5221*
loc13Rv	CACCGTCAGCGGACCAA	qRT-PCR; *Z5221*
loc14Fd	TGCACAGTTTCCACCACCAA	qRT-PCR; *Z5917*
loc14Rv	CCGGCAGACATCAGACTGAA	qRT-PCR; *Z5917*
loc15Fd	CGTGGCTATCGAGGGTGACT	qRT-PCR; *Z0360*

loc15Rv	CTGGGTTAATGTGTTGGTGATAAGA	qRT-PCR; *Z0360*
loc16Fd	GCACTCACCGACATGCTACAA	qRT-PCR; *Z0118*
loc16Rv	CCAGCGCGCACAACTCT	qRT-PCR; *Z0118*
Z3599 RT Fd	GGCATGCCGGATATCAACA	qRT-PCR
Z3599 RT Rv	TCACGCACGTTGAAATAGAACAC	qRT-PCR
Z3600 RT Fd	GGTGCGGTGGCCTTTG	qRT-PCR
Z3600 RT Rv	TCCCGTAGGCGGTCTCTTTA	qRT-PCR
Z3601 RT Fd	CGCGGGTATCGAACAGAAA	qRT-PCR
Z3601 RT Rv	CAGCGTCTGCTTCGCTTTG	qRT-PCR
ilvC RT Fd	TCGACAAGCTGGTGGAAGAA	qRT-PCR
ilvC RT Rv	TCCCAGCCGAACTGAATCAG	qRT-PCR
Z3595 Fd	GCATGTAGAATCAATTGGCTCACGG	RT-PCR; *loc10.5*
Z3596 Rv	AGTGGTCTGATATTGTTCAG	RT-PCR; *loc10.5*
Z3597 Fd	CCACCTGTAACGTAGCCCAT	RT-PCR; *loc10.4* and *loc10.8*
Z3598 Rv	TACCTGTCAGGTGGACGAAGAGC	RT-PCR; *loc10.4*
Z3599 Fd	TCTTCGTCCACCTGACAGGTA	RT-PCR; *loc10.3* and *loc10.7*

Z3599 Rv	AACGACGCTGGCGTTACTGA	RT-PCR; *loc10.3* and *loc10.8*
Z3600 Fd	TCATGAGGCATCGAAGGAGA	RT-PCR; *loc10.2* and *loc10.6*
Z3600 Rv	GTACTTCCCTGCTGCGTTGC	RT-PCR; *loc10.2* and *loc10.7*
Z3601 Fd	TCTGGAAGGTCGTGTTGGCT	RT-PCR; *loc10.1*
Z3601 Rv	AGCTATTGCTGCAACAATGG	RT-PCR; *loc10.1* and *loc10.6*
Z4498 Fd	GTATCAACCGTTGAAGCATC	RT-PCR; *loc11.1* and *loc11.2*
Z4499 Rv	ATCATCGCCAGCTTCAAGAC	RT-PCR; *loc11.1*
Z4500 Rv	TTAATACCCAGCTCACGTAG	RT-PCR; *loc11.2*
Z4500 Fd	TCAGGCAGATCAACAATGTC	RT-PCR; *loc11.3*
Z4504 Rv	TGCGGGTGGTAAACTTCTGA	RT-PCR; *loc11.3*

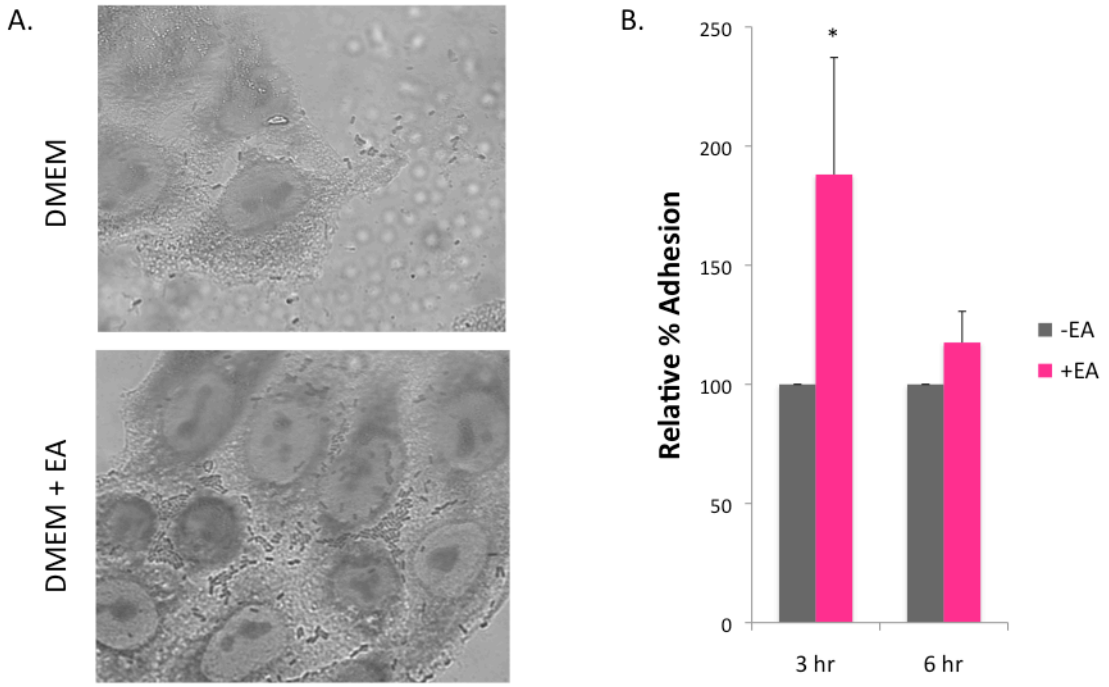

Figure 2.1 EA influences EHEC adhesion to epithelial cells. A. Adhesion assays with HeLa cells showing the different adhesion phenotypes for WT EHEC grown in the absence or presence of EA. B. Percentages of adhering bacteria when grown with or without EA. *, $p < 0.05$ as determined by Student's *t* test.

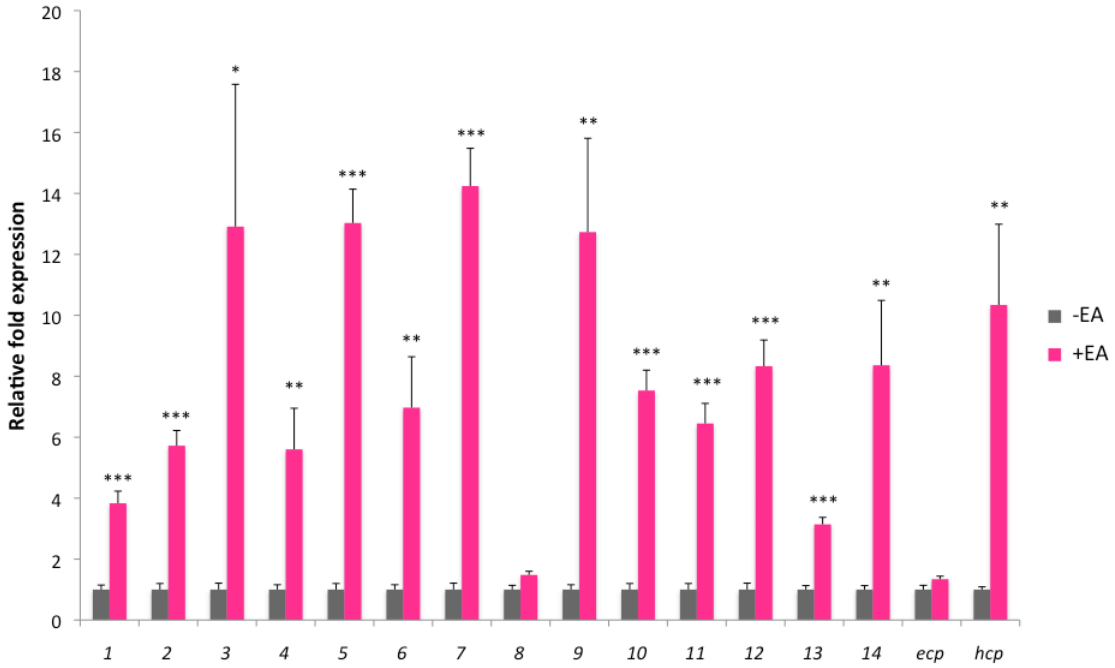

Figure 2.2 EA promotes fimbrial gene expression in EHEC. qRT-PCR of one gene from each fimbrial locus from EHEC grown to early-logarithmic growth phase in minimal media. *, p <0.05; **, p <0.01; ***, p <0.001. qRT-PCR expression values are presented as relative values compared to WT EHEC strain 86-24 grown with NH_4.

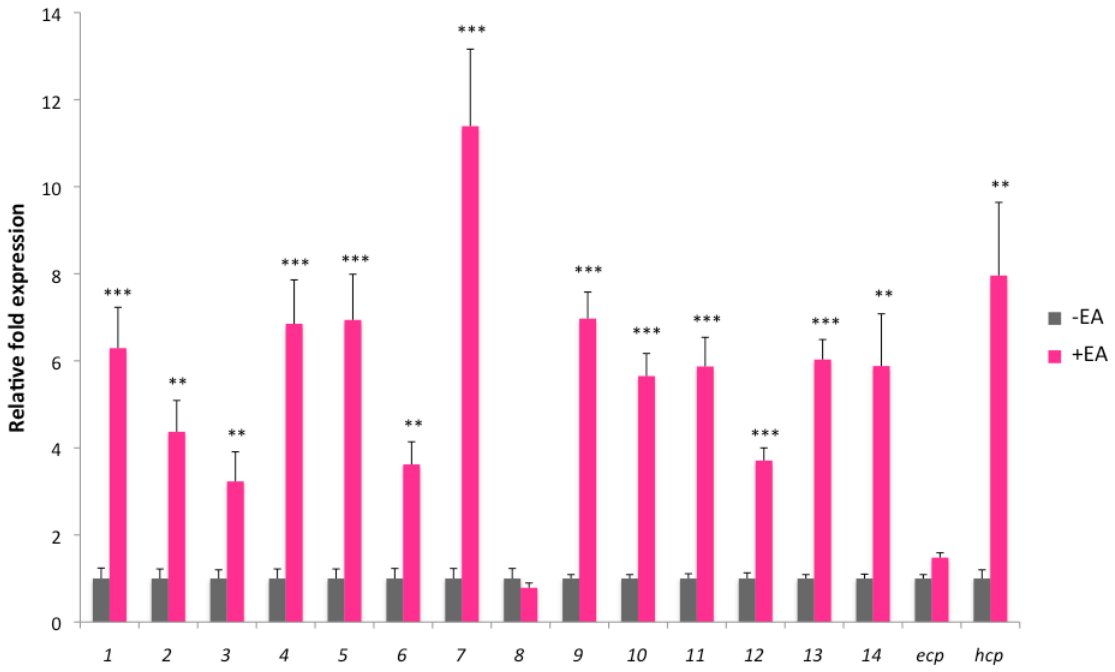

Figure 2.3 EA promotes fimbrial gene expression in EHEC. qRT-PCR of one gene from each fimbrial locus from EHEC grown to mid-logarithmic growth phase in minimal media. *, p <0.05; **, p <0.01; ***, p <0.001. qRT-PCR expression values are presented as relative values compared to WT EHEC strain 86-24 grown with NH_4.

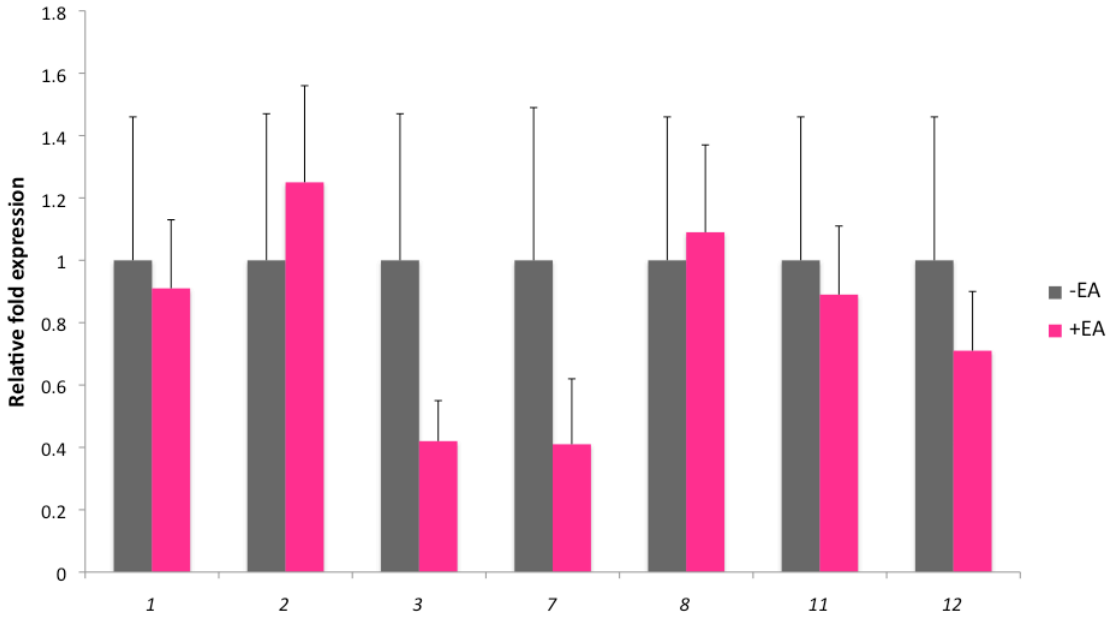

Figure 2.4 EA does not promote fimbrial gene expression in EHEC during late-logorithmic growth. qRT-PCR of one gene from each fimbrial locus from EHEC grown to late-logarithmic growth phase in minimal media. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. qRT-PCR expression values are presented as relative values compared to WT EHEC strain 86-24 grown with NH_4.

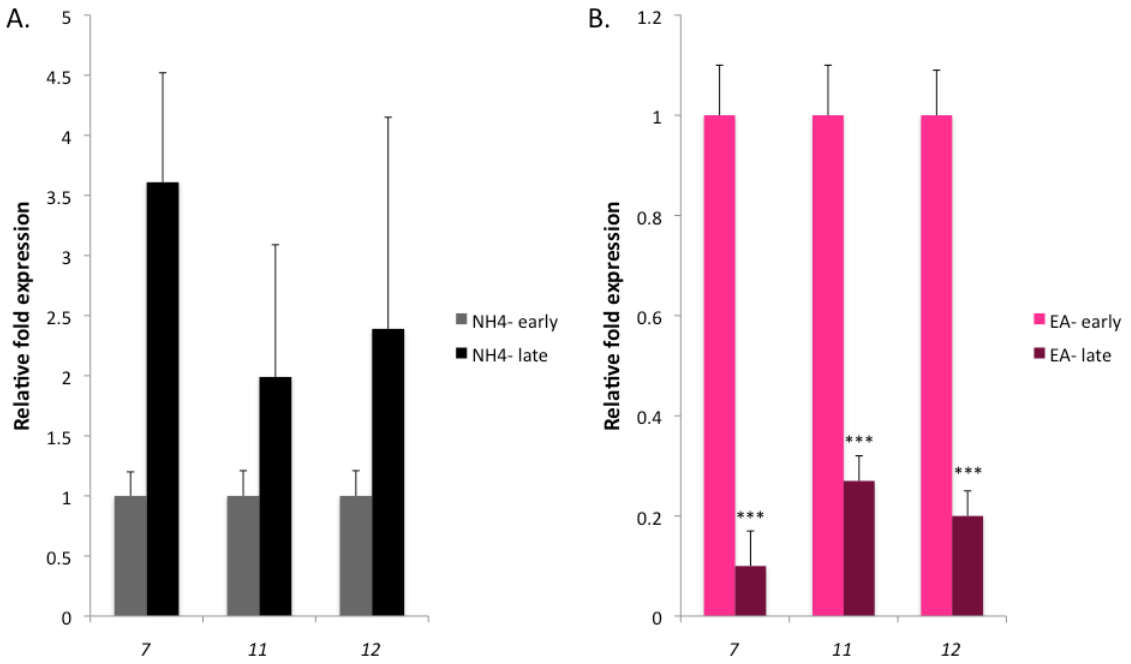

Figure 2.5 Expression of fimbrial genes is decreased in late logarithmic phase relative to early logarithmic phase during growth with EA. A. qRT-PCR comparison of indicated fimbrial loci from EHEC grown to early- or late-log in minimal medium with NH_4. B. qRT-PCR comparison of indicated fimbrial locus from EHEC grown to early- or late-log in minimal medium with EA. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$. qRT-PCR expression values are presented as relative values compared to cells grown to early-log.

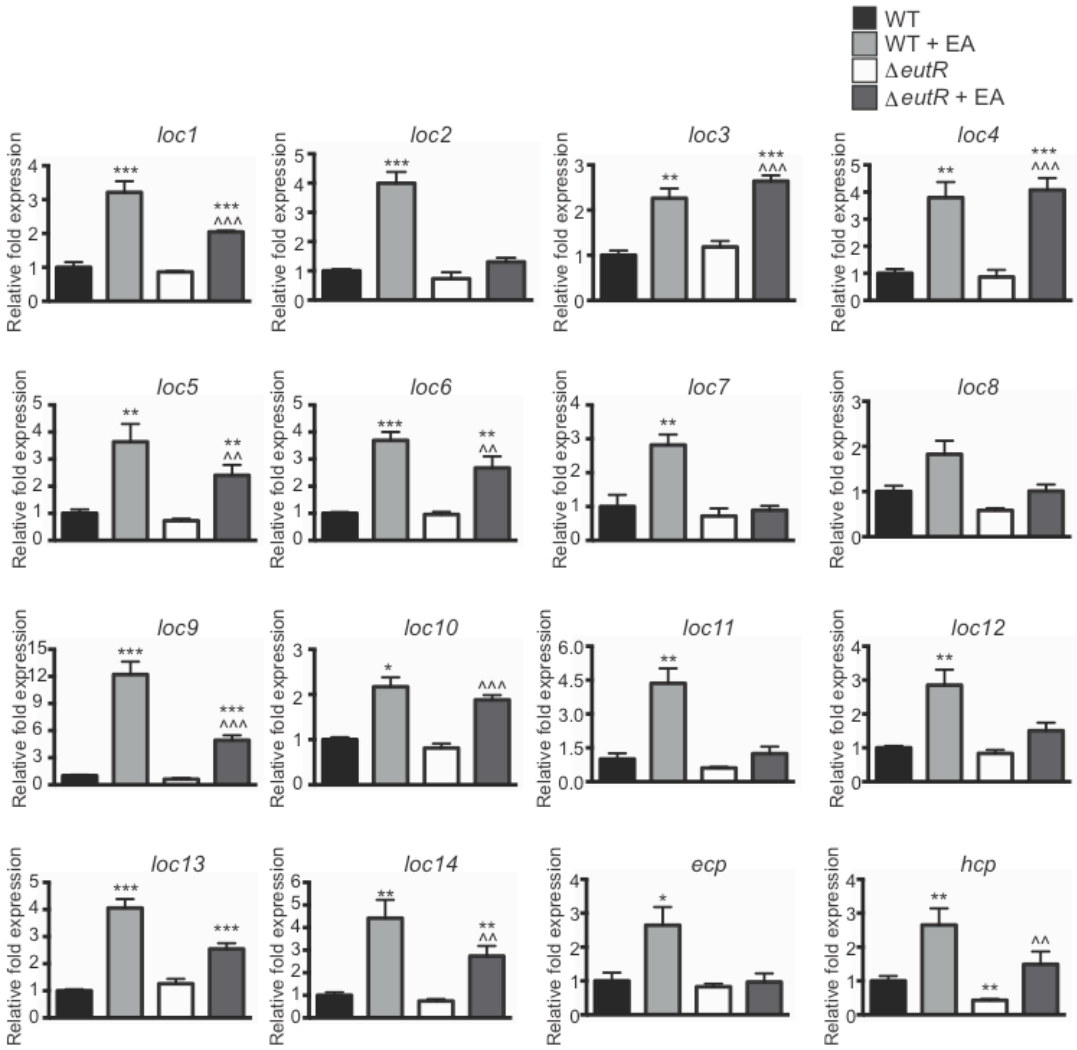

Figure 2.6 The role of EutR in fimbrial gene expression. qRT-PCR of WT EHEC and the Δ*eutR* strain grown in DMEM under anaerobic conditions with or without EA. Asterisks indicate significance in gene expression compared to WT EHEC grown in the absence of EA. *, p <0.05; **, p <0.005; ***, p <0.0005. Carets indicate significance in gene expression between the Δ*eutR* strain grown in the absence of EA and the Δ*eutR* grown in the presence of EA. ^^, p <0.005; ^^^, p <0.0005.

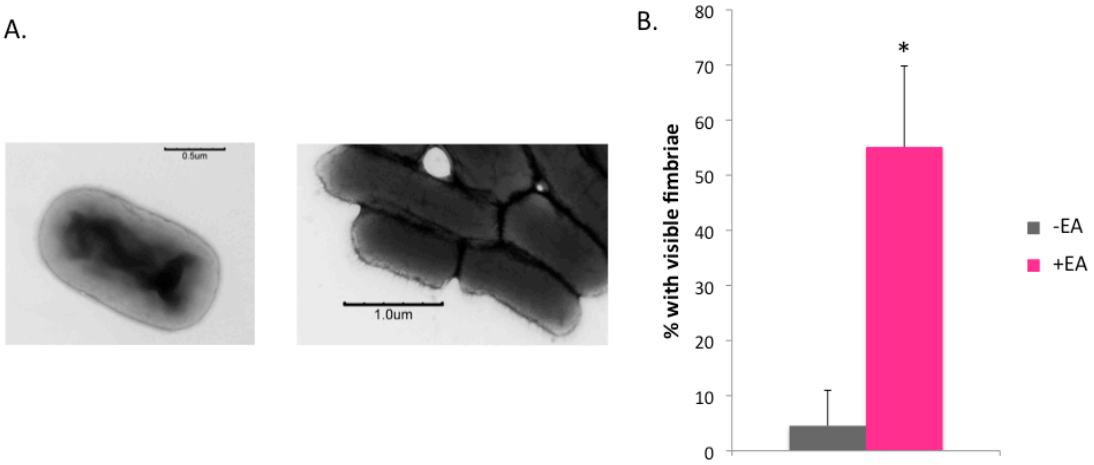

Figure 2.7 EA promotes fimbrial expression on the surface of EHEC cells. A. Electron micrograph of EHEC grown in minimal medium with NH4 (left). Original magnification, 20,000x. Electron micrograph of EHEC grown in minimal medium with EA (right). Original magnification, 10,000x. B. Percentage of cells displaying fimbriae when grown with or without EA. *, $p < 0.05$ as determined by Student's *t* test.

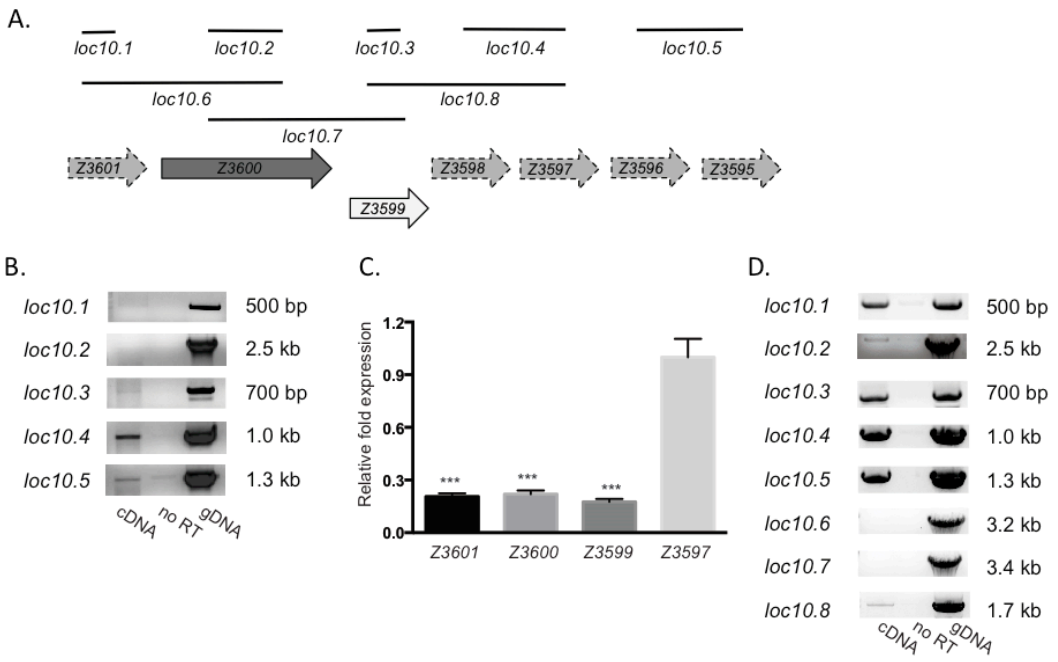

Figure 2.8 Operon mapping of *loc10*. A. Schematic representation of *loc10*. The locations of the primer pairs used in the RT-PCR analysis are indicated. The operon is coded as follows: the putative chaperone is indicated by the white arrow, the putative usher is indicated by the dark grey arrow, and the major and minor subunits are indicated by light grey, dashed arrows. B. RT-PCR showing that the putative fimbrial subunits *Z3598* and *Z3597* are co-transcribed and that *Z3596* and *Z3595* are co-transcribed. PCR products were not visualized in the negative control lanes (no RT), and genomic DNA (gDNA) was used as a positive control. C. qRT-PCR of Z3599, Z3600, and Z3601 as compared to Z3597. ***, $p < 0.0005$. D. RT-PCR with increased cycles demonstrating transcripts of *Z3599, Z3600*, and *Z3601* as well as that *Z3599-Z3597* are co-transcribed.

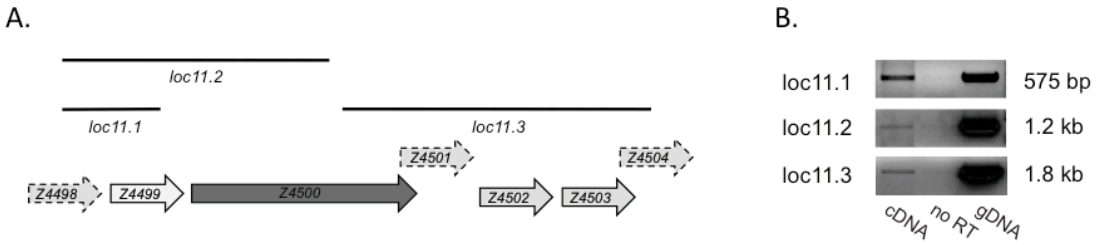

Figure 2.9 Operon mapping of *loc11*. A. Schematic representation of *loc11*. The locations of the primer pairs used in the RT-PCR analysis are indicated. The operon is coded as follows: the putative chaperone is indicated by the white arrow, the putative usher is indicated by the dark grey arrow, putative transposase is indicated by a light grey, solid arrow, and the major and minor subunits are indicated by light grey, dashed arrows. B. RT-PCR showing that all the genes encoded within *loc11* are co-transcribed. PCR products were not visualized in the negative control lanes (no RT), and genomic DNA (gDNA) was used as a positive control.

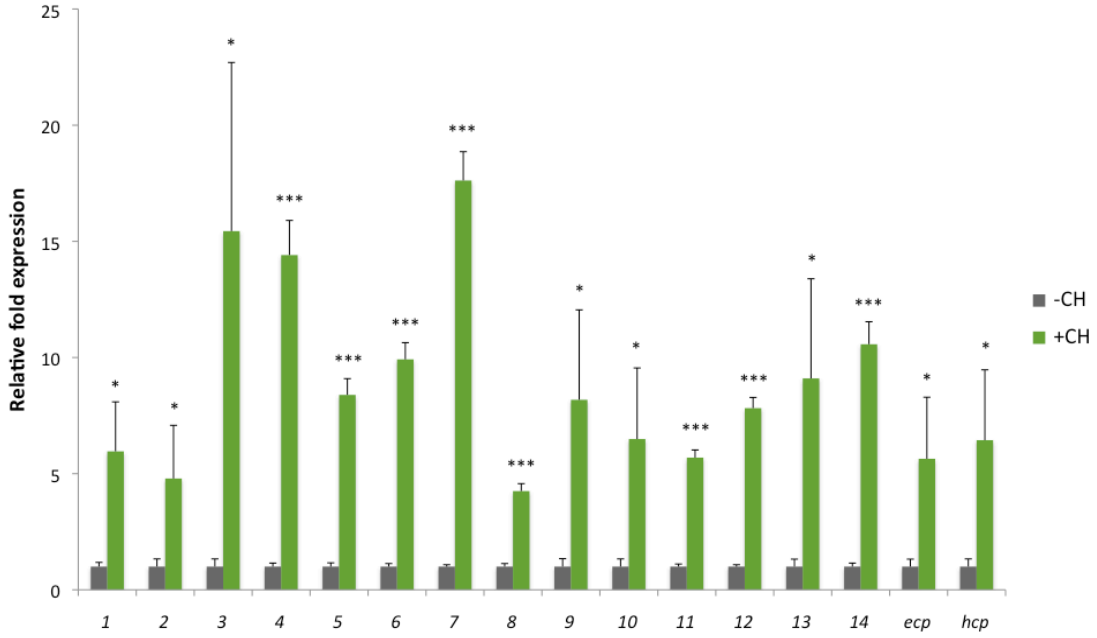

Figure 2.10 Choline promotes the expression of EHEC fimbriae in minimal media. qRT-PCR of one gene from each fimbrial locus from EHEC grown to early-logarithmic growth phase with NH_4 or choline as the sole nitrogen source in minimal medium. *, p <0.05; **, p <0.01; ***, p <0.001. qRT-PCR expression values are presented as relative values compared to WT EHEC strain 86-24 grown without choline.

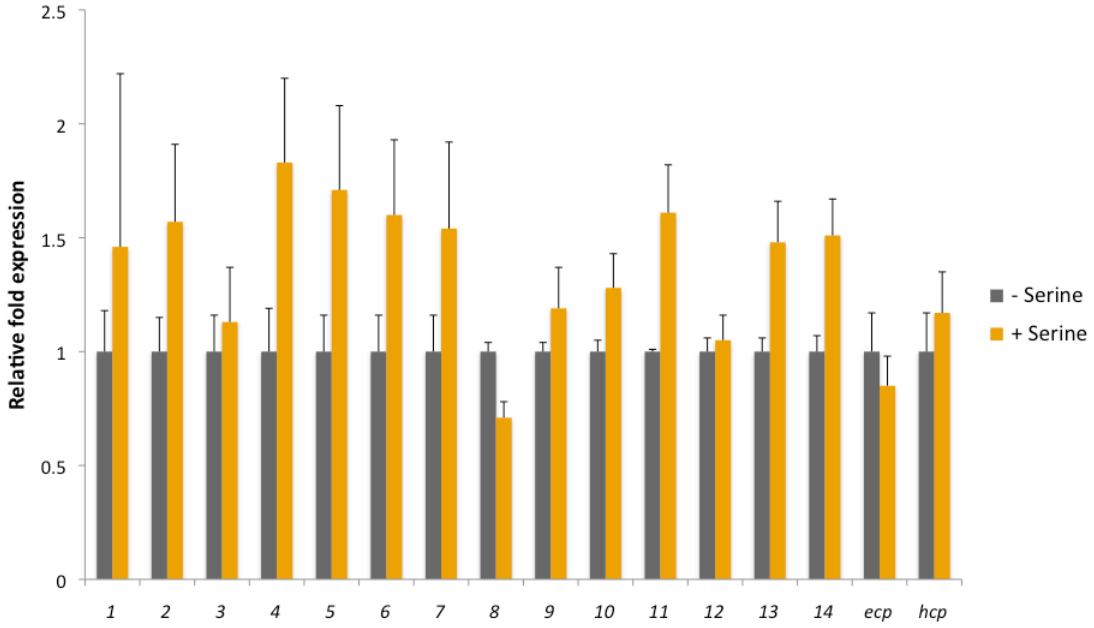

Figure 2.11 Serine does not promote the expression of EHEC fimbriae. qRT-PCR of one gene from each fimbrial locus from EHEC grown to early-logarithmic growth phase with NH_4 or serine as the sole nitrogen source in minimal medium. qRT-PCR expression values are presented as relative values compared to WT EHEC strain 86-24 grown without serine.

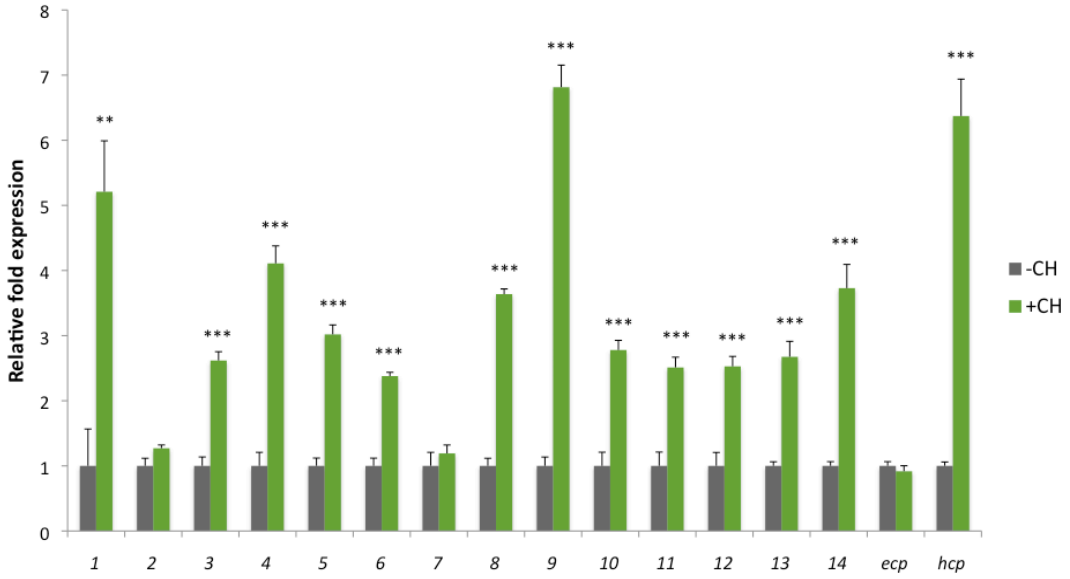

Figure 2.12 Choline promotes the expression of EHEC fimbriae in DMEM. qRT-PCR of one gene from each fimbrial locus from EHEC grown in DMEM in the presence or absence of choline. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. qRT-PCR expression values are presented as relative values compared to WT EHEC strain 86-24 grown without choline.

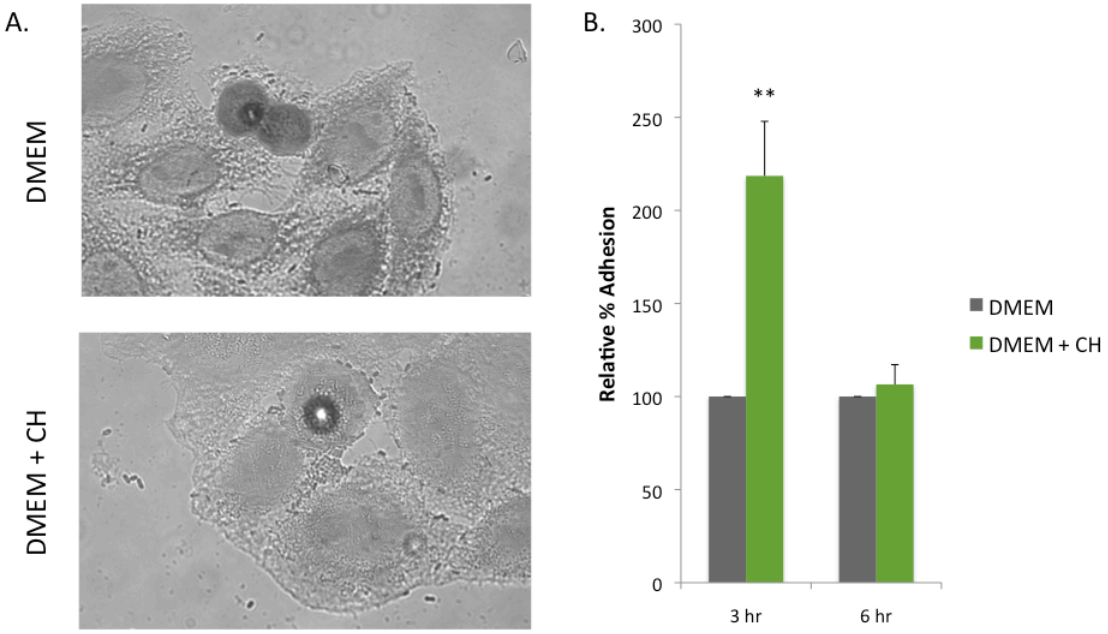

Figure 2.13 Adherence of EHEC to epithelial cells in the presence of choline. A) Adhesion phenotype for WT EHEC grown in the presence of choline. B) Percentage of total bacteria adherent to epithelial cells when grown in the presence of choline relative to the absence of choline. *, $p < 0.05$ as determined by Student's *t* test.

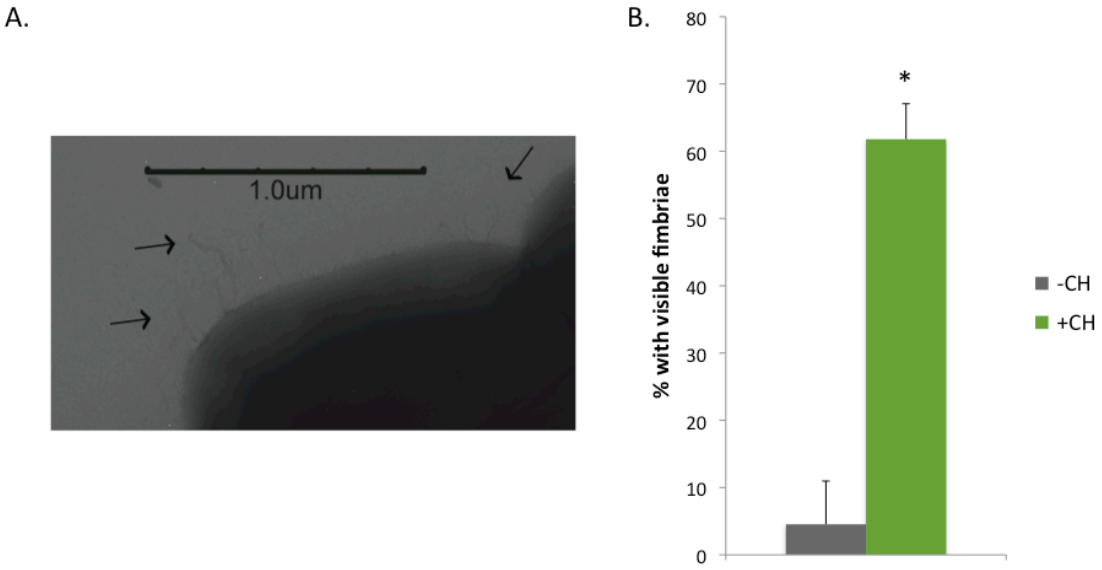

Figure 2.14 Fimbriae are expressed when EHEC is grown with choline. A) Fimbrial structures on the surface of EHEC grown with choline. Original magnification, 15,000x. Arrows indicate choline-induced fimbriae. B) Percentage of cells producing fimbriae when grown without or with choline. *, P <0.05 as determined by Student's t test.

Chapter Three

Ethanolamine-regulated fimbriae are more than just adhesins and control subsequent pathogenic steps

Abstract

The foodborne pathogen enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 responds to the host derived molecule ethanolamine (EA) to activate expression of fimbriae. Fimbriae are surface-exposed structures involved in bacterial adherence; in the context of EHEC pathogenesis, they may mediate early attachment to gastrointestinal (GI) epithelial cells. This study sought to characterize the contribution of two EA-regulated fimbriae, Erf1 and Erf2, to EHEC attachment in the GI tract. Erf1 and Erf2 were not essential for early attachment to epithelial cells *in vitro*; however, these two EA-regulated fimbriae directed the expression of other important EHEC virulence determinants, including Shiga toxin and the locus of enterocyte effacement (LEE). While there was no role for Erf1 and Erf2 in EHEC early adhesion, both were required for the later stages of EHEC adhesion through the formation of AE lesions. Additionally, Erf1 is critical for robust EHEC colonization of the murine GI tract. Our findings demonstrate a novel role for fimbriae in pathogenesis, beyond adhesion, as regulators of virulence.

Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 is a food-borne pathogen that causes hemorrhagic colitis and the fatal complication hemolytic uremic syndrome (HUS). EHEC has a very low infectious dose; less than 100 CFUs are required to cause disease [41]. This low dose, coupled with limited treatment options due to contraindication of antibiotic use, emphasizes the critical importance of studying factors involved in EHEC colonization [36, 39, 40]. Fimbriae are surface-exposed, proteinaceous structures that function in attachment to surfaces and are hypothesized to mediate early attachment of EHEC to epithelial cells in the gastrointestinal (GI) tract, but their contribution to pathogenesis is not well understood. Fourteen of the 16 EHEC fimbriae are assembled through the chaperone-usher pathway, which is the largest and most conserved class of fimbriae in Gram-negative bacteria [5]. The required subunits for chaperone-usher fimbrial assembly are a chaperone, an usher, and a major subunit, but some loci also encode other components, including tip adhesins and regulators [8].

Later EHEC adhesion events involve intimate attachment to the GI epithelium through the formation of attaching and effacing (AE) lesions. AE lesions are characterized by an effacement of the microvilli, an intimate receptor-mediated attachment of EHEC to host epithelial cells, and the formation of an actin-rich pedestal through manipulation of the host cell actin cytoskeleton. Many of the genes required for AE lesion formation are encoded in the locus of enterocyte effacement (LEE). The LEE encodes intimin (*eae*), which is the bacterial receptor for intimate

attachment, the translocated intimin receptor (Tir), a type III secretion system (T3S), and effectors that contribute to colonization and disease manifestation.

Shiga toxin (Stx) and the LEE are the two defining virulence traits for EHEC [25]. Stx inhibits protein synthesis in target cells and contributes to local damage in the GI tract that manifests as bloody diarrhea, hemorrhagic colitis, necrosis, and intestinal perforation [43, 48, 49]. The major receptor for Stx is the glycolipid Gb3, and the kidneys are susceptible to intoxication due to high levels of Gb3 expression on kidney epithelial cells [46, 47]. HUS is caused by the effects of Stx intoxication in the kidneys.

Ethanolamine (EA) is a signal that EHEC has co-opted to recognize the host environment and upregulate virulence genes [81]. EA is a component of both bacterial and mammalian cell membranes and is abundant in the GI tract due to constant cell turnover and the diet [110-112]. Expression of the LEE, Stx, and fimbriae are increased when EHEC is grown in the presence of EA in comparison to the absence [81, 149]. EHEC adherence to epithelial cells is increased during growth in the presence of EA in comparison to growth in the absence of EA, and growth in EA promotes a clustering phenotype by EHEC cells that correlates with the increase in fimbrial expression [149]. The goal of this study was to identify and characterize specific fimbrial loci that mediate EA-responsive adhesion in EHEC. We selected two fimbrial loci, *erf1 (loc9)* and *erf2 (loc2)*, to investigate based on their high level of expression during growth in EA. Here we show that Erf1 and Erf2 play an essential

role in EHEC pathogenesis, independent of adhesion, through modulating the expression of genes involved in toxin production and AE lesion formation.

Materials and Methods

Strains and growth conditions

The strains used in this study are described in Table 3.1. EHEC cultures were grown at 37˚C with shaking in LB, low glucose Dulbecco's modified Eagle's medium (DMEM; Invitrogen), or M9 minimal medium [129] prepared without addition of a nitrogen source to the minimal salts. The nitrogen sources used in this study were EA (ethanolamine hydrochloride, Sigma) and NH_4Cl (Sigma). Vitamin B12 (cyanocobalamin) was supplemented at 150 nM into the media whenever EA was used. EHEC strains were grown aerobically in M9 minimal media supplemented with 2 mM EA at 37˚C with shaking until early logarithmic phase (optical density at 600nm (OD_{600}) of 0.2) for RT-PCR. For all other experiments, EHEC strains were grown in DMEM supplemented with 10 mM EA without shaking for 6 hours at 37˚C . Streptomycin was added to overnight cultures of EHEC at a final concentration of 50 μg/ml.

Construction and complementation of deletion strains

Deletion strains were constructed using λ-Red as previously described [150]. To create the Δ*erf1*strain, *erf1*_LRF and *erf1*_LRR primers were used to delete *Z3279-Z3276*. The Δ*erf2* strain was created using *erf2*_LRF and *erf2*_LRR primers to delete *Z0152-Z0146*. The kanamycin (Δ*erf1*) and chloramphenicol (Δ*erf2*) cassettes were resolved using pCP20 [150].

To complement the fimbrial mutants, *erf1* and *erf2* constructs were amplified using JumpStart Accutaq LA DNA polymerase reagents (Sigma) using the primers indicated in Table 3.2. Both amplified products and pGEN-MCS (Addgene) were digested with BamHI and EcoRI then ligated overnight. Ligations were transformed and screened in *E. coli* DH5-alpha, and then pGEN-*erf1* and pGEN-*erf2* were electroporated into Δ*erf1* and Δ*erf2*, respectively.

RNA extraction, RT-PCR, and qRT-PCR

EHEC strains were grown aerobically in LB overnight at 37˚C with shaking and then diluted 1:100 in DMEM as previously indicated. Bacterial cells from three biological replicate cultures were pelleted and suspended in Trizol. RNA was extracted using the RiboPure bacteria kit (Ambion) following manufacturer's instructions.

For RT-PCR, SuperScript II reverse transcriptase (Invitrogen) and random primers (Invitrogen) were used to synthesize cDNA. A reaction that lacked reverse transcriptase was used as a negative control and genomic DNA was used as a

positive control. PCR was preformed on the cDNA template using JumpStart Accutaq LA DNA polymerase reagents (Sigma). Standard gel electrophoresis methods were used.

Primers used in real-time qPCR assays were designed using Primer Express v1.5 (Applied Biosystems) and validated before use. Reaction mixtures were prepared as previously described [151]. qRT-PCR was performed using a one-step reaction in an ABI 7500-FAST sequence detection system (Applied Biosystems). All data were normalized to the levels of *rpoA* and analyzed using the comparative cycle threshold (C_T) method (manual). The relative quantification method was used to determine the expression level of target genes in various growth conditions. Statistical significance was determined by an unpaired *t*-test, and a p value of ≤0.05 was considered significant.

SDS-PAGE and immunoblotting

Secreted proteins were harvested as previously described [132]. Whole cell lysates were prepared from cells grown for 6 hours in DMEM at 37°C without shaking. SDS-PAGE and immunoblotting were performed as previously described [129]. Samples were probed by Western blot analysis using polyclonal antisera to EspA, monoclonal antisera to Stx2a (Santa Cruz Biotechnology), or monoclonal antisera to RpoA (Neoclone).

Adhesion Assays

Adhesion assays were performed as previously described [132]. Briefly, EHEC strains were grown aerobically in LB overnight at 37 °C without shaking. HeLa cells were washed before infection and placed in low glucose DMEM without antibiotics. The bacterial overnight cultures were diluted 1:100 to approximately 1.0 x 10^9 colony forming units (CFUs)/ ml to infect the HeLa cells. HeLa cells were incubated with the bacteria for 3 hours at 37 °C in 5% CO_2 in the presence of 10 mM EA. The coverslips were washed, the HeLa cells were lysed with Triton-X 100 (Sigma), and then bacterial cells were plated to determine CFU/ml. Adherence is presented as the percentage of the indicated fimbrial mutant that adhered to epithelial cells in the presence of EA relative to WT.

Fluorescein actin staining (FAS) assays

FAS assays were performed as previously described [152]. Briefly, overnight bacterial cultures were grown aerobically in LB overnight at 37˚C without shaking. HeLa cells were washed before infection and placed in low glucose DMEM without antibiotics. The bacterial overnight cultures were diluted 1:100 to infect the HeLa cells. HeLa cells were incubated with the bacteria for 6 hours at 37˚C in 5% CO_2 in the presence and absence of 10 mM EA. The coverslips were then washed, fixed with formaldehyde, permeabilized with 0.2% Triton, and stained. Fluorescein isothiocyanate-labeled phalloidin was used to visualize actin accumulation and

propidium iodide was used to visualize bacteria. For quantification, 400 epithelial cells (100 each from 4 separate experiments) were assessed for the number of AE lesions per epithelial cell.

Animal infection studies

Animal infections were performed as described previously [153, 154] and were approved by the University of Virginia Animal Care and Use Committee. Male, 5 to 6 week old CD-1 mice (Harlan) were given streptomycin-treated water 24 hours prior to infection. Bacteria were grown in LB overnight and then washed once with PBS. Mice were infected by oral gavage with 200 ul containing approximately 4 x 10^8 CFUs of each strain (8 x 10^8 CFUs total). An EHEC 86-24 *ΔlacZ* strain was used as the WT strain in order to differentiate between WT and mutant colonies by pink/white screening on MacConkey agar. The *ΔlacZ* strain and WT EHEC colonize equivalently [86]. Fecal samples were collected daily from each mouse, diluted, and plated on MacConkey Agar (Sigma) with added streptomycin. Mice were monitored for weight loss and signs of morbidity. Random colonies were PCR-screened to verify that isolated bacteria were EHEC. Competitive indices were calculated using the following formula: CI= (CFUs of mutant bacteria shed in feces/CFUs of WT bacteria shed in feces)/(CFUs of mutant bacteria in inoculum/CFUs of WT bacteria in inoculum). A Wilcoxon signed-ranks test was used to determine statistical significance using 1.0 as a theoretical mean.

Results

Characterization of erf1 and erf2

EA is a potent signal for EHEC fimbrial transcription [149]. Genes encoded in 15 of the 16 EHEC fimbrial loci are upregulated during growth with EA in comparison to growth without EA [149]. To better understand how EA-regulated fimbriae contribute to EHEC pathogenesis, we selected two fimbrial loci for further investigation based on expression levels during growth in EA.

During static growth of EHEC in DMEM, *Z3279* from *loc9* was the most highly induced fimbrial gene during growth with EA in comparison to growth without EA [149]. Because of the robust induction in expression during growth with EA, we characterized *loc9*, which encodes a putative chaperone-usher pathway fimbriae. We used RT-PCR and qRT-PCR to confirm that all genes encoded in *loc9* were expressed during growth in EA. RT-PCR demonstrated that the genes are expressed as two transcripts: *Z3279-Z3278* (fimbrial subunit and chaperone) and *Z3277-Z3276* (usher and fimbrial subunit) (Figure 3.1A and B). qRT-PCR measured equivalent expression of all four genes, suggesting that they are regulated together (Figure 3.1C). These results demonstrate that all subunits in *loc9* are expressed during growth in EA and suggests that *loc9* encodes a functional fimbrial structure.

Additionally, we compared the relative expression levels of one fimbrial gene from each locus compared to the transcript level of *Z0024* from *loc1*. *Z0146* from *loc2* was the most highly expressed fimbrial gene with a 46-fold increase in

expression compared to *Z0024* and a 3.8-fold increase in expression from the second most highly expressed gene (*Z0360*). Because of the significantly higher expression level of *loc2* during growth with and without EA in comparison to other fimbrial loci, we further characterized *loc2,* which encodes a putative chaperone-usher pathway fimbriae. RT-PCR indicated that the genes are transcribed in three units: *Z0152-Z0151* (fimbrial subunit and chaperone), *Z0150* (usher), and *Z0149-Z0146* (four fimbrial subunits) (Figure 3.2A and B). The qRT-PCR data showed an increase in transcript level for the fimbrial subunits *Z0149* and *Z0146* compared to other genes in *loc2* (Figure 3.2C), which indicates that additional promoters may exist within the Z0149-Z0146 transcript. However, all subunits in *loc2* are expressed during growth in EA and could form a functional fimbrial structure. These two fimbrial loci were renamed ethanolamine-regulated fimbriae 1 (*erf1, loc9*) and 2 (*erf2, loc2*). To study the contribution of *erf1* and *erf2* to EHEC adhesion and pathogenesis, we constructed targeted deletions of the entire *erf1* and *erf2* operons.

Erf1 and Erf2 influence adherence to epithelial cells

Next, we investigated whether Erf1 and Erf2 mediate early adhesion to epithelial cells. We used an adhesion assay at an early time point to quantify fimbrial-mediated adherence before significant AE lesion formation occurs under our conditions. We did not observe any significant differences in early adhesion in the Δ*erf1* and Δ*erf2* strains in comparison to WT during growth in EA (Figure 3.3).

We performed a fluorescein actin staining (FAS) assay to further determine whether fimbrial mutants were defective in later adherence to epithelial cells in the presence of EA. This assay is used to quantify AE lesion formation through staining of the actin-rich pedestal. We measured AE lesion formation in WT, Δ*erf1*, and Δ*erf2* strains, and both Δ*erf1* and Δ*erf2* formed significantly fewer AE lesions than WT during growth in EA, indicating that both Erf1 and Erf2 promote AE lesion formation (Figure 3.4). We were surprised that Erf1 and Erf2 impacted AE lesion formation but not the prototypical early adhesion associated with fimbrial expression. It is possible that other EHEC fimbriae function redundantly with Erf1 and Erf2 in the mutant strains to mediate early adherence.

Erf1 and Erf2 direct T3S, fimbrial, and toxin expression

Our data suggest that Erf1 and Erf2 have additional, non-redundant functions beyond adherence that impact AE lesion formation. To investigate further, we probed whether Erf1 or Erf2 altered expression of genes involved in AE lesion formation. We performed qRT-PCR on representative genes encoded within the LEE (Figure 3.5). In the Δ*erf1* strain, expression of *ler (LEE1)*, *grlA*, *escC (LEE2)*, *escV (LEE3)*, *eae (LEE5)*, and *espA (LEE4)* was significantly decreased compared to expression in WT (Figure 3.5B). This explains the defect in AE lesion formation in Δ*erf1* and suggests that Erf1 promotes the expression of genes encoded in all operons of the LEE in WT EHEC. In the Δ*erf2* strain, expression of *escC (LEE2)* and

espA (LEE4) was significantly reduced when compared to expression in WT (Figure 3.5C). This suggests a different mechanism for Erf2 LEE regulation in WT EHEC compared to Erf1 regulation, since Erf1 is able to influence expression of all tested genes in the LEE while Erf2 only promotes expression of *escC* and *espA*. Repression of EspA in Δ*erf1* and Δ*erf2* was confirmed by Western blot analysis (Figure 3.5D). The Δ*erf1* and Δ*erf2* strains were complemented by expression of pGEN-*erf1* and pGEN-*erf2,* respectively. Expression of *espA* was detected by qRT-PCR and EspA probed through Western blotting was returned to WT levels in complemented Δ*erf1* and Δ*erf2* strains, confirming that Erf1 and Erf2 are specifically mediating the LEE expression phenotypes observed (Figure 3.6).

Since Erf1 and Erf2 were able to alter LEE gene expression, we next wanted to test whether they more broadly impact adherence by influencing the expression of other EHEC fimbriae. It has previously been suggested that expression of alternative EHEC fimbriae are overexpressed and can complement adhesion in fimbrial mutant strains [101]. This would suggest that fimbrial expression may be increased in the Δ*erf1* and Δ*erf2* strains in order to complement fimbrial adhesion. Conversely, we found that expression of genes encoded in *erf2*, *loc5*, *loc6*, *csg*, *loc10*, *loc11*, *lpf1*, *loc14*, and *ecp* was significantly decreased in Δ*erf1* compared to WT (Figure 3.7). This indicates that Erf1 induces the expression of nine EHEC fimbrial loci. In Δ*erf2*, expression of *erf1* and *ecp* was significantly reduced in comparison to WT, demonstrating that Erf2 influences EHEC fimbrial expression but to a lesser extent than Erf1 (Figure 3.8).

We continued to examine the role of Erf1 and Erf2 in EHEC virulence gene regulation by probing for Shiga toxin (Stx) expression. Stx is an important EHEC virulence determinant and the cause for fatality due to EHEC infection. We determined whether Erf1 and Erf2 alter Stx expression using qRT-PCR and Western blot analysis. Transcription of *stx2a* was significantly increased in Δ*erf2* in comparison to WT, and *stx2a* expression was not significantly different between WT and Δ*erf1* (Figure 3.9A). Western blotting for Stx2a supported the qRT-PCR data, showing an increase in Stx expression in Δ*erf2* but not Δ*erf1* in comparison to WT (Figure 3.9B). These results suggest that Erf2 in WT EHEC represses Stx expression. Together, our findings identify a new role for Erf1 and Erf2 in regulation of gene expression and suggest that these fimbriae act through distinct signaling pathways.

Role of Erf1 and Erf2 in vivo in a murine infection model

Because Erf1 and Erf2 had broadly altered EHEC virulence gene expression, we hypothesize that they impact host colonization *in vivo*. We performed *in vivo* competition assays to determine the ability of Δ*erf1* and Δ*erf2* to colonize the murine GI tract. Mice were infected with WT Δ*lacZ* and Δ*erf1* or Δ*erf2* at a 1:1 ratio. We measured colonization through bacterial shedding by counting colony forming units in fecal samples collected throughout infection. Using these data, we quantified the relative colonization of the two competing strains using pink/white colony screening and determined the colonization fitness of Δ*erf1* or Δ*erf2* relative to WT.

When infected with equal amounts of WT and Δ*erf1* bacteria, significantly more WT bacteria than Δ*erf1* were isolated from the feces of infected animals starting at day 7 post-infection (Figure 3.10A). These findings indicate that Erf1 contributes significantly to EHEC colonization of the murine gastrointestinal tract. At the day of euthanasia, the competitive indices of WT and Δ*erf2* in the feces and in the colon were not significantly different (Figure 3.11A and B), supporting that fecal shedding reflects the relative colonization abilities of WT and Δ*erf2* in the colon.

While there was a trend towards a decrease in colonization by Δ*erf2* in comparison to WT on days 9 and 11, the difference in fitness was not statistically significant (Figure 3.10B). Interestingly, more Δ*erf2* than WT bacteria were isolated from the feces on days 1 and 3 (Figure 3.10B). This could indicate that Δ*erf2* adhered less well than WT and was shed more easily during the first stages of colonization. Alternatively, Δ*erf2* could be initially colonizing the GI tract more effectively than WT, suggesting that Erf2 actually hinders early colonization. Fecal shedding reflected colon colonization when mice were euthanized at 12 days post infection but not 14 days (Figure 3.11C and D). The significant difference at 14 days appeared to be due to inconsistency between the colon and feces in one mouse, and the fecal shedding and colon colonization were consistent in the other seven mice (Figure 3.11C and D).

Discussion

Both Gram-positive and Gram-negative bacteria encode fimbriae to mediate attachment and increase bacterial persistence in target niches [11, 155, 156]. We demonstrated that Erf1 and Erf2 are dispensable for initial adherence to epithelial cells. The adhesion assay utilized measures the total bacterial population adhered to epithelial cells but does not distinguish how the bacteria are adhered. Fimbriae may mediate interactions between epithelial cells and bacterial cells, between multiple bacterial cells, or both; also, multiple fimbriae may be expressed simultaneously that individually mediate one of these interactions. A cluster of cells may result in only one CFU if not mixed thoroughly during epithelial cell lysis, which may underestimate the number of bacteria adhered. Even with these limitations, we postulate that our inability to detect significant changes in adherence between the WT, Δ*erf1*, and Δ*erf2* strains suggests that Erf1 and Erf2 do not individually contribute to early, fimbriae-mediated adherence to epithelial cells under the described conditions. However, Erf1 and Erf2 are critical for later attachment to epithelial cells through the formation of AE lesions.

Erf1 and Erf2 direct T3S, AE lesion formation, fimbrial expression, and toxin production at the transcriptional level through distinct, uncharacterized signaling pathways. Erf1 mediates increased expression of the entire LEE, likely through Ler, and Erf2 contributes to increased expression of only *LEE2* and *LEE4* (Figure 3.5). *LEE2* and *LEE4* encode many of the structural genes required for T3S [157], and regulation of only these two LEE operons by Erf2 may allow for controlled, increased expression of structural proteins independent of effector proteins. If the

Erf1 and Erf2 signaling pathways were active in the same cell, this would allow for increased expression of the entire LEE with a larger induction of the T3S structural genes, which may be important for activating T3S to transition between early and late adhesion.

Outside of the LEE, Erf1 increases fimbrial expression to a greater extent than Erf2, and Erf2 decreases Shiga toxin expression. We do not yet understand the mechanisms for the dissimilar gene regulation phenotypes or the relative contributions of these two pathways in WT EHEC to pathogenesis. Since Erf1 and Erf2 are both induced in our experimental conditions, we postulate that they may be expressed simultaneously and that the sum of these signaling pathways aids in colonization of the GI tract.

We observed decreased shedding of the Δ*erf1* strain in comparison to WT EHEC when infected at a 1:1 ratio in our murine model. Fecal shedding has previously been demonstrated to reflect EHEC colonization in Streptomycin-treated mice [153, 154]. To confirm that shedding reflects colonization, bacterial load in the GI tract was quantified at time of euthanasia. Because these analyses are limited to the terminal time point, we rely on shedding as an output for colonization throughout infection. As colonization progresses, strains that weakly adhere will be cleared with the flux of intestinal contents and lost from the adherent population, which should be reflected in reduced shedding over time. Decreased shedding could also indicate more efficient adherence, especially at earlier time points before colonization is firmly established. Since we observed a consistent decrease in

shedding of the Δ*erf1* strain in comparison to WT EHEC on days 7-11, we hypothesize that Erf1 promotes EHEC colonization in our model.

Although we confirmed that the WT, Δ*erf1*, and Δ*erf2* strains grew at equivalent rate *in vitro* under several conditions, it is possible that they may grow or survive differently in the host. Although we hypothesize that the difference in fitness observed between WT and *erf1* is due to a decreased ability of *erf1* to colonize the GI tract, we cannot exclude that other factors may impact bacterial survival and shedding. Some factors, differences in immune activation or tissue damage for example, are controlled for by inoculation of the two strains into the same animal. Since this model has been established to measure EHEC colonization, we still hypothesize that our findings support a role for Erf1 in colonization of the murine GI tract.

The increased colonization fitness mediated by Erf1 in the WT strain may be through increased fimbrial attachment or altered virulence gene expression. Although we did not observe defects in fimbrial attachment to epithelial cells in our *in vitro* system, Erf1 and Erf2 could bind target molecules present in the murine gastrointestinal tract but absent in the HeLa cells used in our *in vitro* studies. AE lesion formation has not been demonstrated in murine infection models of EHEC and may not contribute to the differences in colonization fitness observed in our study [158]. However, the alterations in expression of T3S effectors, intimin, fimbrial adhesins, and Stx could influence EHEC colonization independent of AE lesion formation. Regardless of whether the effects are direct or indirect, Erf1 is

critical for EHEC colonization fitness in the mammalian GI tract, which has not previously been demonstrated for other characterized EHEC fimbriae.

Both fimbriae and the LEE are colonization factors, however the role of Stx in colonization is unclear. Stx causes significant local damage in the GI tract through increased immune cell recruitment and alteration of epithelial barrier function [43]. Stx-mediated damage to the epithelium during the early stages of infection could interfere with EHEC colonization, leading to decreased bacterial persistence. The damage and inflammation caused by Stx is thought to be more important for later dissemination to new hosts through onset of diarrhea [43]. Our findings indicate that Erf2 reduces Stx expression in WT EHEC. The simultaneous increase in colonization factors and decrease in toxin production through Erf2 may aid in initial colonization.

The prototypical role for chaperone-usher fimbriae in bacterial pathogenesis is to mediate initial attachment to host tissue. The observation that EA-regulated fimbriae influence expression of virulence genes required for later stages of EHEC infection expands on the classical function for fimbriae in pathogenesis. Increasing fimbrial and LEE expression during early interactions with host tissue may enhance bacterial adherence and increase persistence. We hypothesize that linking the initial steps in colonization with expression of virulence determinants critical for later stages of infection is essential for coordinated regulation of virulence. Our findings suggest a novel role for fimbriae in pathogenesis that warrants further investigation

to determine how these fimbriae alter the expression of genes required for subsequent steps in pathogenesis.

Table 3.1 Strains used in this study		
Strain name	Description	
EHEC		
86-24	Wild-type	[57]
Δ*erf1*	Deletion of *Z3276yehBCD*	This study
Δ*erf2*	Deletion of *yadNecpDhtrEyadMLKC*	This study
Δ*lacZ*	Deletion of *lacZ*	[86]
Δ*erf1* pGEN	Empty vector control	This study
Δ*erf2* pGEN	Empty vector control	This study
Δ*erf1* pGEN-*erf1*	Complementation with *Z3276yehBCD*	This study
Δ*erf2* pGEN-*erf2*	Complementation with *yadNecpDhtrEyadMLKC*	This study

Table 3.2 Oligonucleotide primers used in this study

Primer name	Primer sequence	Primer use; gene
erf1_LRF	ATCATGAAACATTCAATTATTGCTGTCGCTGTCTTATCT TCTGTATTTATGTGTAGGCTGGAGCTGCTTC	Δ*erf1*
erf1_LRR	TTCTTATTATTAGCCACTTGCTCATCTTGCTTGTTATTAA TCGTATTTCACATATGAATATCCTCCTTAG	Δ*erf1*
erf2_LRF	AGGATGCATGTAATGAAAAAAGCACTTCTCGCAGCCGCT CTGGTTATGGCTTGTGTAGGCTGGAGCTGCTTC	Δ*erf2*
erf2_LRR	CGTTTTACTTATTCGTAGGTAAAGGAGAAGGTCGCGTTA CCTGAAAATGTTCCAACACCATATGAATATCCTCCTTAG	Δ*erf2*
erf1_CF	CTAGGAATTCTTGCCAACACCGTTTTAAGCAT	Δ*erf1* complement

erf1_CR	CTAGCCTAGGGATCAGCGACACCGACGGTA	Δ*erf1* complement
erf2_CF	CTAGGAATTCAGCACTTCATGCAAATAGATTAGGC	Δ*erf2* complement
erf2_CR	CTAGCCTAGGAGCAGAACTCTGGTGCGATG	Δ*erf2* complement
loc1Fd	CCAAGTATCGAAGTACTAGCATCAGAGT	qRT-PCR; *Z0024*
loc1Rv	CCCTGGTCTCATTCTGGTCAA	qRT-PCR; *Z0024*
erf2Fd	TCTGGGTGCTTGGTATGAAGTTAT	qRT-PCR, RT-PCR; *Z0146*
erf2Rv	TGTGGTGCCGCAGATGAA	qRT-PCR, RT-PCR; *Z0146*
loc3Fd	TCTCAAACCTGATGGGAATAGCTT	qRT-PCR; *Z0686*
loc3Rv	CGTGCAGAAAAATGAAGAACGT	qRT-PCR; *Z0686*

loc4Fd	CGCCCTGGCCATATCTCTAC	qRT-PCR; *Z0872*
loc4Rv	TTTAAAGTGGATTTCACCAGATCCT	qRT-PCR; *Z0872*
loc5Fd	GCAGATACCCGGTGGCATAT	qRT-PCR; *Z1290*
loc5Rv	TCCCTGGCCGCCTAAAC	qRT-PCR; *Z1290*
loc6Fd	ACGCCGTTTACGGTTTCTGT	qRT-PCR; *Z1538*
loc6Rv	GGTGGTGTTGATTGCCTGATC	qRT-PCR; *Z1538*
csgFd	GCGTTGTCAATGGATTGCAA	qRT-PCR; *csgD*
csgRv	TCAGGTAACTGGCAAGCTTTTG	qRT-PCR; *csgD*
loc8Fd	TGCCAGACAATTTCCAACGA	qRT-PCR; *Z2204*

loc8Rv	GTCCCTTTGCTGCATTCACTTA	qRT-PCR; *Z2204*
erf1Fd	TGGCGGTGATTCAGTCAGTATT	qRT-PCR RT-PCR; *Z3279*
erf1Rv	ACCAACCGCGCCATCATA	qRT-PCR, RT-PCR; *Z3279*
loc10Fd	ACGGCCGAATCGTCAAAG	qRT-PCR; *Z3597*
loc10Rv	CGTGACCCTCAAATGGAAATG	qRT-PCR; *Z3597*
loc11Fd	GCGCTTATGCCTCCTCAGAA	qRT-PCR; *Z4501*
loc11Rv	CTGGTTCACCGTAACATCATCAA	qRT-PCR; *Z4501*
lpf1Fd	TCTGTATTTGCTGCGGTTGGT	qRT-PCR; *Z4971*
lpf1Rv	GGTATCGCAATCTTCCAGTTTGA	qRT-PCR; *Z4971*

lpf2Fd	TGGTTTCGCCGCTAATGG	qRT-PCR; *Z5221*
lpf2Rv	CACCGTCAGCGGACCAA	qRT-PCR; *Z5221*
loc14Fd	TGCACAGTTTCCACCACCAA	qRT-PCR; *Z5917*
loc14Rv	CCGGCAGACATCAGACTGAA	qRT-PCR; *Z5917*
ecpFd	CGTGGCTATCGAGGGTGACT	qRT-PCR; *Z0360*
ecpRv	CTGGGTTAATGTGTTGGTGATAAGA	qRT-PCR; *Z0360*
hcpFd	GCACTCACCGACATGCTACAA	qRT-PCR; *Z0118*
hcpRv	CCAGCGCGCACAACTCT	qRT-PCR; *Z0118*
lerFd	CGACCAGGTCTGCCC	qRT-PCR
lerRv	GCGCGGAACTCATC	qRT-PCR

grlAFd	CCGGTTGTTCCAGGACTTTC	qRT-PCR
grlARv	TAAGCGCCTTGAGATTTTCATTT	qRT-PCR
escCFd	GCGTAAACTGGTCCGGTACGT	qRT-PCR
escCRv	TGCGGGTAGAGCTTTAAAGGCAAT	qRT-PCR
escVFd	TCGCCCCGTCCATTGA	qRT-PCR
escVRv	CGCTCCCGAGTGCAAAA	qRT-PCR
eaeFd	GCTGGCCCTTGGTTTGATCA	qRT-PCR
eaeRv	GCGGAGATGACTTCAGCACTT	qRT-PCR
espAFd	TCAGAATCGCAGCCTGAAAA	qRT-PCR
espARv	CGAAGGATGAGGTGGTTAAGCT	qRT-PCR
Stx2aFd	ACCCCACCGGGCAGTT	qRT-PCR
Stx2aRv	GGTCAAAACGCGCCTGATA	qRT-PCR
Z3278 RTF	CCACCGAATAGCCCAGAAGA	qRT-PCR, RT-PCR
Z3278 RTR	TACCGGAGCAATACCAGCAG	qRT-PCR, RT-PCR

Z3277 RTF	ATTGCTCGGCATTGAAGCAC	qRT-PCR, RT-PCR
Z3277 RTR	CAATGTCGTACTGCCCAGGT	qRT-PCR, RT-PCR
Z3276 RTF	GCGGGGGATACATTTACGCT	qRT-PCR, RT-PCR
Z3276 RTR	TCCTTACTGTTCGCCGCAAT	qRT-PCR, RT-PCR
Z0152 RTF	GGCGTGGTTACCCTGGATAC	qRT-PCR, RT-PCR
Z0152 RTR	GGAGCACCTGGATCACACTC	qRT-PCR, RT-PCR
Z0151 RTF	CTTTCCGCACACGCATCAAA	qRT-PCR, RT-PCR
Z0151 RTR	GGCGTTGGGTTATTTGCCTG	qRT-PCR, RT-PCR
Z0150 RTF	TCCGCCCGGTGAATTTGTTA	qRT-PCR, RT-PCR

Z0150 RTR	AGAAGGGGTGCGTAAAGGTG	qRT-PCR, RT-PCR
Z0149 RTF	ATCCGCGTTAATAGGCCTGG	qRT-PCR, RT-PCR
Z0149 RTR	AGTTATCGTGCAGGTGGTGG	qRT-PCR, RT-PCR
Z0148 RTF	AAACAGCGCCCAAATAGTGC	qRT-PCR, RT-PCR
Z0148 RTR	AGTCCATACTTCCACAGCCG	qRT-PCR, RT-PCR
Z0147 RTF	ATTGCCAGGTGCTACCGAATA	qRT-PCR, RT-PCR
Z0147 RTR	GAAAAACGTCGGCATTGTGA	qRT-PCR, RT-PCR

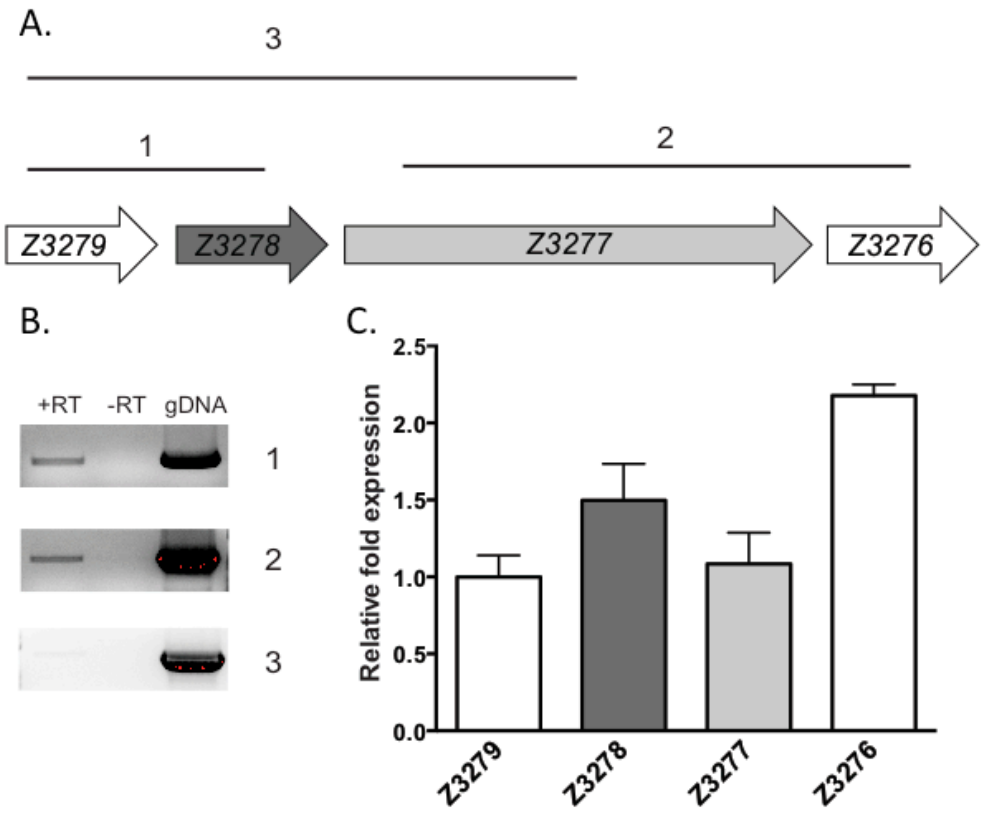

Figure 3.1 The *erf1* locus encodes and expresses all required subunits for the assembly of functional fimbrial structures. A. Schematic of *erf1* showing fimbrial subunits (white), chaperone (dark gray), and usher (light gray). B. RT-PCR was performed to map the transcriptional units in *erf1*. Genomic DNA was used as a positive control and a cDNA synthesis reaction without reverse transcriptase (RT) added was used as a negative control. C. Expression of each gene encoded within *erf1* relative to *Z3279* was assessed by qRT-PCR. Error bars indicate SD from three independent samples.

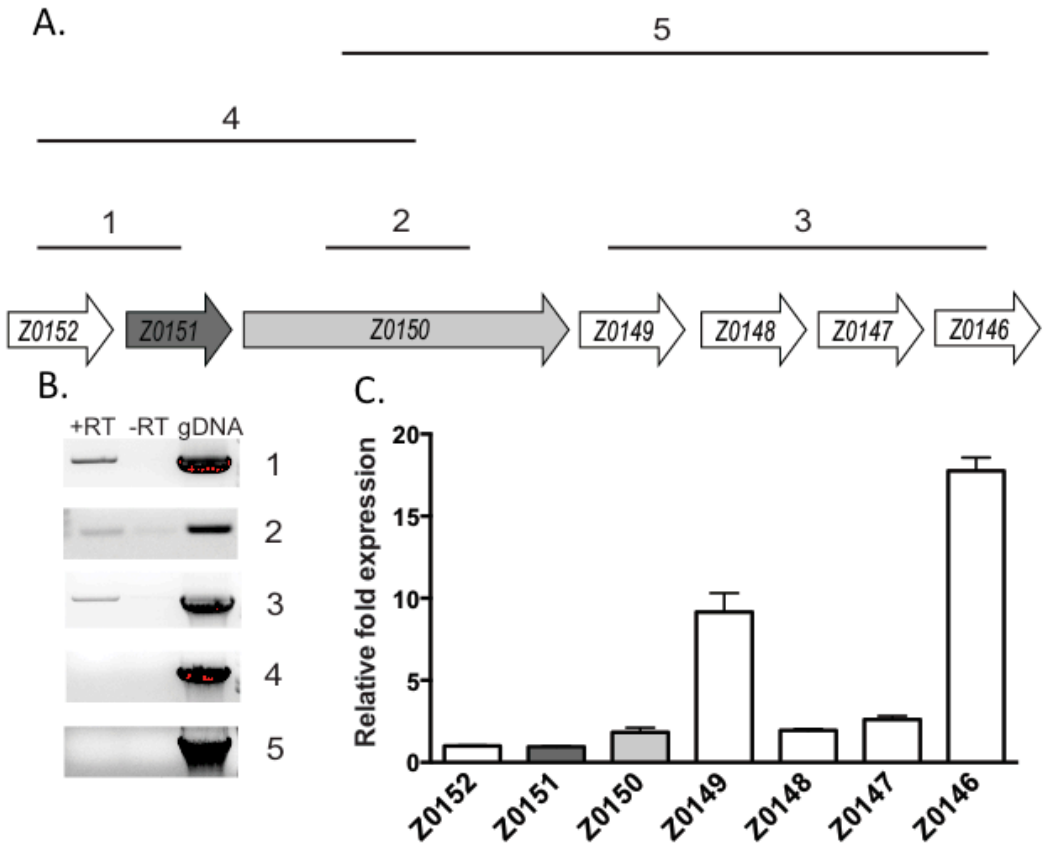

Figure 3.2 All required subunits encoded in *erf2* are expressed and could assemble a functional fimbriae. A. Schematic of *erf2* showing fimbrial subunits (white), chaperone (dark gray), and usher (light gray). B. RT-PCR was performed to map the transcriptional units in *erf2*. Genomic DNA was used as a positive control and a cDNA synthesis reaction without RT added was used as a negative control. C. Expression of each gene encoded within *erf2* relative to *Z0152* was assessed by qRT-PCR. Error bars indicate SD from three independent samples.

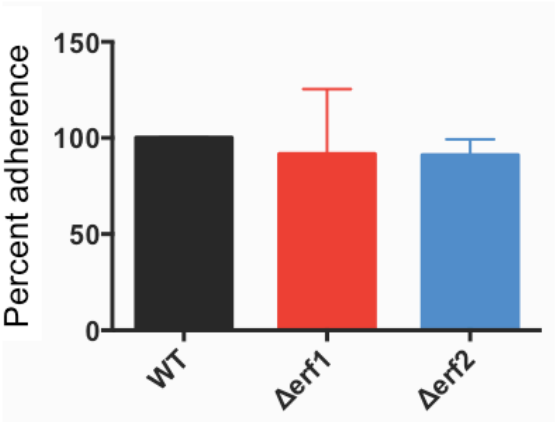

Figure 3.3 Erf1 and Erf2 do not alter initial EHEC adherence. Quantification of total early adherence by WT EHEC, Δ*erf1*, and Δ*erf2* using an adhesion assay. HeLa cells were infected with EHEC, washed, and then lysed; lysates were then dilulted and plated to determine the total number of bacteria adhered per well. Adhesion of Δ*erf1*, and Δ*erf2* is shown as relative to WT. Error bars indicate SD.

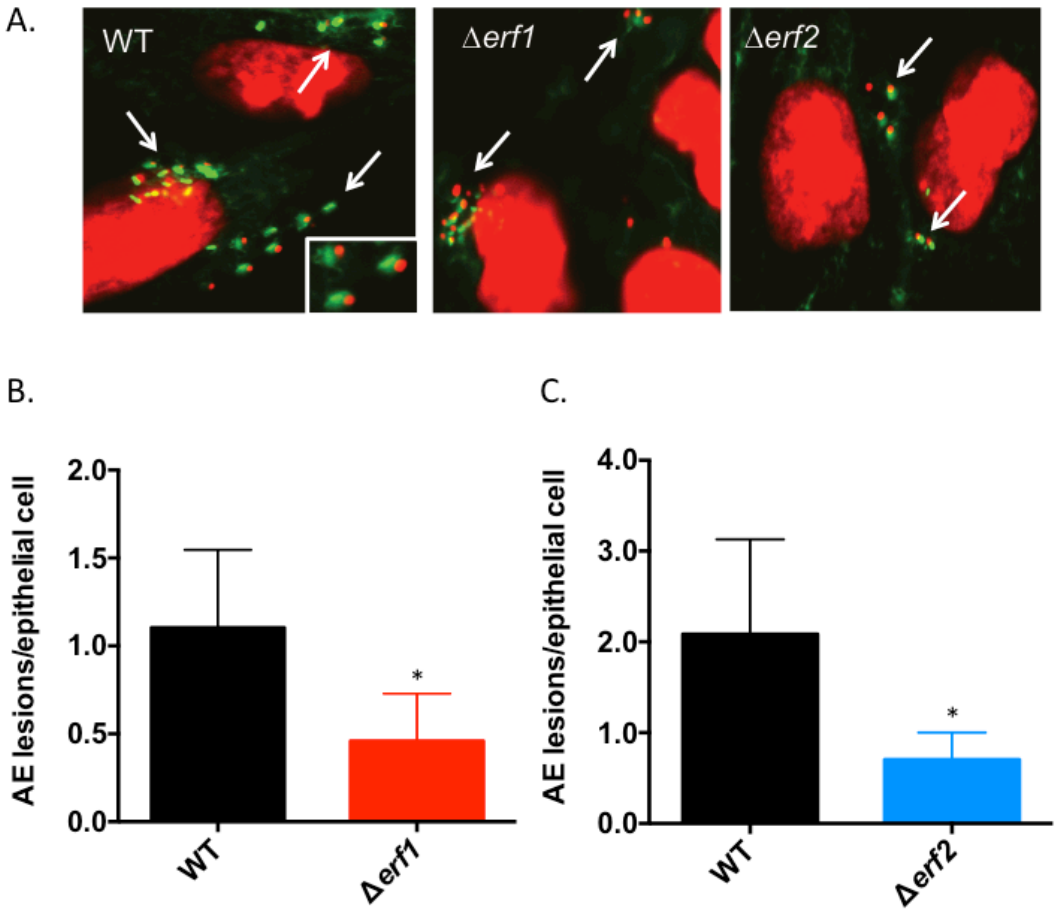

Figure 3.4 Erf1 and Erf2 influence the formation of AE lesions by EHEC. A. Representative images showing AE lesion formation by WT EHEC, Δ*erf1*, and Δ*erf2*. Bacterial cells and epithelial cell nuclei are shown in red; actin staining is shown in green. WT panel contains an inset with three AE lesions at higher magnification. B. Quantification of AE lesion formation by WT EHEC and Δ*erf1*. Error bars indicate SD. An unpaired *t*-test was performed to determine a p-value of 0.0474. C. Quantification of AE lesion formation by WT EHEC and Δ*erf2*. Error bars indicate SD from four independent experiments. An unpaired *t*-test was performed to determine a p-value of 0.0443.

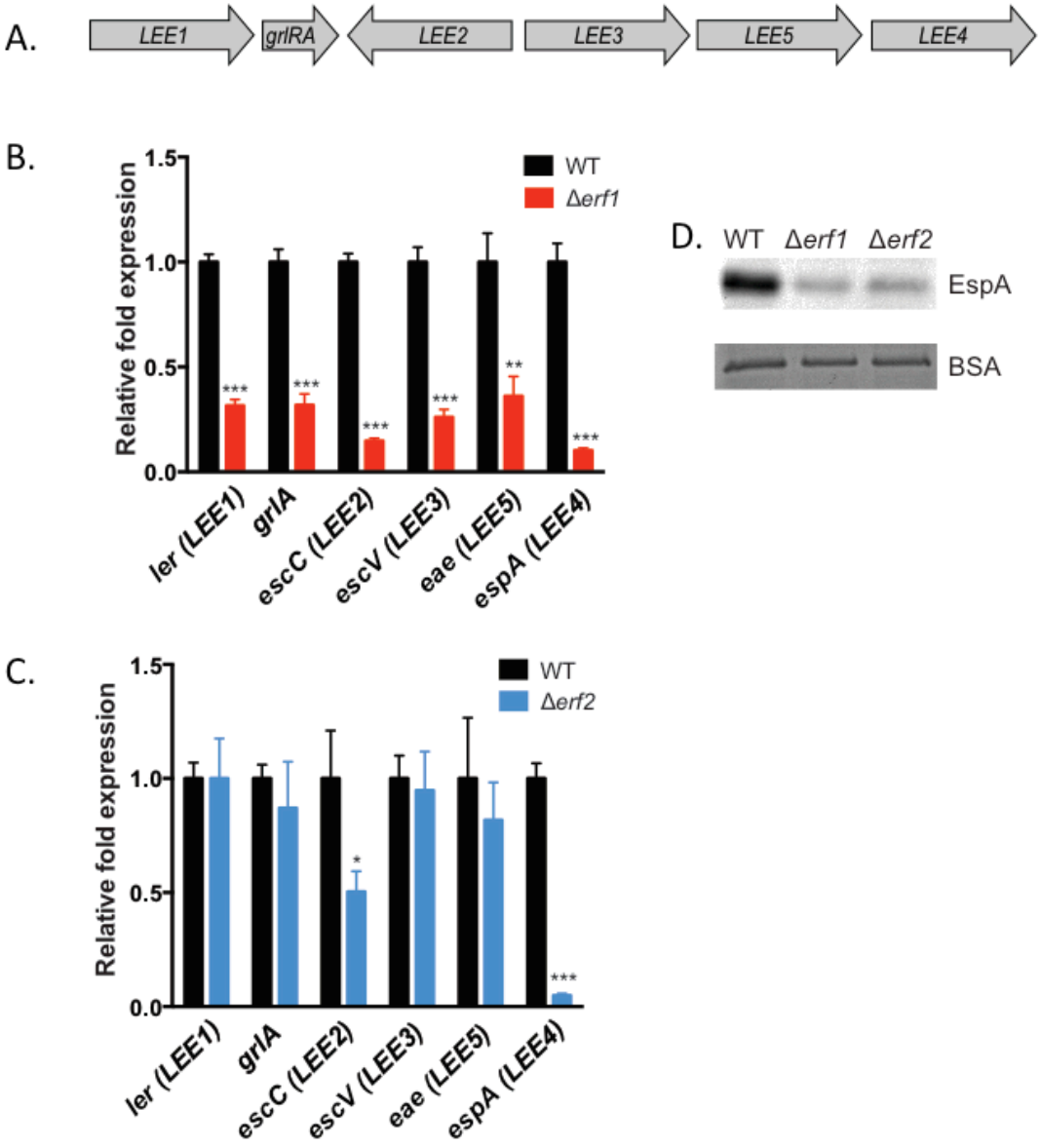

Figure 3.5 Erf1 and Erf2 promote expression of genes encoded within the LEE. A. Schematic of the LEE. B. Expression of a representative gene from each LEE operon in Δ*erf1* relative to WT EHEC was assessed by qRT-PCR. Error bars indicate SD from three independent samples. An unpaired *t*-test was performed to determine significance. ** p≤0.01, *** p≤0.001. C. Expression of a representative gene from each LEE operon in Δ*erf2* relative to WT EHEC was assessed by qRT-PCR. Error bars

indicate SD from three independent samples. An unpaired *t*-test was performed to determine significance. * $p \leq 0.05$, *** $p \leq 0.001$. D. EspA expression in WT EHEC, Δ*erf1*, and Δ*erf2* was probed by Western blotting of secreted protein samples. BSA (bovine serum albumin) was used as a loading control. This analysis was repeated and confirmed with a second set of samples.

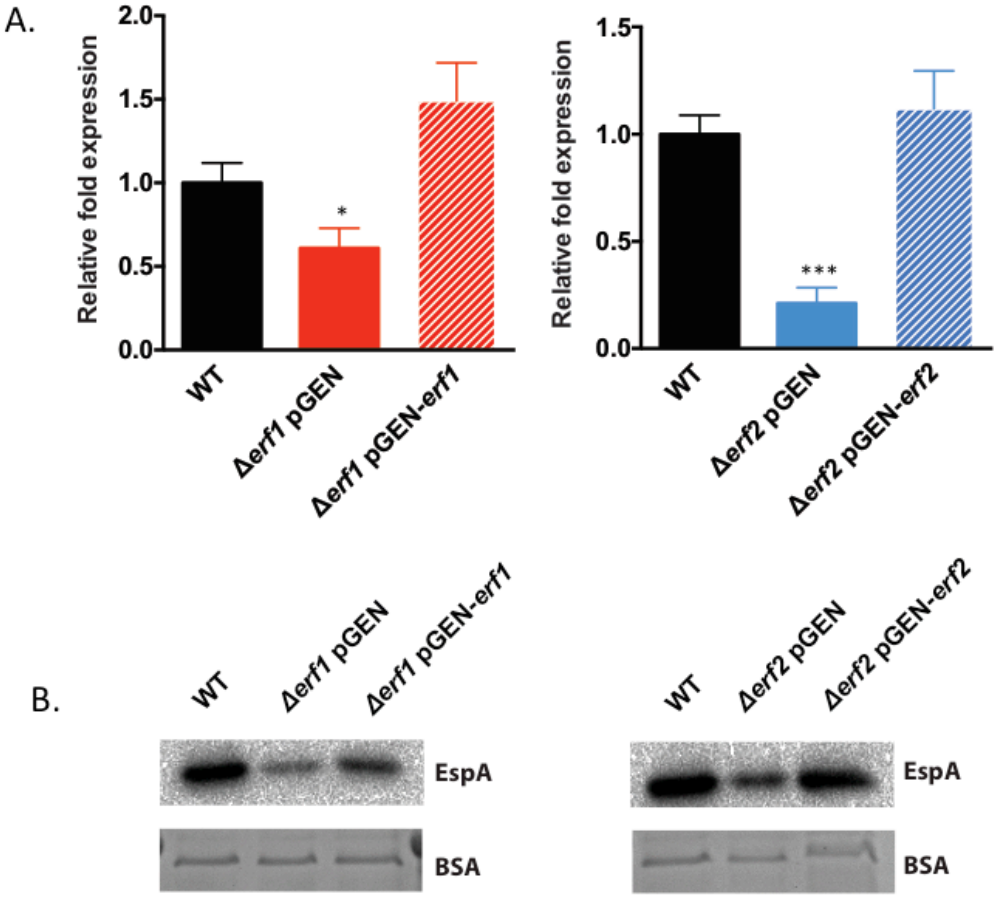

Figure 3.6 Complementation of Erf1 and Erf2 in Δ*erf1* and Δ*erf2* restores EspA expression. A. Expression of *espA* in Δ*erf1* and Δ*erf2* with pGEN empty vector or pGEN containing fimbrial constructs (pGEN-*erf1* and pGEN-*erf2*). *espA* expression was assessed by qRT-PCR and is shown relative to WT EHEC. Error bars indicate SD from three independent samples. An unpaired *t*-test was performed to determine significance. * $p \leq 0.05$, *** $p \leq 0.001$. B. EspA expression in WT EHEC, Δ*erf1* or Δ*erf2* with empty vector, and Δ*erf1* or Δ*erf2* with complements determined by Western blot analyses. Coomassie staining for BSA was used as a loading control. This analysis was repeated and confirmed with a second set of samples.

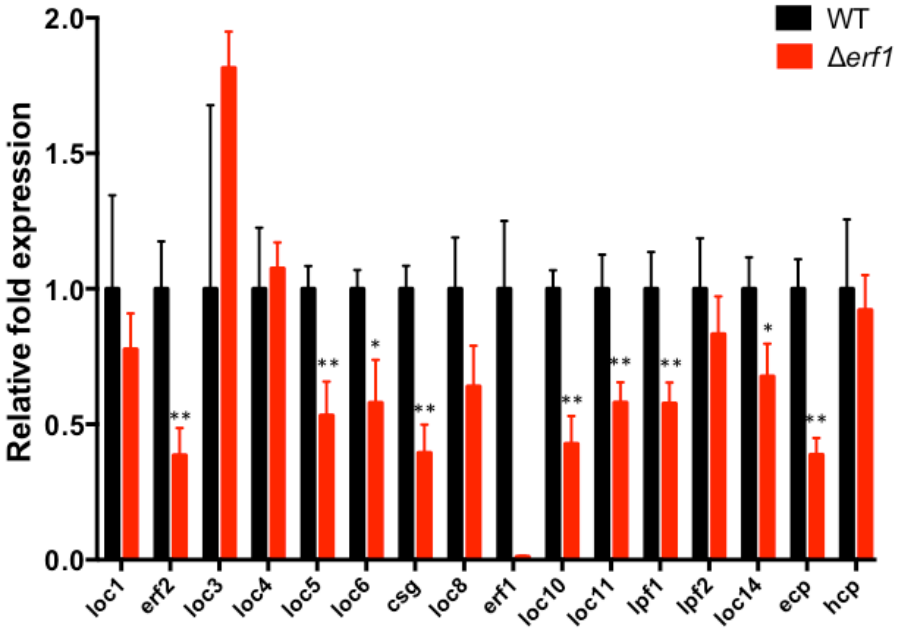

Figure 3.7 Erf1 promotes fimbrial gene expression. Expression of representative genes from each fimbrial loci in Δ*erf1* relative to WT EHEC was assessed by qRT-PCR. Error bars indicate SD from three independent samples. An unpaired *t*-test was performed to determine significance. * p≤0.05, ** p≤0.01.

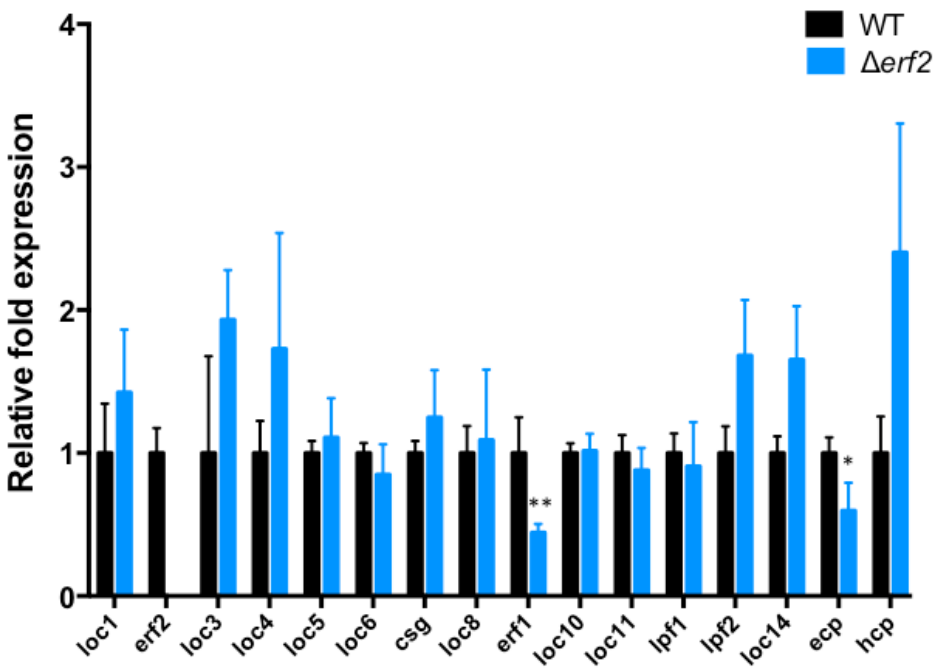

Figure 3.8 Erf2 promotes expression of *erf1* and *ecp*. Expression of representative genes from each fimbrial loci in Δ*erf2* relative to WT EHEC was assessed by qRT-PCR. Error bars indicate SD from three independent samples. An unpaired *t*-test was performed to determine significance. * p≤0.05, ** p≤0.01.

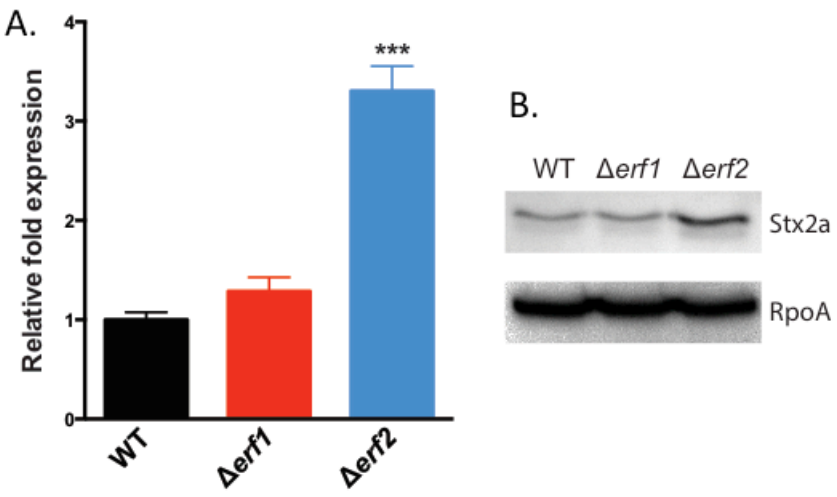

Figure 3.9 Erf2 influences Shiga toxin expression. A. Expression of *stx2a* determined by qRT-PCR in WT EHEC, Δ*erf1*, and Δ*erf2*. Error bars indicate SD from three independent samples. An unpaired *t*-test was performed to determine significance. *** p≤0.001. B. Stx2a expression in WT EHEC, Δ*erf1*, and Δ*erf2* was probed by Western blotting of whole-cell lysate samples using antisera against Stx2a and RpoA (loading control). This analysis was repeated and confirmed with a second set of samples.

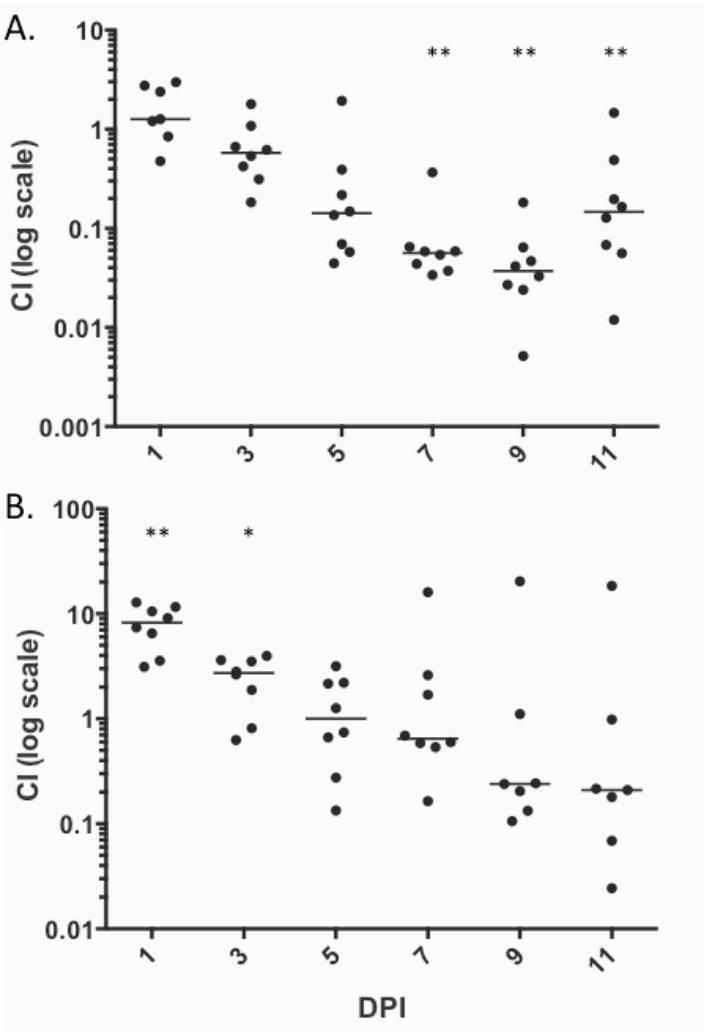

Figure 3.10 Erf1 is critical for EHEC colonization of the murine gastrointestinal tract. A. Colonization fitness determined through calculation of competitive indices (CI) of WT and Δ*erf1* shed in the feces at indicated days post infection (DPI). A Wilcoxon signed ranks test was used to assess significance comparing to a theoretical mean of 1. * p≤0.05, ** p≤0.01, *** p≤0.001. A total of 8 mice were infected in two independent experiments. Lines represent the median. B. Colonization fitness determined through calculation of competitive indices of WT

and Δ*erf2* shed in the feces at indicated DPI. A Wilcoxon signed ranks test was used to assess significance comparing to a theoretical mean of 1. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. A total of 8 mice were infected in two independent experiments. Lines represent the median. Debi Luzader assisted with these experiments.

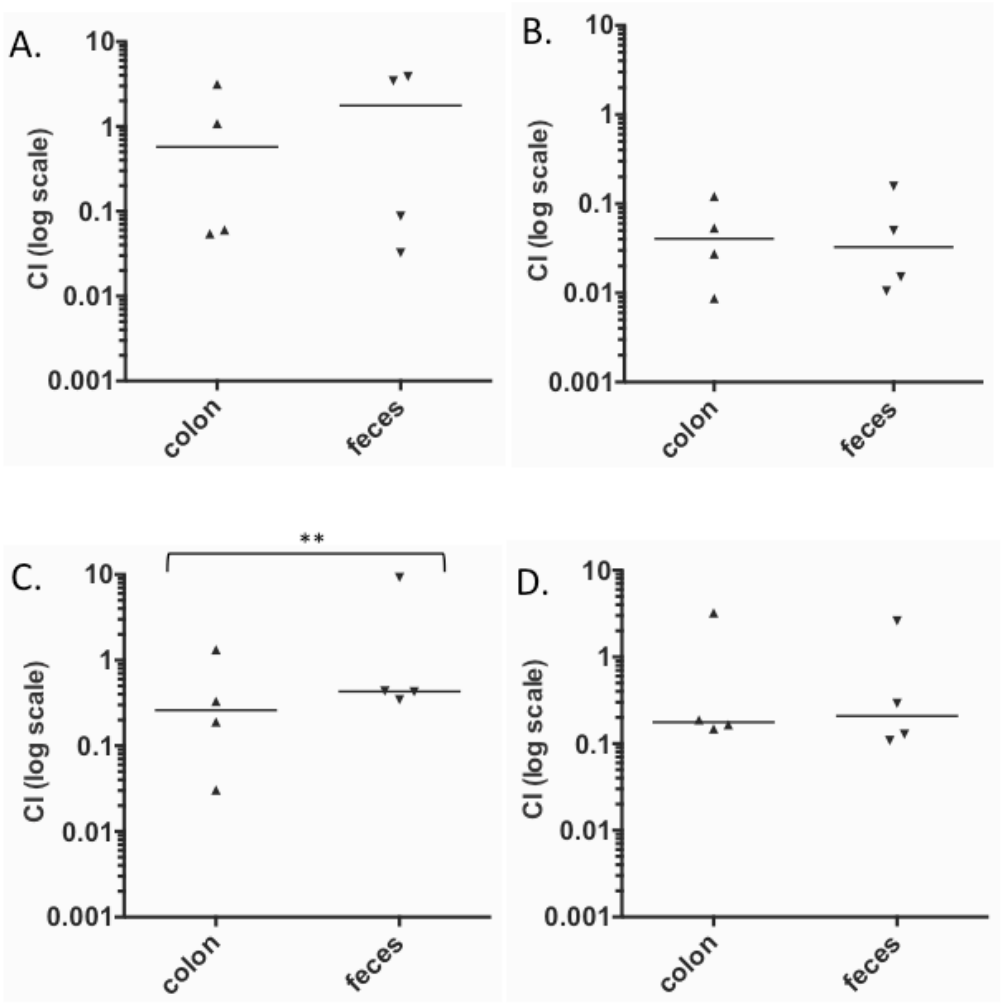

Figure 3.11 Comparison between fecal shedding and colon colonization.

Competitive indices (CI) were compared on the day of euthanasia between the colon and the feces. Data from individual experimental groups is shown separately because of variation in the number of days post infection (DPI) that euthanasia occurred. There was no difference in the CI observed in the feces and the colon for mice infected with WT and Δ*erf1* euthanized on day 14 (A) or day 12 (B). There was a difference in the CI observed in the feces and the colon for mice infected with WT and Δ*erf2* euthanized on day 14 (C) but not day 12 (D). An unpaired t-test was used

to determine statistical significance and even standard deviation was not assumed. Lines represent the median. ** $p \leq 0.01$. Debi Luzader assisted with these experiments.

Chapter Four

Discussion and Future Directions

Summary

Previously, the expression of many EHEC fimbriae has not been documented *in vitro*, and it has been suggested that most EHEC fimbriae may be cryptic or nonfunctional [91]. Our work has identified that the biologically relevant signals EA and choline induce the expression of fifteen and thirteen EHEC fimbrial loci, respectively [149]. Additionally, we characterized four of these EA-regulated fimbrial loci and found that all of the subunits required for forming a functional fimbrial structure are expressed during growth in EA. Together, these data demonstrate that EHEC fimbriae are expressed and could be functional *in vivo*. The simultaneous induction of fifteen fimbrial loci led us to inquire which of these EA-responsive fimbriae contribute to host colonization. We determined that one EA-regulated fimbriae, Erf1, contributes to colonization of the murine GI tract. We also made an intriguing discovery that Erf1 as well as the EA-regulated fimbriae Erf2 direct the expression of genes involved in later pathogenic steps, specifically T3S, AE lesion formation, and toxin production. These findings in EHEC expand on the role of fimbriae as not simply mediating bacterial attachment but also modulating virulence.

Determining the mechanism for gene regulation by Erf1 and Erf2

The most urgent future directions are inspired by our findings identifying a new role for EA-regulated fimbriae in virulence gene regulation. Fimbriae

prototypically facilitate the first interactions between bacteria and their hosts. We postulate that linking these initial steps in colonization with expression of virulence determinants critical for later steps in pathogenesis is an advantageous strategy for coordinated regulation of virulence. While our data do not demonstrate that Erf1 or Erf2 mediate EHEC early attachment to epithelial cells, they may still be important for strong adherence to the host mucosa. Promoting T3S, AE lesion formation, and additional fimbrial expression during early interactions with host tissue could strengthen bacterial adherence and increase persistence. This effective coordination of adherence may be essential during colonization of the GI tract to resist clearance with the normal flux of intestinal contents.

While we hypothesize that fimbrial regulation of virulence gene expression is important for EHEC pathogenesis, we do not yet understand how Erf1 and Erf2 are mediating these effects. Some fimbrial loci encode regulators, which provide an intuitive mechanism for gene regulation. Examples of fimbrial genes that act as regulators include MrpJ from *Proteus mirabilis* and PapX from uropathogenic *E. coli* [159, 160]. These two regulators function similarly to inhibit motility during fimbrial expression by transcriptionally repressing *flhDC*, which encodes the master regulator for flagella expression [159, 160]. Additionally, a fimbrial gene product (*Z0021* from *loc1*) in EHEC has been implicated in repressing motility downstream of *flhDC* transcription. Z0021 is a hypothetical protein that lacks homology with any known proteins, and it is therefore unclear if Z0021 is a regulator or how Z0021 influences motility mechanistically [161].

No putative regulators are encoded in *erf1* or *erf2*, leading us to hypothesize that effects on gene regulation are indirect and require the involvement of one or more signaling intermediates. We propose to identify these intermediates using unbiased transcriptional and proteomic approaches. We have submitted WT, Δ*erf1,* and Δ*erf2* samples for microarray analyses to evaluate differences in transcription in WT EHEC compared to the Δ*erf1* and Δ*erf2* strains. From these data, we aim to identify target regulators as well as characterize the global gene expression phenotypes in Δ*erf1* and Δ*erf2.* If transcript levels of candidate regulators are not altered, protein levels will be investigated. We have begun to investigate broad changes in protein levels by Coomassie staining whole cell lysates and have observed differences in the intensity of specific protein bands. However, it is difficult to identify affected proteins without quantitative analyses. To identify proteins with differential expression in WT versus Δ*erf1* or Δ*erf2*, we will submit the whole cell lysate samples for tandom mass spectrometry. This unbiased approach would provide more information and be more cost effective than individually excising bands. We anticipate that the combination of both microarray and protein analyses will define the Erf1 and Erf2 regulons as well as identify candidates for signaling intermediates.

Our data do not distinguish between which aspect of fimbrial expression (transcription, translation, or assembly) affects the expression of the LEE, fimbriae, and Stx in the Δ*erf1* and Δ*erf2* strains. We hypothesize that regulation is occurring at the level of translation or assembly, specifically through either the presence of

fimbrial proteins or the assembly of fimbrial structures. A related mechanism for regulation of T3S effector transcription is through interactions between chaperones and transcriptional regulators. The first report of the dual role of chaperones in both controlling secretion of effectors and regulating gene expression demonstrated a physical interaction between the chaperone SicA and T3S regulator InvF in *Salmonella typhimurium* that activated transcription of effector genes [162, 163]. Similarly, the transcriptional regulator MxiE and the T3S chaperone IpgC act together to increase transcription of effectors in *Shigella flexneri* [164]. Coordination between the T3S chaperone and transcriptional regulator in these two enteric pathogens connects the assembly of a secretion-competent structure to the expression of secreted effectors. It is possible that the accumulation of chaperones or fimbrial proteins during assembly is involved in a similar signaling mechanism.

To begin to decipher which fimbrial components, if not all, are essential for modulating virulence gene expression, we will create a set of strains and plasmids to identify the roles of the chaperone, major subunit, and usher. We will start by complementing the Δ*erf1* and Δ*erf2* strains with their encoded usher, chaperone with or without major subunit, or all three components together (Figure 4.1). Evaluating EspA expression (as a representative from the LEE) in these complements will suggest whether it is the insertion of the usher in the outer membrane (usher alone), presence of fimbrial subunits and/or chaperones in the periplasm (chaperone with or without major subunit), or assembly of a complete structure (usher, major subunit, and chaperone, lacks tip adhesin) that contributes to the observed changes in gene expression.

If assembly of fimbrial structures is required to modulate virulence gene expression, information about fimbrial assembly at the outer membrane would need to reach the cytoplasm for activation of gene expression. Logistically, intermediates localized in the periplasm, inner membrane, and/or cytoplasm would be necessary. One likely candidate is CpxAR, a two-component system that responds to membrane stress [165]. CpxA is a histidine kinase that resides in the inner membrane, and CpxR is a response regulator that localizes to the cytoplasm and promotes gene expression when activated by CpxA [166, 167]. CpxAR has been implicated in regulation of fimbrial assembly both at the transcript and protein level [168-171]. In different systems, CpxAR has been shown to promote or inhibit fimbrial expression as well as respond to the assembly of fimbrial structures [168-171]. Notably, CpxR directly regulates expression of VirF, the master regulator of virulence genes in *Shigella* [172]. Because of these examples in the literature, we hypothesize that CpxAR could be the link between Erf1/2 expression and regulation of virulence genes. CpxA may interact with fimbrial subunits in the periplasm or sense fimbrial assembly through changes in the outer membrane, which would lead to gene expression regulation through CpxR (Figure 4.2). To test this, we propose to first probe for the expression of *cpxR* and *spy*, which are both transcriptionally upregulated in response to CpxA activation, in the WT, Δ*erf1*, and Δ*erf2* strains. If Erf1 and Erf2 regulation of virulence genes in EHEC involves CpxAR as an intermediate, we would expect that expression of *cpxR* and *spy* would be reduced in the Δ*erf1* and Δ*erf2* strains in comparison to WT.

An alternative mechanism for modulation of gene expression by fimbrial structures is through ligand binding. Our gene expression experiments were conducted in the absence of host epithelial cells. We have previously shown that EHEC cells grown in the presence of EA display a bacteria-to-bacteria clustering pattern that we hypothesize is due to fimbrial expression [149]. Erf1 and/or Erf2 could bind to ligands on other bacterial cells, but we do not know if this binding occurs in our growth conditions for gene expression. We did not observe any bacterial clumping or settling during growth with EA for gene expression. Although our observations suggest otherwise, signaling events could be initiated through fimbrial binding to other bacterial cells, either through engaging bacterial receptors or by alterations in membrane tension due to binding. Examples of characterized contact-dependent regulation mechanisms include CrgA in *Neisseria* and the Cpx two-component system. In *Neisseria meningitidis,* type IV pili expression is induced during early interactions with target cells and is turned off during intimate adherence [173]. The CrgA regulatory protein downregulates pili after contact with epithelial cells, which contributes to the switch from initial to intimate adhesion [174]. In this system, cell contact is an important factor in the coordination of steps involved in early and late adhesion. Additionally, the Cpx system (described above) senses cell contact and adhesion through disturbances in the membrane and then responds to regulate gene expression [175].

Independently of translation, assembly, or function of fimbrial proteins and structures, transcription of *erf1* or *erf2* may contribute to the changes in gene

expression we observed in WT, Δ*erf1*, and Δ*erf2* strains. One putative mechanism is through the expression of non-coding RNAs encoded within the fimbrial loci. Non-coding RNAs contribute to hierarchal control of fimbrial expression in Salmonella [176]. It is possible that *erf1* and/or *erf2* encode non-coding RNAs that may impact expression of the LEE, other fimbriae, and Stx. To test this, we could modify or delete the native fimbrial promoters, which would inhibit transcription of fimbrial genes. A non-coding RNA would likely be expressed under a separate promoter and therefore would still be expressed. We could then determine, using EspA expression, whether a putative non-coding RNA encoded within the fimbrial loci impacts LEE expression. To identify non-coding RNAs encoded within *erf1* or *erf2*, we could evaluate the RNA transcriptome [177]. Alternatively, there may be global effects on gene expression of deleting entire fimbrial loci (5-7 kb). Local and global chromosomal structure may be altered by excision of these large regions of fimbrial genes. Chromosomal position can alter gene expression [178], and shifts in chromosomal position and structure may influence gene expression in the Δ*erf1* and Δ*erf2* strains. Our finding that complementation of *erf1* and *erf2* on a plasmid restores *espA* expression (Figure 3.6) suggests that alterations in gene expression are not due to changes in chromosomal structure due to *erf1* or *erf2* deletion.

In summary, we anticipate that the combination of unbiased and targeted approaches described herein should provide us with new insights into the mechanism for fimbrial regulation of virulence gene expression in EHEC.

Understanding this mechanism will broaden our knowledge both on the role of EHEC fimbriae in virulence as well as additional levels of regulation of important virulence determinants (Stx and the LEE).

Understanding the contribution of additional EA-regulated fimbriae to EHEC pathogenesis

We characterized two EA-regulated fimbriae, Erf1 and Erf2, and discovered a unique role for these fimbriae in promoting the expression of genes involved in later steps in pathogenesis. Thirteen more fimbrial loci are upregulated during growth in EA, and we do not know if these fimbriae mediate similar effects. Future plans include constructing mutants in additional EA-regulated fimbriae, initially focusing on uncharacterized loci, and elucidating their roles in adherence and EHEC virulence gene regulation. Both Erf1 and Erf2 direct gene regulation through distinct mechanisms, and it is plausible that other EHEC fimbriae could also contribute to modulation of virulence gene expression.

Our transcriptional analysis was performed on a population of cells and does not describe whether each cell in the population was transcribing single, multiple, or all fifteen fimbriae. Our data suggest that additional fimbriae mediate initial attachment to epithelial cells in the *Δerf1* and Δ*erf2* strains and may function redundantly; therefore, we may not be able to observe an early adherence phenotype for any of the single fimbrial mutants. An interesting tool to evaluate the

contribution of the entire repertoire of EA-regulated fimbriae to EHEC early adhesion would be to create a deletion strain lacking all sixteen fimbrial loci. Single fimbrial loci could then be added back to assess their individual roles in EHEC adherence. These experiments would definitively characterize the role of EHEC fimbriae in promoting early adherence and colonization.

Implications of this study to the field

We have shown that EA induces the expression of fifteen EHEC fimbriae, and that four of these EA-regulated loci are complete and could encode functional fimbriae. The increase in fimbrial transcription correlated with an increase in adhesion to epithelial cells and the presence of fimbrial structures on the bacterial surface during growth in EA. Previously, expression of most EHEC fimbriae had not been observed *in vitro*. This led investigators to hypothesize that EHEC may only possess a limited repertoire of functional fimbriae. However, our work suggests that EHEC fimbriae are expressed, functional, and contribute to EHEC adhesion to epithelial cells.

Although our characterization of two EA-regulated fimbriae, Erf1 and Erf2, did not show a role in fimbrial-mediated adherence to HeLa cells *in vitro*, we cannot rule out the possibility that Erf1, Erf2, or other EA-regulated fimbriae are essential for adhesion in the human GI tract. Notably, we found that Erf1 is critical for robust colonization of the murine GI tract, which is the first evidence that a single fimbrial

locus contributes to EHEC host colonization. Overall, our findings emphasize the importance of fimbriae in EHEC pathogenesis, which was not previously recognized.

Additionally, we have shown a novel role for EA-regulated EHEC fimbriae in directing later steps in pathogenesis, including T3S, toxin production, and intimate attachment. These results are not only important for our understanding of EHEC virulence but may also provide insight into how fimbriae function in diverse bacterial pathogens. Both Gram-positive and Gram-negative bacteria encode fimbriae [11, 155, 156]; therefore, regulation of bacterial gene expression through fimbriae could be a widespread mechanism for coordination of virulence.

In summary, our data provide the first evidence that EHEC fimbrial expression is induced by a signal present in the host environment and that fimbriae contribute to EHEC pathogenesis both through increased adherence as well as modulation of expression of virulence determinants. The critical role of EA-regulated fimbriae in EHEC colonization and gene regulation of virulence factors could be exploited by the development of new therapeutic interventions that target fimbrial expression or function. Further characterization into the contribution of individual fimbriae as well as EA signaling to colonization in a human host would identify targets for development of novel treatment options. Examples could include molecules that block EA signaling or fimbrial adherence.

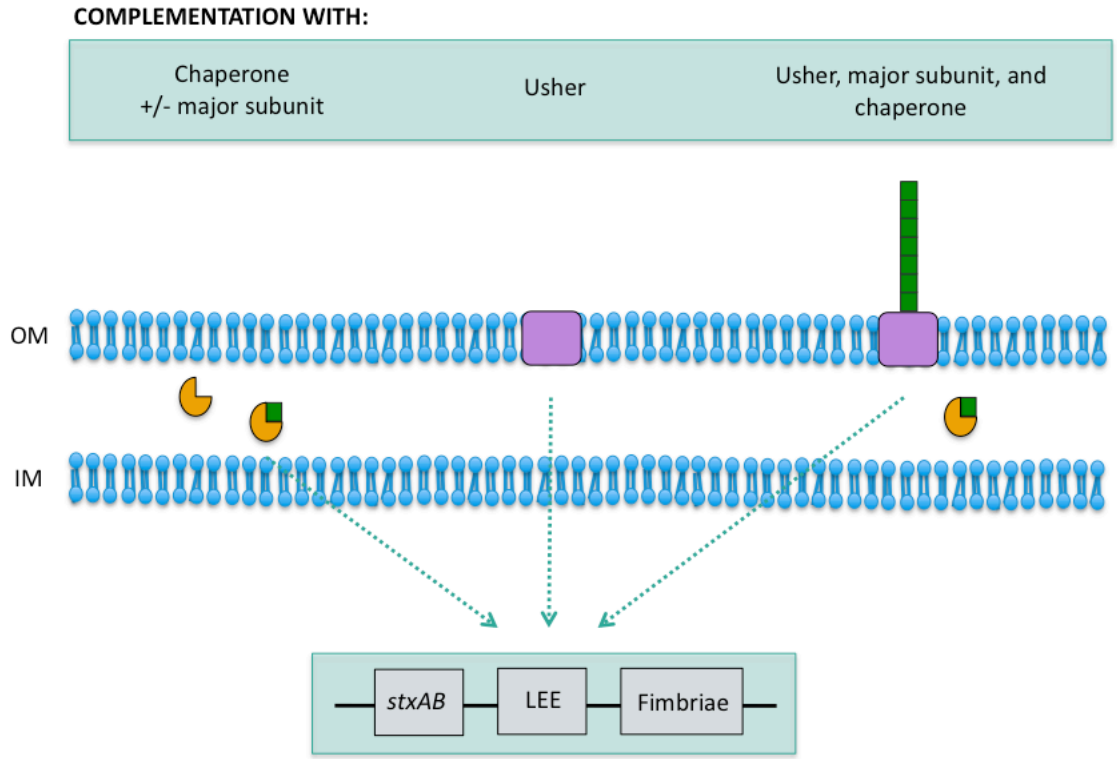

Figure 4.1 Complementation strategy to determine which fimbrial components are required for regulation of gene expression. Expressing individual and groups of fimbrial components in the Δ*erf1* and Δ*erf2* strains will identify which fimbrial components mediate gene regulation of virulence factors. Evaluating gene expression in these complements could suggest whether integration of the usher in the outer membrane (usher alone), the presence of fimbrial subunits in the periplasm (chaperone with or without major subunit), or the presence of a complete fimbrial structure (usher, major subunit, and chaperone) mediate the altered expression of virulence genes observed in Δ*erf1* and Δ*erf2* compared to WT. Dotted arrows highlight proposed targets for gene expression analyses.

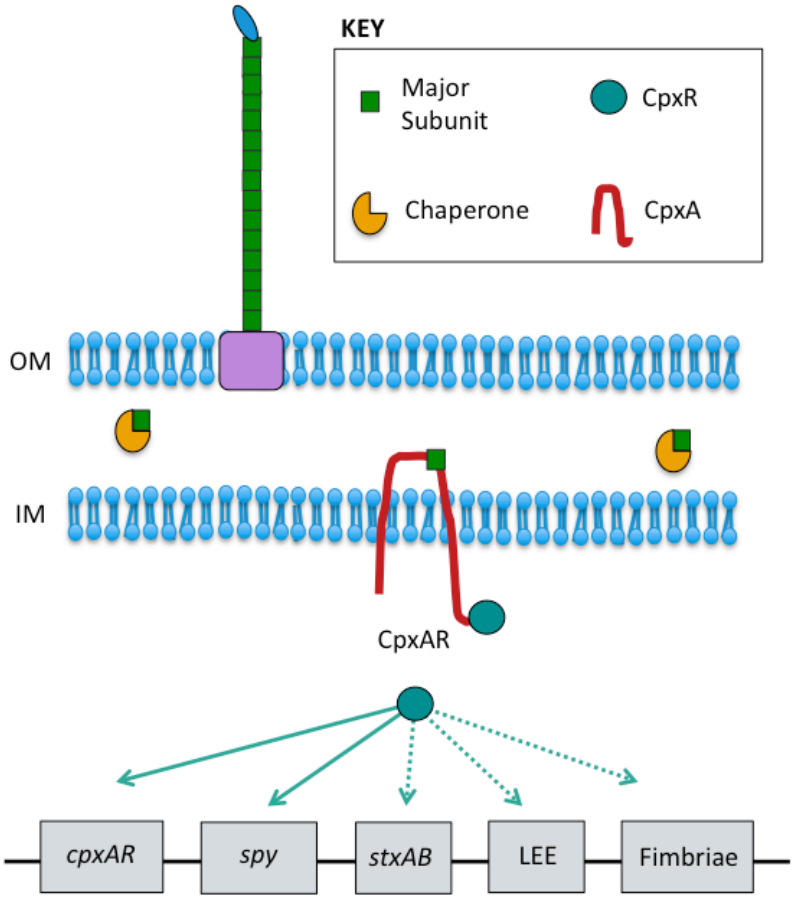

Figure 4.2 Model for how CpxAR could act as an intermediate between fimbrial assembly and virulence gene regulation. We hypothesize that CpxA may be able to interact with fimbrial subunits in the periplasm or sense fimbrial assembly through changes in the outer membrane. CpxA activation leads to phosphorylation of CpxR, which can activate gene expression. Solid arrows indicate known targets of CpxR and dotted arrows highlight proposed targets.

Appendix One

Influence of neutrophil defects on *Burkholderia cepacia* complex pathogenesis

Adapted from "Influence of neutrophil defects on *Burkholderia cepacia* complex pathogenesis"

Laura A. Porter and Joanna B. Goldberg

Frontiers in Cellular and Infection Microbiology. 2011; 1:9.

Abstract

The *Burkholderia cepacia* complex (Bcc) is a group of Gram-negative bacteria that are ubiquitous in the environment and have emerged as opportunistic pathogens in immunocompromised patients. The primary patient populations infected with Bcc include individuals with cystic fibrosis (CF), as well as those with chronic granulomatous disease (CGD). While Bcc infection in CF is better characterized than in CGD, these two genetic diseases are not obviously similar and it is currently unknown if there is any commonality in host immune defects that is responsible for the susceptibility to Bcc. CF is caused by mutations in the CF transmembrane conductance regulator, resulting in manifestations in various organ systems, however the major cause of morbidity and mortality is currently due to bacterial respiratory infections. CGD, on the other hand, is a genetic disorder that is caused by defects in phagocyte NADPH oxidase. Because of the defect in CGD, phagocytes in these patients are unable to produce reactive oxygen species, which results in increased susceptibility to bacterial and fungal infections. Despite this significant defect in microbial clearance, the spectrum of pathogens frequently implicated in infections in CGD is relatively narrow and includes some bacterial species that are considered almost pathognomonic for this disorder. Very little is known about the cause of the specific susceptibility to Bcc over other potential pathogens more prevalent in the environment, and a better understanding of specific mechanisms required for bacterial virulence has become a high priority. This review will summarize both the current knowledge and future directions

related to Bcc virulence in immunocompromised individuals with a focus on the roles of bacterial factors and neutrophil defects in pathogenesis.

Introduction

The *Burkholderia cepacia* complex (Bcc) is a group of Gram-negative bacilli that have emerged over the past thirty years as opportunistic pathogens in immunocompromised populations, specifically cystic fibrosis (CF) and chronic granulomatous disease (CGD). Bcc has recently been recognized as an emerging nosocomial pathogen in patients without CF and CGD, and its spread in the hospital setting has been associated with cross-transmission, frequent pulmonary procedures, and central venous access [179]. This complex of at least seventeen distinct species was first identified as a plant pathogen in 1950's and was shown to be the causative agent of onion rot [180-182]. Bcc is also present in many non-pathogenic, ecologically beneficial relationships with plants, where it can be advantageous through the production of antimicrobial compounds, degradation of contaminants, and fixation of nitrogen [182, 183].

Since *B. cenocepacia* and *B. multivorans* are the two most prevalent species in Bcc infections of both CGD and CF patients [179, 184], most investigations into the mechanisms of virulence have focused on these two species. Although we will attempt to more broadly apply these data to Bcc, it is currently unknown whether the recognized virulence factors always function similarly in all species of the

complex during their interactions with the host innate immune system. It is also unknown whether there are unique and distinct bacterial factors that are required for disease in CGD versus CF, considering that these two diseases do not share any significant known immune defects. Due to the specific sensitivity of these two patient populations to Bcc, there may be unrecognized commonalities in host defects that allow for Bcc infection.

Bcc as opportunistic pathogens

Because Bcc infections are primarily associated with individuals with CGD and CF, it is interesting to compare these two not obviously related genetic diseases. Table A1.1 summarizes the disease manifestations and defects in phagocyte function associated with CGD and CF.

B. cepacia (previously referred to as *Pseudomonas cepacia*) was recognized as an important pathogen in CGD in 1975 [185]. Bcc is now the leading cause of fatality from bacterial infections in CGD patients Winkelstein et al 2000 [186]. CGD is a genetic disorder caused by the inactivation of the phagocyte nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex. Because of this defect, some cells (neutrophils, mononuclear cells, macrophages, and eosinophils) in CGD patients are unable to produce reactive oxygen species, which results in ineffective clearance of some pathogens. These patients present with recurrent bacterial and fungal infections, which is frequently accompanied by uncontrolled inflammation

and granuloma formation. The rate of incidence in the United States is 1/200,000 live births, or at least 20 patients per year [187].

CGD can be caused by mutations in any of the four components of NADPH oxidase. Flavocytochrome b_{558} is the catalytic center of the complex, and is a heterodimer composed of $p22^{phox}$ and $gp91^{phox}$. It is contained on the membrane of specific granules and is incorporated into the phagosome membrane upon fusion [188]. It provides a channel for electrons to be transferred through from NADPH in the cytosol to oxygen that is contained in the phagosome [188]. The complex also contains two cytosolic components: $p67^{phox}$ and $p47^{phox}$ [189]. These cytoplasmic components are essential for stabilizing flavocytochrome b_{558} and facilitating the transfer of electrons. A defect in any one of these four components leads to inactivation of the complex and an inability to produce reactive oxygen species. X-linked recessive CGD is caused by defects in $gp91^{phox}$. Autosomal recessive CGD is caused by defects in $p47^{phox}$, $p67^{phox}$, or $p22^{phox}$. Approximately 65% of CGD cases are X-linked (defects in $gp91^{phox}$), 25% of cases are due to defects in $p47^{phox}$, and the rest are split evenly between defects in $p67^{phox}$ and $p22^{phox}$ [187]. There are two other genetic disorders with similar presentations to CGD that are not caused by mutations in NADPH oxidase components. Inhibitory mutations in Rac or deficiencies of glucose-6-phosphate dehydrogenase can also result in an inability to produce reactive oxygen species (ROS) [190].

Overall mortality for CGD patients is around 2-5% per year, but morbidity remains a major issue for these patients and their families, as most patients have

least one severe infection every 3-4 years [186, 191]. In the initial cases of CGD diagnosed between 1957-1976, *Staphylococcus* was the most common infectious agent, followed by the common enteric pathogens *Klebsiella* species and *Escherichia coli* [190]. Now *Aspergillus* species are the most common causative infectious agents, followed by Bcc and *Staphylococcus aureus* [186]. The most common presentations of infection include pneumonia, infectious dermatitis, and recurrent/severe abscess formation beneath the skin and in organs [186]. Other symptoms have been described that are not caused directly by infection, including enteritis/colitis, discoid lupus, and chorioretinitis [186, 192, 193]. The treatment regimen for these patients includes interferon-γ, antibacterial prophylaxis, and antifungal prophylaxis. Prophylactic treatment usually includes trimethoprim-sulfamethoxazole and itraconazole [194]. Bone marrow transplantation can cure the disease if a histocompatible donor is available, which is not an option for most patients. As with other monogenetic diseases, gene therapy has the potential to be an effective curative treatment for CGD, however, to date, the clinical trials have not been successful. The promise and problems associated with this approach have been recently reviewed [195].

CF is caused by mutations in the CF transmembrane conductance regulator (CFTR), which functions as an ion channel. This defect results in the accumulation of mucus on epithelial surfaces, which contributes to ineffective mucocilliary clearance in the lungs. CF is one of the most common inherited genetic diseases in the world with an annual frequency of approximately 1/3,000 live births [196]. In

fact, one out of every twenty-five Caucasians is a heterozygous carrier of a mutated CFTR gene [197]. The airway in CF patients is chronically colonized with potentially pathogenic bacteria, specifically *Pseudomonas aeruginosa, S. aureus,* and *Haemophilus influenzae*. Colonization and frequent infection of the CF airway leads to an excessive neutrophil (polymorphonuclear leukocyte, PMN) driven inflammatory response, which results in host tissue damage [198, 199]. These patients experience recurrent respiratory infections and chronic lung inflammation that eventually culminates in respiratory failure [200]. Pulmonary inflammation in CF is associated with high levels of inflammatory cytokines (IL-8, IL-6, TNF-alpha, and leukotriene B_4), reduced levels of anti-inflammatory cytokines and antiproteases, and a rapid and substantial influx of PMNs [201].

Bcc infections in both the CGD and CF patient populations initially present as a respiratory tract infection. An interesting distinction between infections in these two patient populations is the clinical presentation of this infection. In CF, pulmonary infection with Bcc generally causes endobronchial disease, and diagnosis is made by identification of bacteria in the sputum. In CGD, Bcc lung infection results in a pneumonic process associated with nodular infiltrates and diagnosis requires isolation following lung biopsy. Antibiotic treatment is generally able to clear Bcc in CGD, although recurrent re-infections do occur. In CF, Bcc infections are usually seen in older patients that are already chronically colonized with other CF pathogens, notably *P. aeruginosa* [197]. According to the 2009 Cystic Fibrosis Foundation Registry Report, about 2.7% of CF patients are infected with Bcc, and

these infections are associated with earlier fatality than non-Bcc infected patients. Potentially due to the chronic nature of Bcc infections in CF and the sustained presence of the microbial community in the form of biofilms in the CF lung, Bcc isolates associated with CF show a higher degree of antibiotic resistance than Bcc isolates from CGD infections [202]. Bcc infections in CF patients can result in a severe, necrotizing pneumonia, referred to as "cepacia syndrome", that is not observed in Bcc infections in CGD patients. However, Bcc infections in CGD patients can become invasive and result in septicemia.

CGD and CF are both rare genetic diseases and, while infections by Bcc are prominent in these populations, Bcc infections are still relatively infrequent. Studies investigating Bcc virulence in CGD and CF aim to decrease morbidity and mortality in these patient populations, but they can also increase our understanding of how healthy individuals are able to fight off these rare infections. Since Bcc infections are very uncommon in immunocompetent individuals, the normal host immune response must be able to efficiently remove these pathogens, and the immune response or environment in CGD and CF, as well as other immunocompromised patients, would appear to be defective in this clearance. It is the investigation into the distinction between these normal and susceptible hosts that gives insight into normal and defective immune function in bacterial infections. Previous studies investigating CGD and other rare genetic diseases have contributed greatly to knowledge in the field on normal phagocyte function and bacteria-phagocyte interactions [203-205]. A better understanding of the infectious processes in

patients with rare genetic defects in phagocyte function contributes to current knowledge on how normal, healthy individuals resist infections.

Functions of normal PMNs and defects in CGD and CF patients

CGD manifests as a defect in all phagocytes normally capable of producing ROS. Macrophages and PMNs are normally the focus of study of CGD host phagocyte-pathogen interactions because of their essential roles as professional phagocytes. A recent review by Saldias and Valvano has summarized the current literature on the interactions between Bcc and macrophages [206], thus here we will focus specifically on Bcc interactions with PMNs. Bcc infections are characterized by a rapid and robust recruitment of PMNs to the site of infection. Normal PMNs have the ability to phagocytose and kill bacteria via oxidative and non-oxidative mechanisms, but CGD PMNs are only capable of utilizing non-oxidative killing mechanisms, leading to ineffective clearance of some pathogens. Because of the essential role of PMNs in normal host responses to bacteria and the substantial contribution of defective PMN function in CGD to pathogenesis, this review will focus on the defects associated with CGD PMNs and will provide comparison with potential defects in CF PMN function.

PMNs are highly motile phagocytes that comprise the first line of defense of the innate immune system against bacteria and play a crucial role in host defense in the lung. Four percent of the adult body mass is devoted to the production of PMNs,

and as a result, PMNs are the most common nucleated cell in blood. Although PMNs are terminally differentiated once they leave bone marrow, they are still able to act autonomously as wandering phagocytes in all tissues, requiring highly specific and specialized responses [189]. They kill bacteria by fusing granules containing antimicrobial compounds with phagocytic compartments containing bacteria. These granules can also fuse with the plasma membrane to kill extracellular bacteria.

There are three types of granules that PMNs synthesize, and these different granule types contain molecules with different antimicrobial activities that often work synergistically to kill microbes once fused with the phagosome. Granules contain components that kill bacteria through both oxidative and non-oxidative mechanisms. Azurophil or primary granules contain myeloperoxidase, three neutral proteinases (cathepsin G, elastase, and proteinase 3), bactericidal permeability-increasing protein (BPI), and defensins. Specific or secondary granules contain lactoferrin, transcobalamin II, lysozyme, gelatinase-associated lipocalin, and flavocytochrome b_{558}. Gelatinase or tertiary granules are classified by the presence of gelatinase but not lactoferrin and can contain any of the other components in specific granules. The matrix in granules contains negatively charged sulphated proteoglycans that sequester antimicrobial components until the granule is fused with the phagosome [188, 207].

Oxidative killing relies on the generation of ROS, primarily through NADPH oxidase. Electron transfer to oxygen through NADPH oxidase generates superoxide

(O_2^-) in the phagosome. Superoxide is rapidly degraded into hydrogen peroxide (H_2O_2) by superoxide dismutase. Hydrogen peroxide can readily cross bacterial membranes and cause intracellular damage. CGD PMNs are defective in their ability to assemble functional NADPH oxidase complexes and therefore are unable to utilize oxidative killing mechanisms.

In addition to oxidative killing mechanisms, normal PMNs can also use non-oxidative killing, which includes interactions between cationic regions of the granule proteins and the negatively-charged bacterial membrane, as well as bacterial degradation by granule proteases. Examples of cationic peptides and proteins involved in non-oxidative killing of bacteria are BPI, LL-37, defensins, and azurocidin. These cationic granule peptides and proteins bind to and accumulate on the bacterial surface, leading to membrane permeabilization and osmotic lysis. These components are extremely important in non-oxidative killing of Gram-negative pathogens by PMNs [208]. Granules also contain other components that possess different antimicrobial activities. Serine proteases degrade phagocytosed bacteria [209]. Lactoferrin sequesters iron away from pathogens, but also possesses direct antimicrobial activity [210]. Many of the factors that function in non-oxidative killing strategies act synergistically or depend on each other for full activity after fusion or exocytosis. The lack of functional NADPH oxidase in CGD results in the reduced flux of potassium ions into the phagolysosome [189]. This influx plays a role in the release of cationic proteins from the matrix, and because of this CGD PMNs may also exhibit defects in non-oxidative killing. In CGD PMNs, the

phagolysosome additionally does not increase pH and does not acidify as profoundly, which also has negative effects on the activity of antimicrobial peptides and enzymes [211].

Normal PMNs also use neutrophil extracellular traps (NETs) as a mechanism to kill extracellular microbes. These NETs are composed of PMN chromatin coated in granule components that have been expelled from the cell. The chromatin binds extracellular bacteria and fungi, trapping them in close contact with high concentrations of antimicrobial granule contents. NET formation is dependent on the generation of reactive oxygen species by NADPH oxidase, and therefore CGD PMNs are also deficient in their ability to produce NETs [212]. It has been shown that this defect also contributes to the inability of CGD PMNs to kill pathogens commonly associated with infections in CGD, specifically *S. aureus* and *Aspergillus* species [213, 214].

Most prior research has focused on the lung epithelial cell defects in CF [215, 216]. However more recently, there has been an emphasis in the field on defective immune cell function in the CF lung environment, particularly on potential defects in PMN function. These studies may assist in bridging the gap in understanding the specific susceptibilities of these two patient populations to Bcc infections. Defects in CF PMN function could contribute to ineffective clearance of lung pathogens in the presence of zealous PMN recruitment and inflammation [196]. It is already well established that PMN necrosis and release of proteolytic enzymes contribute significantly to the tissue damage in the CF lung. It has been shown that PMNs can

become trapped in bacterial biofilms in the CF lung and undergo necrosis; after necrosis, their biomass, NETs, and intracellular leakage can contribute biofilm formation and lung injury [214, 217, 218]. CF PMNs display excessive and prolonged superoxide production when stimulated, are constitutively primed (likely via the presence of IL-8, TNF-alpha, and *P. aeruginosa* alginate in the CF airway), and are unresponsive to anti-inflammatory signals through IL-10 [219-221]. Another observed potential defect is an alteration in degranulation; greater levels of primary granule components are secreted extracellularly by CF PMNs than by normal PMNs, possibly due to effects of intracellular pH on signaling [220-223]. This release of toxic granule contents contributes to the extensive lung damage characteristic of CF. The absence of CFTR has also been shown to have direct effects on PMN function. The addition of chloride ion to hydrogen peroxide to produce hypochlorous acid (HOCl) through myeloperoxidase is another ROS-dependent killing mechanism, and CF PMNs display a decreased ability to chlorinate bacteria and a decreased ability to kill *P. aeruginosa* that is exacerbated by a chloride-deficient microenvironment [224, 225].

There are also indications that the environment in the CF lung may significantly impair PMN function. High concentrations of neutrophil elastase present in the CF lung cleave CXCR1, Fcγ receptors, and iC3b, as well as lead to the loss of CD16 and CD14 expression [199, 226-229]. The loss of these receptors leads to deceased complement-dependent phagocytosis. Hypersalinity of the airway surface fluid has been shown to inhibit the killing actions of epithelial cells in the CF

lung through inactivation of β-defensin-1 and potentially other antimicrobials, although these findings are considered somewhat controversial in the CF field [230-232]. Peripheral PMNs isolated from CF patients show comparable NET formation to normal PMNs, but there is also evidence that PMNs in the CF lung environment display increased NET formation that contributes to the pulmonary obstruction typical in CF [233, 234]. Further investigation into defective phagocyte function in CF could provide insight into links between the susceptibilities of both these patient populations to Bcc infections.

Although resident and migrating phagocytes play an essential role in bacterial clearance in the airway, there are other important components of the host defense at the mucosal airway surface. The airway epithelium protects the host from infection at the interface with the environment, where it is constantly exposed and bombarded with microbes. Epithelial cells produce cytokines, chemokines, and antimicrobial peptides, either constitutively or upon stimulation with microbial products [235]. Examples of antimicrobial products produced by airway epithelial cells include β-defensins, LL-37, and lactoferrin [235]. Dual oxidases (Duox) are also expressed by epithelial cells in the airways and produce hydrogen peroxide, which contributes to antimicrobial activities through the lactoperoxidase system [236]. This theoretically allows for partial reconstitution of ROS production in the lung in response to bacterial infection, but interesting it has been shown that Bcc does not stimulate hydrogen peroxide production by bronchial epithelial cells through Duox [237].

Models used to assess virulence of Bcc in CGD and CF

Many previous studies have focused on Bcc virulence in models of CF, and these models have been well described in other reviews [183, 238, 239]. Therefore, this section will focus on describing models of CGD and their potential for use in studies of Bcc virulence. Many *in vitro* and *in vivo* models of CGD are available, and some of these models have been adapted to assess microbial virulence in this patient population. There are two characterized, commercially available mouse models of CGD. The first is a model of X-linked CGD, which lacks the mouse equivalent of human $gp91^{phox}$ [240]. This strain of mouse exhibits a complete lack of functional protein, an inability to form a functional NADPH oxidase complex, and phagocytes that are unable to produce a respiratory burst. Bcc are virtually avirulent in wild-type mouse strains but are able to cause lethal infections in mouse models of CGD [241, 242]. Bcc CF isolates have been tested in this model, and the infecting bacteria multiplied five orders of magnitude in 3-6 days after intratracheal infection. The infected mice displayed neutrophil-dominated lung inflammation and abscesses, sepsis, and eventual death, which models a disease progression reflective of that observed in CGD human hosts. Isogenic mutants in some known virulence factors of *B. cepacia* and *B. cenocepacia*, in particular in quorum sensing and exopolysaccharide production, as well as a panel of less virulent isolates were also examined, and a loss or decrease in virulence was observed [241].

The autosomal recessive mouse model of CGD lacks the mouse equivalent of $p47^{phox}$, which is the complex component most commonly associated with human cases of autosomal recessive CGD. Characterization of this model showed a phenotype similar to human CGD; these mice spontaneously developed severe infections, usually by the same or related pathogens that are associated with infections in human CGD hosts [243]. Phagocytes from these animals display an inability to produce an oxidative burst and are unable to kill *S. aureus* and other pathogens characteristic of CGD [243]. This model has also been used to study the virulence of Bcc in CGD. *B. multivorans* clinical isolates from both CGD and CF patients were shown to establish lethal infections when injected intraperitoneally, while an environmental isolate was avirulent [244].

Mouse models allow analysis of Bcc virulence in a CGD host, but additional approaches are necessary to directly investigate Bcc interactions with host phagocytes. Both cell lines and primary cells have been used in assessment of bacterial virulence in interactions with CGD phagocytes. PMNs and macrophages can be derived from CGD patients, when available, or from CGD animals [244-246]. Because these cells are isolated directly from patients or animals with CGD, these cells most closely replicate the CGD defect, but unfortunately are unable to be genetically modified or adequately controlled experimentally. Also, the use of primary cells adds an additional level of variation to experiments due to donor-specific genetic and environmental factors. Cell lines that are genetically modified to lack functional NADPH oxidase proteins have also been constructed. PBL-985, a

human myeloid leukemia cell line, is deficient in gp91^{phox} and can be differentiated into monocytes or granulocytes [247]. These cells can be further genetically modified and lack the donor variability of primary cells. Unfortunately, PMNs are terminally differentiated cells, and precursor immortal cell lines must be differentiated *in vitro* into a PMN-like state. In a related promyelocytic cell line, HL-60, it has been demonstrated that *in vitro* differentiation from the promyelocytic stage results in an inability to synthesize secondary and tertiary granules [248]. Therefore, these precursor cell lines lack the full array of antimicrobial activities associated with mature, terminal PMNs. There are also variants of the J774.16 murine macrophage cell line that are deficient in gp91^{phox}, and do not need to be differentiated [249]. These immortal macrophage lines can be used to broadly study phagocyte interactions but are unable to utilize the full repertoire of killing mechanisms employed by PMNs.

Normal cell lines and primary cells can also be treated with inhibitors of NADPH oxidase function to create a CGD phenotype. Diphenyleneiodonium (DPI) is the most commonly used inhibitor and acts on flavoproteins, such as flavocytochrome b_{558} of NADPH oxidase. An electron is extracted from flavin adenine dinucleotide (FAD) by DPI to form a radical, which is then added back to protein groups in or near the active site of the flavoprotein to form covalent, phenylated adducts. Studies have also shown that DPI can also act on and inhibit nitric oxide synthase and xanthine oxidase [250], while anaerobic killing, phagocytosis, and motility are not significantly affected by DPI-treatment [251].

There is a slight decrease in degranulation of azurophilic and specific granules after DPI-treatment, which is similar to what has been observed in PMNs isolated from CGD patients [251]. Another well-studied NADPH oxidase inhibitor is apocynin (4-hydroxy-3-methoxyacetophenone, also known as acetovanillone), which is a methoxy-substituted catechol that inhibits superoxide production by phagocytes while not affecting phagocytosis. It likely inhibits complex assembly by blocking sulfhydryl groups [252]. Although use of these inhibitors produces a CGD phenotype and creates an *in vitro* system amenable to controlled experimentation, the cells may exhibit additional defects due to non-specific action of these inhibitors or may not manifest completely the defects of CGD phagocytes. Also, *in vitro* experimentation with either primary cells or immortal cell lines can only be used to assess virulence factors that directly interact with host cells in comparison to factors that may indirectly affect cells through modulating the host environment.

Resistance of Bcc to killing by PMNs

Bcc is a very diverse group of pathogens, both genetically and phenotypically. Studies have shown remarkable differences in virulence between clinical isolates and environmental isolates of Bcc, and even clinical strains of the same species have been observed to have varying virulence *in vitro* and in mouse models. In the context of Bcc infections in CF [238], factors have been shown to have direct effects on interactions with host innate immune cells. Both Figure A1.1 and Table A1.2

illustrate the contribution of Bcc factors to bacterial survival in interactions with phagocytes. Most of the factors described in this review have been investigated mainly in models of CF, but these factors may have broad roles for protection from host phagocyte killing and are likely also important in Bcc survival in interactions with CGD PMNs.

B. cenocepacia has been shown to be readily ingested by normal PMNs and phagocytosis is associated with a robust oxidative burst. In PMN bactericidal assays, Bcc is killed by normal PMNs but is able to persist when incubated with CGD PMNs [244, 253]. Similarly *B. cenocepacia* has been found to induce PMN apoptosis, an effect dependent on viable bacteria. CGD PMNs were also equally apoptotic in comparison to normal PMNs after phagocytosis of *B. cenocepacia*, but *B. cenocepacia* also induced PMN necrosis in a subset of CGD PMNs, which could contribute to increased virulence in this population. This was true in both primary CGD PMNs and PMNs treated with DPI [254]. Another study found that a CGD clinical isolate of *B. multivorans* showed a higher association with CGD PMNs than with PMNs from normal, healthy donors, which suggests an additional distinction between the interactions of Bcc with normal and CGD PMNs [244].

Defenses against host oxidative killing

In the course of an infection, successful pathogens must evade the innate immune system in order to persist. Because of the prevalence of Bcc as CGD

pathogens, oxidative killing mechanisms by host phagocytes, specifically neutrophils, are thought to be essential for Bcc clearance in healthy individuals. Superoxide dismutases and catalases protect bacteria from oxidative damage during normal respiration and in interactions with the host innate immune response. Bcc has been shown to be resistant to concentrations of ROS *in vitro* that are equivalent to an oxidative burst and this is due to periplasmic superoxide dismutase (Sod), catalase-peroxidases (Kat), and an antioxidant melanin-like pigment [206, 255]. Both catalase and superoxide dismutase are universal mechanisms of bacterial resistance to host oxidative killing. A pigment produced by *B. cenocepacia* C5424 has been studied, and the absence of the pigment in a strain mutated in *hppD* (4-hydroxyphenylpyruvate dioxygenase) was shown to lead to an increase in sensitivity to extracellular superoxide and hydrogen peroxide in *in vitro* bacterial viability assays [256]. This study also showed that the mutant co-localized to a higher extent than the wild-type strain with more degradative compartments in macrophages, and that this co-localization was dependent on NADPH oxidase activity. This finding suggested that wild-type bacteria that are able to produce the melanin-like pigment are more protected from oxidative killing in the phagosome, and that mutant bacteria that are unable to produce the pigment are associated with a loss of bacterial viability inside the phagosome and an inability to delay progression to a more degradative compartment. Thus, the melanin-like antioxidant pigment produced by *B. cenocepacia* has been shown to be protective against host oxidative killing [256]. However not all strains of Bcc isolated from

patients produce this pigment, suggesting that additional mechanisms of resisting host oxidative killing must exist.

Exopolysaccharides are bacterial virulence factors that have roles in evasion of host defenses, adhesion, and resistance to antimicrobials. Bcc produces at least four different exopolysaccharides, and the predominant exopolysaccharide is the heptasaccharide cepacian [257, 258]. Not all Bcc isolates make cepacian, but most clinical isolates do produce exopolysaccharide [258]. Cepacian produced by *B. cenocepacia* has been shown to scavenge reactive oxygen species and inhibit neutrophil chemotaxis in *in vitro* migration assays [259]. Mutants that are unable to produce cepacian are less virulent than wild-type, cepacian-producing strains in a $gp91^{phox-/-}$ mouse model of CGD, which suggests that this exopolysaccharide is potentially an important bacterial factor for virulence in CGD [241].

Defenses against host non-oxidative killing

Bcc possesses tools to combat non-oxidative killing mechanisms, and these factors likely play an essential role in bacterial pathogenesis in CGD hosts that are defective in their ability to kill bacteria through oxidative killing mechanisms. Zinc metalloproteases are commonly utilized by bacteria to proteolytically degrade host antimicrobials. *B. cenocepacia* zinc metalloproteases ZmpA and ZmpB have been shown to act in resisting bacterial killing by host antimicrobial peptides in *in vitro* bacterial viability assays [260]. ZmpB cleaves β-defensin-1, ZmpA cleaves LL-37,

and both enzymes cleave elafin and secretory leukocyte inhibitor. All of these targets are components of the innate immune response to bacteria. Mutants in both *zmpA* and *zmpB* have been tested in a rat agar bead model, which is commonly used for assessing virulence of pathogens that chronically infect CF patients. Both mutant strains were able to persist and were present in the lung 14 days after infection, but they were less virulent than the wild-type strain. The *zmpA* mutant was characterized by a reduction in bacterial load in comparison to the wild-type strain, and the *zmpB* induced less lung tissue inflammation [261]. This indicates that ZmpA and ZmpB are required for full virulence but are not required for bacterial survival in this model. It would be interesting to evaluate these mutants in a mouse model of CGD, since such an acute model of infection could directly assess the importance of these zinc metalloproteases to infection.

Lipopolysaccharide (LPS) is dually important both in targeting of and protection from non-oxidative killing. LPS is a major component of Gram-negative membrane and is composed of lipid A, core oligosaccharides, and O-antigen. The negative charge of lipid A is an important binding target for cationic antimicrobial peptides. The lipid A of Bcc contains Kdo (3-deoxy-D-*manno*-oct-2-ulosinic acid), Ko monosaccharide (D-*glycero*-D-*talo*-oct-ulosonic acid), and Ara4N (4-amino-4-deoxy-L-arabinose) [262, 263]. Substitution of Ara4N on the lipid A and inner core oligosaccharide is protective against cationic antimicrobials, and synthesis of Ara4N is essential in *B. cenocepacia* for survival [264]. Inner core oligosaccharides are required for *in vitro* resistance to antimicrobial peptides and survival in a rat model

of lung infection. Specifically, a deep rough mutant (lacking both O-antigen and the inner core) of *B. cenocepacia* was more sensitive to killing by polymyxin B, melittin, and HNP-1 in *in vitro* bacterial viability assays [265].

In a signature-tagged mutagenesis screen of *B. cenocepacia* K56-2 in the rat agar bead model of chronic infection, several clones were identified as being avirulent that contained insertions in the genetic region responsible for O-antigen synthesis, indicating that under these conditions O-antigen was essential for virulence [266]. Interestingly, clinical isolates have been isolated with either an LPS-rough (with no O-antigen) or an LPS-smooth (with O-antigen) phenotype, indicating that O-antigen may not be required for virulence in all species of the complex under all conditions. For example, one of the best characterized *B. cenocepacia* isolates, the ET-12 lineage CF isolate J2315, which displays an LPS-rough phenotype. O-antigen has been shown to be essential for serum resistance but not in resistance to host antimicrobials [267].

In addition to factors that have been recognized to act directly to protect against host defenses, genetic regulators can also contribute to virulence. The alternative sigma factor RpoE, a transcriptional regulator, has shown to be required for lysosomal fusion delay, growth under high osmolarity, and growth under high temperature [268]. RpoE has also been shown to play a role in resistance to polymyxin B in *in vitro* bacterial viability assays, which suggests that expression of RpoE may protect the bacteria from non-oxidative killing as well [269]. Collectively,

these two studies do implicate that regulation by RpoE is important for Bcc virulence, but the RpoE regulon is yet to be determined.

Since normal, healthy individuals do not typically contract clinical Bcc infections, the virulence associated with each of these factors likely relies partially on host susceptibility and bacterial factors, which may differ between these two genetic disorders. Although the virulence factors studied in CF likely also play important roles in Bcc pathogenesis in CGD, this is by no means a comprehensive list and research is currently being conducted to learn more about specific roles of both these molecules as well as currently unrecognized factors using models for assessing virulence in CGD.

Concluding Remarks and Future Directions

Despite the extensive amount of focus and new insight into Bcc virulence obtained in the past decade, there is still much to learn. Bcc is a very diverse group comprising of at least seventeen distinct species, and the majority of studies have focused on only the most prevalent three species: *B. cenocepacia*, *B. multivorans*, and *B. cepacia*. Also, many clinical strains and available isogenic mutants within these species have only been investigated in subsets of the models available to assess virulence in either CGD or CF. A more comprehensive view of the virulence of this complex will be obtained through future studies characterizing the similarities and differences between each species and the role of each virulence factor in many

models. It is already recognized that clinical isolates from the same species (example: *B. cenocepacia* J2315 and K56-2) display different virulence phenotypes, and differences between these related isolates could be characterized. In addition, most studies have focused on Bcc virulence in the context of CF. As presented in this review, many of these studies have contributed knowledge that can be applied to potential host-pathogen interactions in CGD. Further characterization of known virulence factors in models of CGD and high-throughput genetic screens to identify virulence factors that are essential specifically in CGD will generate a more complete understanding of Bcc pathogenesis in CGD. Also, as previously mentioned, there is no clear link between the immune defects in CF and CGD, and therefore the progression of disease and factors required could differ greatly between infections in these two patient populations.

A majority of the work investigating Bcc-host phagocyte interactions up to now has focused on macrophages, mainly due to the availability of immortal cell lines. Due to the large influx of PMNs to the site of infection in both CF and CGD, PMNs are playing an extremely important role in pathogenesis. The bacterial killing mechanisms used by PMNs are more numerous and varied than that of macrophages, and the process of phagosomal maturation also varies greatly between PMNs and macrophages. It is thought that Bcc exists in an intracellular Bcc-containing vacuole (BcCV) in macrophages, but it is unknown whether Bcc is able to persist in a similar compartment in PMNs [270]. It has been shown that both the type IV and type VI secretion systems play a role in interactions with

macrophages and may be important for intracellular survival, but it is unknown what their role is in interactions with PMNs [271, 272]. It has been demonstrated that Bcc is ineffectively killed and cleared by CGD PMNs [244, 253], but it is not clear whether the surviving Bcc are intracellular or associated extracellularly with PMNs. This distinction is very important for understanding the mechanism in which Bcc is able to persist in this patient population.

Another avenue for future research is to identify mechanisms of effective killing and clearance of Bcc in normal individuals. Normal PMNs are able to effectively kill Bcc while CGD PMNs are unable to do so. Because of the defect in oxidative killing in CGD, this was suggestive of oxidative killing being important for clearance by normal PMNs. However, *in vitro* studies have demonstrated high resistance of Bcc to both oxidative and non-oxidative killing mechanisms. There are also other defects in non-oxidative killing associated with CGD PMNs, specifically in granule component activation and NET formation. It could also be the synergistic effects of all of these defects together that allow for persistence in this patient population as well as in CF patients. These mechanistic studies are difficult in a terminally differentiated cell that is not amenable to genetic manipulation but could be feasible *in vitro* using purified components or in cell-based assays using combinations of inhibitors.

A more broad implication of understanding Bcc pathogenesis in CGD is to search for commonalities in virulence strategies employed by all CGD pathogens. Since the spectrum of potential pathogens causing infections in this population is

very narrow and most infectious agents are opportunistic, there may be very specific factors or strategies utilized by this group of microbes to cause infections in CGD. Understanding these overarching themes of resistance to killing in the CGD host could lead to the development of both preventative care and treatment options.

Since Bcc infections are typically restricted to patients with CF or CGD, the comparison of the defects in these two genetic diseases remains an interesting venue for future research. As previously described in this review, Bcc infections in CF occur subsequent to colonization with *P. aeruginosa*, and the presence of bacterial biofilms and the effect of these biofilms on PMN function may contribute to the ability of Bcc to persist in CF patients. A recent study confirmed the contribution of the CF lung environment to susceptibility to Bcc by infecting wild-type, normally resistant mice with *B. cenocepacia* and alginate from *P. aeruginosa*; in this case the bacteria were able to persist and cause lung inflammation [273]. It is also hypothesized that the CF lung environment may result in defective PMN function through anaerobic mucus-covered airways, high protease concentrations, and high levels of inflammatory signals [196, 274]. In addition to the lung environment, treatments for CF may also impact PMN function. Macrolide therapy has been shown to significantly improve lung function in CF, but these drugs also accelerate PMN apoptosis, inhibit ROS production by PMNs, and inactivate neutrophil elastase [275-277]. The use of macrolides is largely ineffective in treatment and prevention of Bcc, and its effects on PMN function may actually contribute to the pathogenicity of Bcc in CF [278]. A more complete understanding of PMN defects in CF, whether

attributed to treatment, the inflammatory environment, or the CFTR defect directly, could find a link between immune function in these two patient populations and better define the requirements for Bcc virulence in immunocompromised populations.

Table A1.1 Comparison of disease presentation and phagocyte function in CGD and CF		
	CGD	**CF**
Disease Presentation		
Mutation	NADPH oxidase	CFTR
Prevalence	1/200,000 live births (US)	1/3,000 live births
Clinical presentation	Recurrent bacterial and fungal infections, most commonly pneumonia, infectious dermatitis, and recurrent/severe abscess formation	Chronic and recurrent bacterial colonization and inflammation of the lungs
Infections commonly associated	*Aspergillus, Burkholderia, Serratia, Staphylococcus*	*Pseudomonas, Staphylococcus, Haemophilus*
Defects in phagocyte function		
Chemotaxis	Robust recruitment of PMNs to sites of inflammation, despite potential	Increased presence of and reduced responsiveness to

	effects of ROS defect on PMN chemotaxis	PMN chemoattractants (leukotriene B_4, IL-8), robust recruitment of PMNs to CF lung
Phagocytosis	No known defects.	High concentrations of neutrophil elastase present in the CF lung cleave CXCR1, Fcγ receptors, and iC3b, as well as lead to the loss of CD16 and CD14 expression [196]. The loss of these receptors leads to deceased complement-dependent phagocytosis.
Oxidative Killing	Inability to produce ROS caused by defect/inability to assemble functional NADPH oxidase complex [279].	No physical defects in ability to produce normal quantities of ROS, but the inflammatory CF lung environment may contribute to any abnormal oxidative responses

Non-oxidative killing	Decreased flux of potassium ions into the phagolysosome, lack of increase in pH, and lack of normal acidification [189]. The combination of these factors leads to a decrease in the activity of antimicrobial peptides and enzymes. No NET formation [212].	Potential alteration in degranulation; greater levels of primary granule components are secreted extracellularly by CF PMNs than normal PMNs, possibly due to effects on intracellular pH on signaling [196]. No intrinsic defects in the ability to produce NETs [234].

Table A1.2 Bcc Virulence factors involved in interactions with host phagocytes		
	Role	References
Oxidative killing		
Catalase (KatA, KatB, and KatG)	Catalyzes degradation of hydrogen peroxide into water and oxygen	[280, 281]
Superoxide dismutase (SodB and SodC)	Catalyzes degradation of superoxide into hydrogen peroxide	[282]
Melanin-like pigment	Antioxidant pigment, protects against host oxidative stress	[256]
Cepacian exopolysaccharide	Scavenges ROS, inhibits PMN chemotaxis	[259]
Non-oxidative killing		
Zinc metalloproteases (ZmpA and ZmpB)	Proteolytically degrades host antimicrobials (examples: LL-37, β-defensin-1, and secretory leukocyte inhibitor)	[260, 261]
Lipopolysaccharide core	Confers resistance to polymyxin B and host antimicrobial peptides	[265]

RpoE (σ^E)	Alternative sigma factor that regulates genes required for resistance to polymyxin B, which likely contribute to resistance to host non-oxidative killing	[269]
Intracellular survival		
RpoN (σ^N)	Alternative sigma factor that regulates genes required for delay of phagolysosomal fusion in macrophages, traditionally involved in gene regulation in nitrogen-limited environmental conditions	[283]
RpoE (σ^E)	Alternative sigma factor that is required for delay of phagolysosomal fusion in macrophages, traditionally involved in gene regulation in response to extracytoplasmic/heat stress	[268]
Type IV secretion system	Required for intracellular survival and replication in macrophages, may play a role in normal endocytic processing	[272]
Type VI secretion system	Involved in actin rearrangements in macrophages	[271]

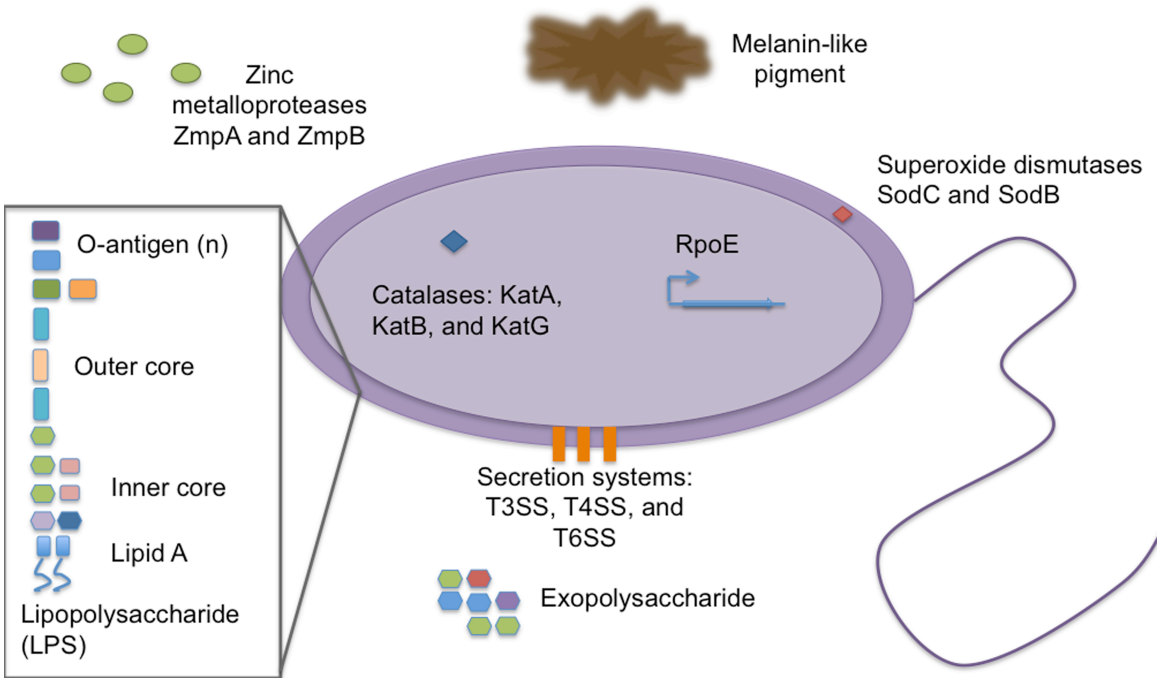

Figure A1.1 Bcc virulence factors important for survival in interactions with host phagocytes. Representation of known virulence factors utilized by members of Bcc in order to persist when confronting host phagocytes. The description and evidence in support of the roles of these factors is described in the text.

Appendix Two

Single amino acid substitution in homogentisate 1,2-dioxygenase is responsible for pigmentation in a subset of *Burkolderia cepacia* complex isolates

Adapted from "Single amino acid substitution in homogentisate 1,2-dioxygenase is responsible for pigmentation in a subset of *Burkolderia cepacia* complex isolates"

Laura A. Gonyar, Sarah C. Fankhauser, and Joanna B. Goldberg

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Abstract

The *Burkholderia cepacia* complex (Bcc) is a group of Gram-negative bacilli that are ubiquitous in the environment and have emerged over the past thirty years as opportunistic pathogens in immunocompromised populations, specifically individuals with cystic fibrosis (CF) and chronic granulomatous disease (CGD). This complex of at least eighteen distinct species is phenotypically and genetically diverse. One phenotype observed in a subset of *Burkholderia cenocepacia* (a prominent Bcc pathogen) isolates is the ability to produce a melanin-like pigment. Melanins have antioxidant properties and have been shown to act as virulence factors allowing pathogens to resist killing by the host immune system. The melanin-like pigment expressed by *B. cenocepacia* is produced through tyrosine catabolism, specifically through the autoxidation and polymerization of homogentisate. *B. cenocepacia* J2315 is a CF clinical isolate that displays a pigmented phenotype when grown under normal laboratory conditions. We examined the amino acid sequences of critical enzymes in the melanin synthesis pathway in pigmented and non-pigmented Bcc isolates, and found that an amino acid substitution of glycine (G) for arginine (R) at amino acid 378 in homogentisate 1,2-dioxygenase (HmgA) correlated with pigment production; we identify this as one mechanism for expression of pigment in Bcc isolates.

Introduction

The *Burkholderia cepacia* complex (Bcc) is a group of Gram-negative bacilli that are found ubiquitously in the environment and have emerged over the past thirty years as opportunistic pathogens in immunocompromised populations, specifically cystic fibrosis (CF) and chronic granulomatous disease (CGD). This complex of at least eighteen distinct species is the leading cause of bacterial infections in CGD patients, who are characterized by increased susceptibility to bacterial and fungal infections caused by genetic defects in phagocyte NADPH oxidase (Phox) [186]. Bcc is a significant cause of morbidity and mortality in both CGD and CF [186, 187, 284] and reviewed in [183]. During the course of an infection, Bcc must combat the innate immune system in order to persist. Because of the prevalence of Bcc as CGD pathogens and the fact that CGD patients have defects in oxidative killing mechanisms, it is hypothesized that this mechanism of killing by host phagocytes is essential for Bcc clearance in healthy individuals [244, 253] and reviewed in [285]. Superoxide dismutases and catalases are enzymes commonly used to protect bacteria from oxidative damage during normal respiration and in interactions with the host innate immune response, but pathogens can also utilize additional factors that assist in protection from host oxidative killing mechanisms.

Melanins are produced by all kingdoms of life and are a normal component of human skin and hair, but they can also be exploited by pathogens to promote their own survival in human hosts. Melanins are characterized as structurally diverse, high molecular weight polymers that are composed of quinolines, which can exist in

three different oxidation states [286-289]. Melanins act as a trap for unpaired elections, and this activity contributes to the antioxidant role in the presence of reactive oxygen species [290]. Different types of melanins are characterized by the pathways from which they are derived. Eumelanins are synthesized from 3,4-dihydroxyphenylalanine (DOPA) by phenoloxidases. Yellow or reddish melanins incorporate cysteine with DOPA and are called pheomelanins. Melanins derived from homogentisic acid (HGA) by tyrosinases are called pyomelanin [291]. Melanins formed from acetate via the polyketide synthesis pathway are called dihydroxynaphthalene melanins [292].

Melanins confer a survival advantage in the environment as well as the host. Melanins have been shown to increase microbe survival through protection from oxidative stress, digestive enzymes (in amoeboid and nematode predators, as well as phagocytes), radiation, extreme temperature, and heavy metal toxicity [292]. *Cryptococcus neoformans*, *Aspergillus* sp., *Wangiella dermatitidis*, *Sorothrix schenckii*, and *Burkholderia cepacia* are all pathogens that produce melanin, but the role of melanin in defense against the host is best characterized in *Cryptococcus neoformans* and *Aspergillus* species [293].

The melanin-like pigment produced by *B. cenocepacia* C5424 was characterized in a study by Keith *et al*., and it was shown *in vitro* that absence of the pigment led to an increase in sensitivity to hydrogen peroxide and extracellular superoxide [256]. This study also showed that the pigment-deficient mutant colocalized to a higher extent than the wild-type strain with more degradative compartments in macrophages, and that this increase in co-localization was

dependent on Phox activity [256]. This suggested that pigment-deficient bacteria were less protected from oxidative killing in the phagosome, and the inability to delay progression to a more degradative compartment was a potential reason for the sensitivity. The study by Keith *et al*. also began to describe the distribution of this pigmented phenotype amongst Bcc strains. They reported that out of the 22 *B. cenocepacia* isolates in their collection, only 4 were pigmented after 72 hours of culture on L-agar. They attempted to complement the non-pigmented *B. cenocepacia* strain K56-2 with the 4-hydroxyphenylpyruvate dioxygenase (*hppD)* gene from the pigmented *B. cenocepacia* C5424 strain, however this did not confer a pigmented phenotype to *B. cenocepacia* K56-2 [256]. These data demonstrate that the pigment is important for *B. cenocepacia* survival in interactions with host immune cells, but it is still not understood what mechanisms allow for pigmentation in a subset of Bcc isolates.

We have identified homogentisate 1,2-dioxygenase (HmgA) as a potentially critical enzyme for regulation of pigment production. We have observed a correlation between a glycine to arginine change at residue 378 of homogentisate 1,2-dioxygenase (HmgA) and pigment production in members of the Bcc. This study tests the hypothesis that one mechanism for pigmentation in Bcc isolates is defective HmgA activity due to a G378R amino acid substitution.

Results and discussion

B. cenocepacia J2315 produces a melanin-like pigment

Because of the established role of the pigment produced by Bcc as a potential virulence factor, we began to characterize the expression of this pigment by *B. cenocepacia* J2315. *B. cenocepacia* J2315 is the sequenced type strain and a member of the ET-12 lineage. *B. cenocepacia* J2315 is a CF clinical isolate that displays a pigmented phenotype when grown under normal laboratory conditions; pigment becomes visually detectable at stationary phase.

B. cenocepacia J2315 encodes homologous genes for all enzymes in the pyomelanin synthesis pathway (Figure A2.1A) but does not encode any genes required for eumelanin or pheomelanin synthesis [294]. Pyomelanins are synthesized through tyrosine catabolism with HGA as an intermediate. Tyrosine is converted to 4-hydroxyphenylpyruvate (4HPP) by the aromatic aminotransferase TyrB. There are two homologues to TyrB encoded in the genome of *B. cenocepacia* J2315: BCAL2303 and BCAM1478. 4HPP is then modified by HppD (BCAL0207) to form HGA. HGA is then released from the cell, oxidized, and polymerized to form pyomelanin. HmgA facilitates diversion of HGA into an alternative pathway resulting in the production of acetoacetic acid and fumaric acid. Previous work by Keith *et al*. defined the pigment produced by *B. cenocepacia* strain C5424 as a pyomelanin synthesized by this pathway [256], and we predicted that the pigment produced by J2315 would be synthesized via the same mechanism. As expected with a pigment synthesized through tyrosine catabolism, growth in tyrosine (Luria broth supplemented with 1 mg per mL L-tyrosine) leads to increased pigment production (Figure A2.1B). Additionally, we created a deletion mutant in *hppD* in *B. cenocepacia* J2315 and this mutant, J2315 Δ*hppD*, exhibited a non-pigmented

phenotype (Figure A2.1C), which corroborates what was shown by Keith *et al.* in *B. cenocepacia* C5424 [256]. Since HppD is required for pyomelanin production and not involved with other melanin synthesis pathways, we concluded that *B. cenocepacia* J2315 is producing a pigment through the pyomelanin synthesis pathway, which had not previously been established.

Identification of amino acid substitution in HmgA

Several studies using other pathogenic bacteria have associated defects in HmgA function with a pigmented or hyperpigmented phenotype [295-298]. Since HmgA catalyzes an enzymatic step that shuttles HGA into another pathway where it is further degraded to acetoacetic acid and fumaric acid, a decrease in HmgA activity would lead to accumulation of HGA and to an increase in pigment production. After aligning the inferred amino acid sequence of HmgA in sequenced, publicly available Bcc strains, we observed a glycine to arginine amino acid change at residue 378 that only occurred in pigmented Bcc strains (Figure A2.2). The glycine at this residue was conserved amongst Bcc isolates and in other bacterial genera (Figure A2.2). To further investigate this correlation, we characterized the pigmentation phenotype and sequenced HmgA in two additional Bcc isolates that did not have public sequence data available (*B. cenocepacia* C3865 and C5424). The *hmgA* genes from *B. cenocepacia* C5424 and C3865 were amplified from genomic DNA using hmgA seq Fd (GCGCGATCCACCTGTATG) and hmgA seq Rv (GTTGCTCCGGATTGAAGTGT). All non-pigmented strains encoded a glycine at residue 378 (Table 2.1). Out of the four pigmented strains in our collection, three encoded an arginine at residue 378 (Table

2.1). *B. cenocepacia* C3865 is pigmented and encodes a glycine at residue 378. We observed differences in this strain in both the timing of pigment production and the hue of the pigment, and we hypothesize that this strain may produce pigment through an alternative, not yet defined mechanism.

The human enzyme (termed HGO) has a crystal structure available [299] and is 52% identical at the protein level to HmgA encoded in *B. cenocepacia* J2315. HGO assembles as a dimer of trimers, and the active site iron ion is coordinated between subunits in the trimer. Two histidines and one glutamic acid are involved in iron binding, and they correspond to H341, E347, and H377 in the *B. cenocepacia* protein (Figure A2.2). The G378R change occurs in this region, and this substitution could potentially disrupt iron-binding and therefore enzyme function. Additionally, mutations in this region have previously been shown to inactivate HGO in the human disease alkaptonuria [300].

Expression of hmgA from a non-pigmented strain results in a non-pigmented phenotype in B. cenocepacia J2315

B. cenocepacia J2315 and *B. cenocepacia* K56-2 both belong to the ET-12 lineage and are closely related. The *hmgA* gene in *B. cenocepacia* J2315 and K56-2 is identical except for a one base pair change (G to C) that results in the glycine to arginine change at residue 378 in the HmgA protein in J2315. To begin to test whether this amino acid change was responsible for the observed pigmented phenotype, we amplified the *hmgA* genes from *B. cenocepacia* J2315 (a pigmented strain that encodes an arginine at residue 378 of HmgA) and *B. cenocepacia* K56-2 (a

non-pigmented strain that encodes an glycine at residue 378 of HmgA), referred to as J*hmgA* and K*hmgA*, respectively. The amplified *hmgA* gene from both of these strains was then cloned into the pUCP18Tc vector and transferred to *B. cenocepacia* J2315 and K56-2. *B. cenocepacia* J2315 complemented with both its own gene (pUCP18TC-J*hmgA*) or the empty vector (pUCP18Tc) maintained a pigmented phenotype as measured by absorbance of the supernatant at OD_{480} after 24-30 hours of growth (Figure A2.3A). On the other hand, complementation of *B. cenocepacia* J2315 with K*hmgA* (pUCP18TC-K*hmgA*) resulted in a non-pigmented phenotype (Figure A2.3A). *B. cenocepacia* K56-2 remained non-pigmented when containing any of the plasmids, but a statistically significant increase in the OD_{480} of the supernatant was observed when expressing J*hmgA* (pUCP18TC-J*hmgA*) in comparison to K*hmgA* (Figure A2.3A). This minor increase in OD_{480} in *B. cenocepacia* K56-2 suggests that there may be competition for substrate between the native KHmgA and the cloned JHmgA enzymes when both are expressed. In these experiments, we did not observe any significant differences in CFU/ml at the 24-hour time point between wild-type and complemented strains (data not shown). Additionally, we expressed both J*hmgA* and K*hmgA* in another pigmented Bcc isolate, *B. cenocepacia* BC7, which also has arginine at position 378 in HmgA. *B. cenocepacia* BC7 expressing J*hmgA* retained a pigmented phenotype, but consistent with what we observed with *B. cenocepacia* J2315, expression of K*hmgA* resulted in a loss of pigmentation (data not shown). These results support the hypothesis that the G378R amino acid change in HmgA encoded by *B. cenocepacia* J2315 and BC7 impairs enzyme activity leading to the accumulation of visibly detectable pigment,

and that an otherwise identical enzyme derived from *B. cenocepacia* K56-2 lacking this amino acid change is functional and eliminates visually detectable pigment production.

Complementation of Pseudomonas aeruginosa PA14 hmgA::Tn with KHmgA but not JHmgA results in restoration of a wild-type nonpigmented phenotype

We transferred the same plasmids expressing either versions of *hmgA* to a *Pseudomonas aeruginosa* PA14 insertional mutant in *hmgA*, PA14 *hmgA*::Tn. Because of this insertion in *hmgA*, *P. aeruginosa* PA14 *hmgA*::Tn displays a hyperpigmented phenotype. Since this mutant has been previously characterized and has known defects in HmgA [297], we utilized it to test the functionality of JHmgA and KHmgA enzymes. Similar to what we had observed in *B. cenocepacia* J2315, KHmgA was able to compensate for the *hmgA* mutation. PA14 *hmgA*::Tn (pUCP18Tc-K*hmgA*) had a non-pigmented phenotype on agar plates and supernatants were non-pigmented after growth in LB for 30-35 hours in LB (Figure A2.3B). On the other hand, expression of J*hmg*A was unable to complement this phenotype (Figure A2.3B). This data suggests that the KHmgA enzyme is functional and leads to an absence of pigment due to conversion of excess HGA to acetoacetic acid and fumaric acid.

Expression of KhmgA in B. cenocepacia J2315 does not alter susceptibility to H_2O_2

In other systems it has been shown that pyomelanin production protects against H_2O_2 [256, 297]. We hypothesized that *B. cenocepacia* J2315 pUCP18Tc-Kh*mgA*

(non-pigmented) would be more sensitive to H_2O_2 than the isogenic pigmented strains (J2315 containing pUCP18Tc or pUCP18Tc-J*hmgA*). We tested *B. cenocepacia* J2315 expressing K*hmgA* or J*hmgA* for sensitivity to H_2O_2 using two different assays. Using a standard disc assay to test sensitivity of stationary phase cultures grown on agar plates, we observed no difference in the diameter of the zones of inhibition with 50, 100, 400 or 800 mM H_2O_2 (data not shown). Using an assay that was previously performed with *P. aeruginosa* PA14 and PA14 *hmgA*::Tn (Rodriguez-Rojas et al., 2009**)**, we then tested whether sensitivity to H_2O_2 during exponential phase growth by measuring percent survival after incubation with H_2O_2. We confirmed that PA14 *hmgA*::Tn was more resistant to 25, 50 mM and 100 mM H_2O_2 than PA14 (Figure A2.3D). However, *B. cenocepacia* J2315 was much more sensitive to H_2O_2; even at 5 mM H_2O_2 we noted less than 10% survival of J2315 (Figure A2.3C). Under these conditions, the expression of K*hmgA* in *B. cenocepacia* J2315 did not increase susceptibility to H_2O_2.

Conclusions

We found that a G378R amino acid substitution in homogentisate 1,2-dioxygenase (HmgA) correlated with pigment production. Complementation of the pigmented phenotype of *B. cenocepacia* J2315 with *hmgA* from K56-2 resulted in a non-pigmented phenotype. These results suggest that the G378R version of HmgA is not functional and we propose that this is one mechanism that allows for pigmentation in some members of the Bcc. Melanins are protective against both oxidative and

non-oxidative stresses, which could contribute to increased fitness of Bcc isolates both during infection and in the environment. While we did not observe differences in susceptibility to H_2O_2, this does not exclude the possibility that pyomelanin production in J2315 could be providing protection against other oxidative species. Future exploration into the correlation of HmgA functionality and fitness in different niches could provide important insight into the mechanisms for Bcc survival in the presence of both host and environmental stresses.

Table A2.1 Pigmentation in Bcc strains

Strain	Amino acid at residue 378	Produces pigment under normal laboratory conditions	Type of isolate
B. cenocepacia			
J2315	R	Yes	CF clinical isolate
BC7	R	Yes	CF clinical isolate
C5424	R	Yes	CF clinical isolate
K56-2	G	No	CF clinical isolate
H111	G	No	CF clinical isolate
MC0-3	G	No	Environmental isolate
AU 1054	G	No	CF clinical isolate
C3865	G	Yes	CF clinical isolate
B. multivorans			
ATCC 17616	G	No	Environmental isolate

CGD2M	G	No	CGD clinical isolate
CGD1	G	No	CGD clinical isolate

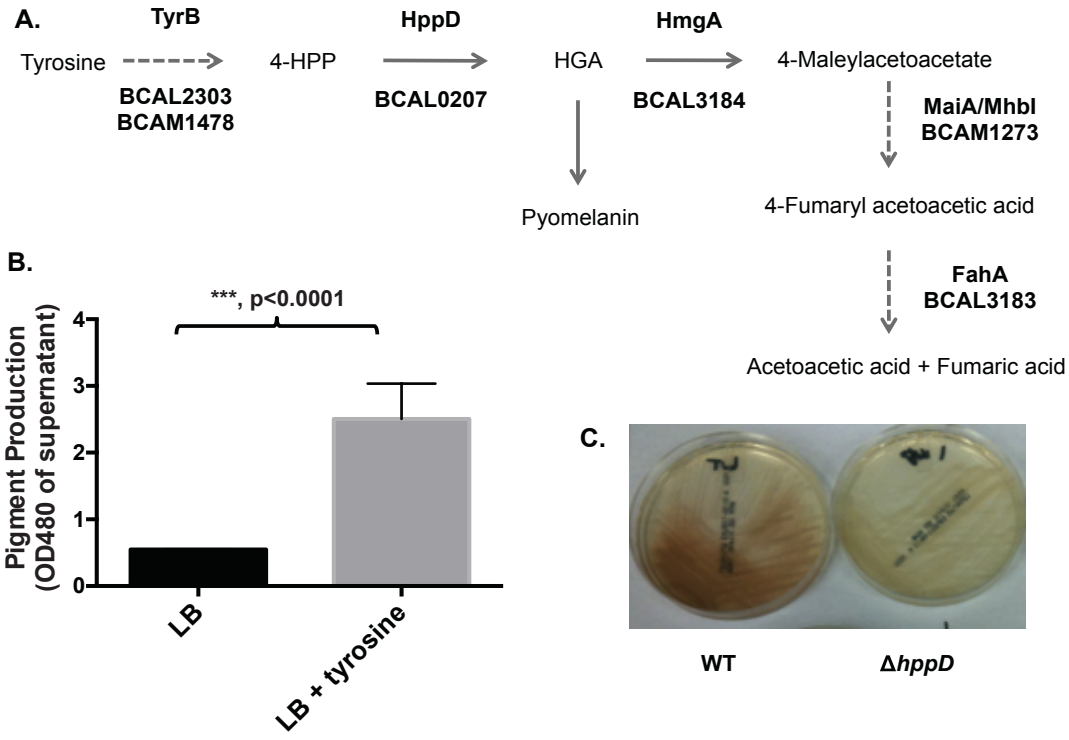

Figure A2.1 The pigment produced by *B. cenocepacia* J2315 is a pyomelanin and is synthesized through tyrosine catabolism. A. Genes homologous to enzymes involved in the pyomelanin synthesis pathway were identified in the *B. cenocepacia* J2315 genome using sequence homology and KEGG pathway annotations. 4-HPP, 4-hydroxyphenylpyruvate; HGA, homogentisic acid. B. Growth of *B. cenocepacia* J2315 in LB with 1 mg per mL tyrosine resulted in increased pigment production in comparison to growth in LB without tyrosine as determined by the OD_{480} of the culture supernatant at 24 hours. C. An unmarked deletion mutant in the gene that encodes the last enzyme in the pathway to make the pigment, *hppD* (BCAL0207), was constructed in *B. cenocepacia* J2315 and this strain was non-pigmented. The system for making unmarked deletions through gene

splicing by overlap extension described by Flannagan et al. [301] was used to create *B. cenocepacia* J2315 Δ*hppD*. The deletion construct was made by amplifying the flanking regions of the gene using the following four primers: F1-XbaI (GGTCTAGAAATCGGCAACGCCGTCGTTTCCTTGAAGC), R1 (TGCCGCGCGGTGCAAGCGGTCGTGTCTCCTGTGCGG), F2 (CCGCACAGGAGACACGACCGCTTGCACCGCGCGGCA), and R2 EcoRV (GGGATATCTTCGCCGGTTTTACGGGATGGTAGCACTGG).

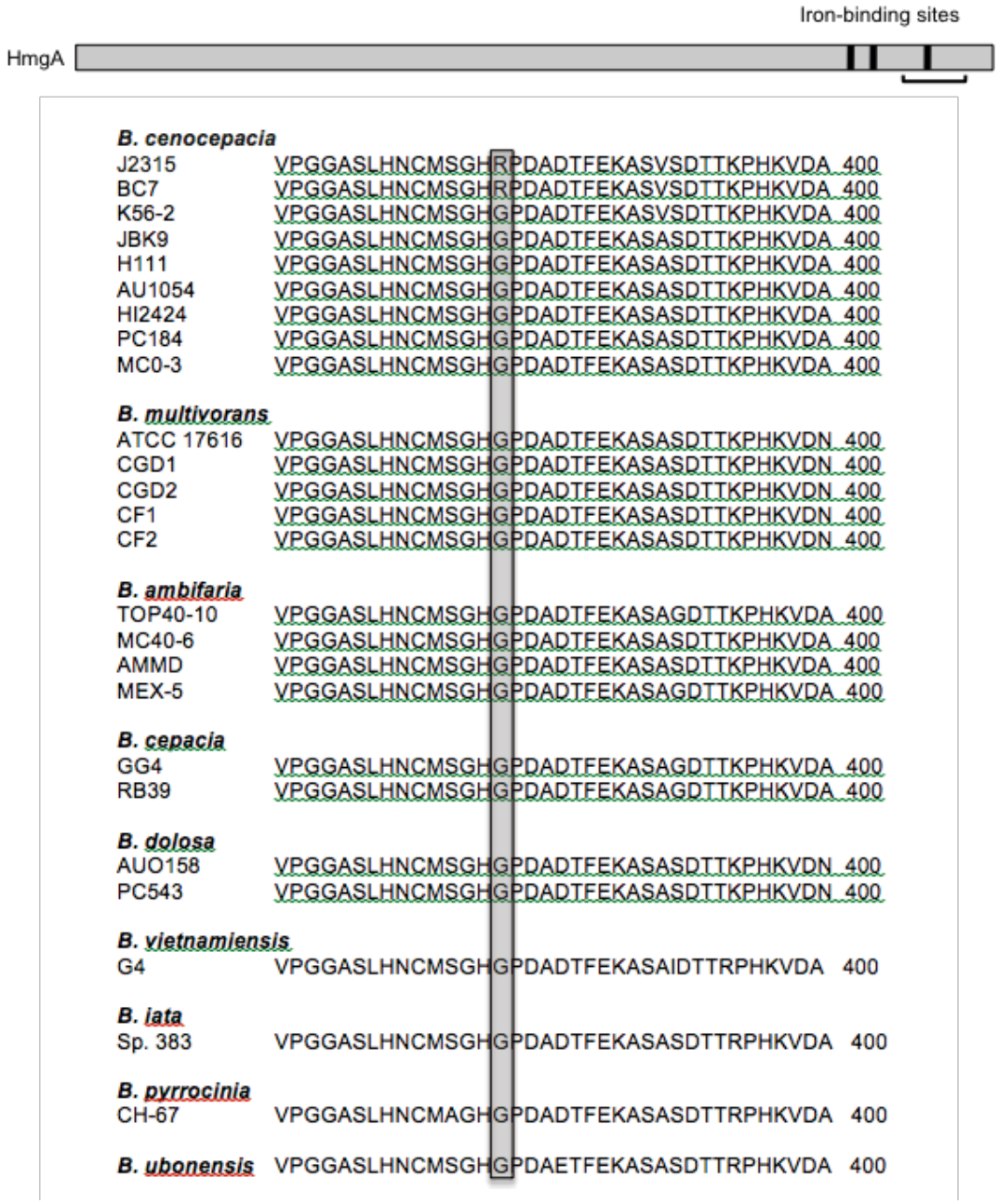

Figure A2.2 A glycine at residue 378 of HmgA is conserved amongst Bcc strains and is changed to an arginine in *B. cenocepacia* J2315. A. Diagram of the HmgA gene of Bcc showing iron-binding sites. The region marked in brackets corresponds

to residues 363-400, which are aligned below. B. Sequences of inferred amino acid sequence of the *hmgA* gene from Bcc strains from residues 364-400 were aligned to show conservation of a glycine at residue 378. Sequence identity of the entire proteins ranged between 94-99%.

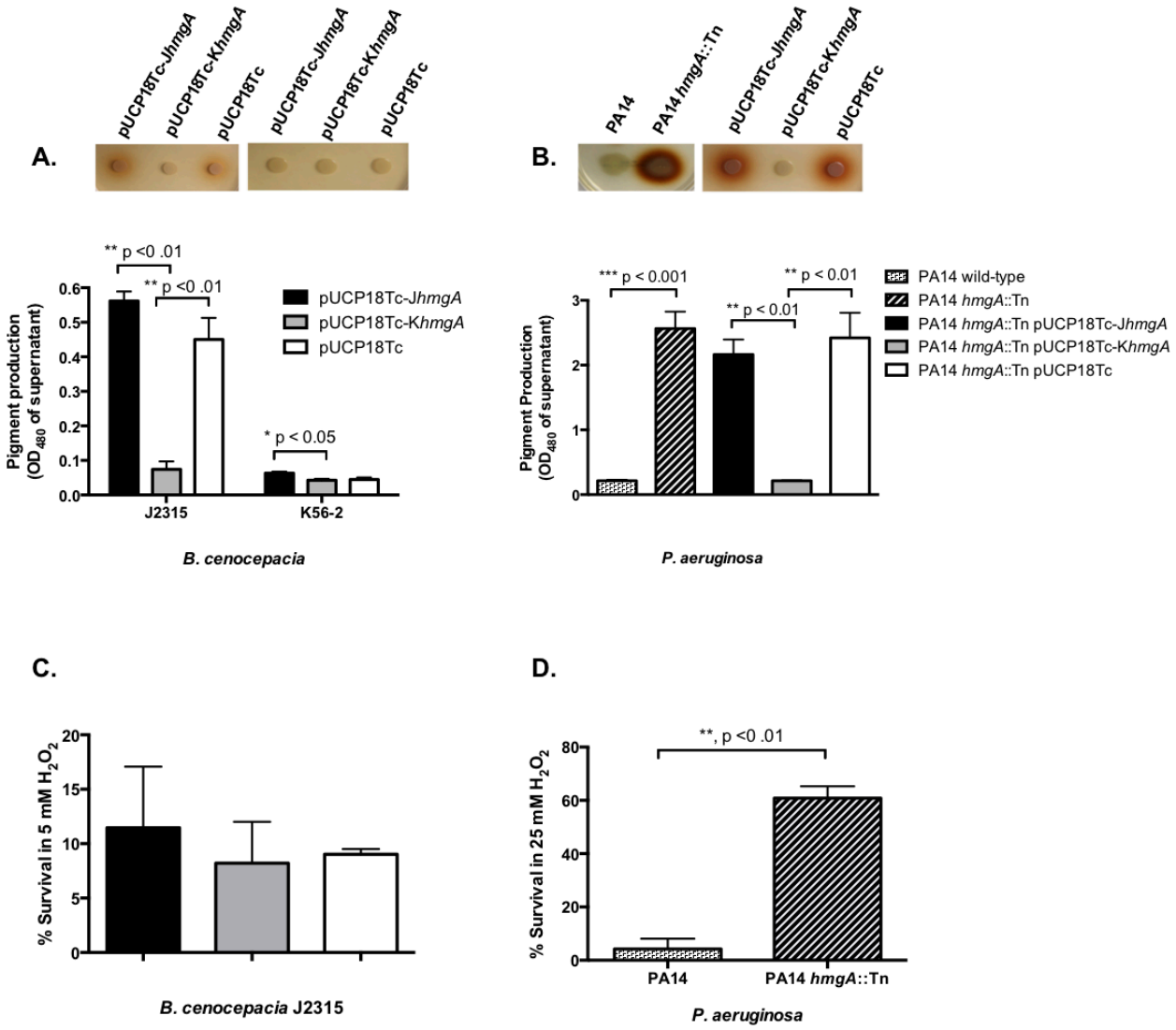

Figure A2.3 Expression of *hmgA* from a non-pigmented strain results in a non-pigmented phenotype in *B. cenocepacia*. A. The *hmgA* genes from *B. cenocepacia* J2315 and K56-2 were amplified from genomic DNA using *hmgA* Fd SacI (CTAGGAGCTCATGACGCTTGACCTGTCGAAACCGGCAA) and *hmgA* Rv XbaI (CTAGAGATCTTCATCGTTGCTCCGGATTGA). The PCR products were cloned into TOPO pCR2.1 (Invitrogen) and then digested with SacI and XbaI. Digested inserts were ligated into similarly digested pUCP18Tc [302] and sequenced to confirm

identity. pUCP18Tc and recombinant *hmgA*-containing plasmids were electroporated into *B. cenocepacia* J2315 and K56-2 using a modified version of a protocol published previously [303]. The resultant plasmid-containing strains were grown in LB with 100 μg/ml tetracycline at 37°C with shaking for 24-30 hours and then evaluated the level of pigmentation by assaying the OD_{480} of the supernatant. B. Recombinant plasmids containing the *hmgA* genes from J2315 (pUCP18Tc-J*hmgA*), K56-2 (pUPC18Tc-K*hmgA*), or the vector (pUCP18Tc) were transferred to *P. aeruginosa* PA14 *hmgA*::Tn. Liquid cultures were incubated at 37°C in LB with 50 μg/ml tetracycline for plasmid-containing strains, and without for non-plasmid containing strains with shaking for 30-35 hours and then evaluated for the level of pigmentation by assaying the OD_{480} of the supernatant. C. Susceptibility to H_2O_2 of J2315 strains expressing pUCP18Tc, pUCP18Tc-K*hmgA*, pUCP18Tc-J*hmgA* was measured after exponential phase cultures were incubated for 45 minutes with 5 mM H_2O_2 and then grown out for 48 hours on LB. Susceptibility was measured by calculating CFU after culture sample was incubated with 5 mM H_2O_2 divided by CFU of culture sample incubated in LB. D. Susceptibility to H_2O_2 of PA14 WT or PA14 *hmgA*::Tn was measured after exponential phase cultures were incubated for 45 minutes with 20mM H_2O_2 and then grown out for 24 hours on LB. Susceptibility was measured by calculating CFU after culture sample was incubated with 20 mM H_2O_2 divided by CFU of culture sample incubated in LB.

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