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ROLE OF A NOVEL FIMBRIAL ADHESIN IN ACID-INDUCED HOST ADHESION OF *ESCHERICHIA COLI* O157:H7

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Bachelors of Science Biology

University of Maryland Baltimore County, MD, USA 2007

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A thesis presented to Ryerson University
in partial fulfillment of the requirements for the degree of
Master of Science in the Program of

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Molecular Science

Toronto, Ontario, Canada, 2009

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Abstract

Role of a Novel Fimbrial Adhesin in Acid-Induced Host Adhesion of *Escherichia coli* O157:H7

Frances B. Chingcuanco, Ryerson University, 2009, Master of Science in Molecular Science

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 must be able to survive the acid stress of gastric passage. Previous studies show that adhesion of EHEC O157:H7 to human epithelial cells is increased if EHEC was pre-exposed to acid. Whole genome array analysis of EHEC reveals that the putative fimbrial gene *yadK* is significantly upregulated after EHEC exposure to acid treatments. In this study, a *YadK* deficient mutant ($\Delta yadK$) in EHEC O157:H7 wild-type (WT) strain 85-170 was constructed and phenotyped. The results of this study demonstrate that $\Delta yadK$ exposed to acid stress showed no acid-induced increase in bacteria-host cell adhesion observed in similarly acid-induced wild type cells. This study concludes that *YadK* plays a role in acid-induced host adhesion of EHEC O157:H7. It also indicates that acid stress, which is a part of the host's natural assault to resist invasion, may regulate factors responsible for enhanced bacteria-host attachment, resulting in increased EHEC virulence.

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List of Abbreviations Used

AA	Acid adapted/acid stressed cells
A/E	Attaching and effacing lesions
AR	Acid response
ATCC	American Type Culture Collection
ATR	Acid tolerance response
CaCo-2	Colonic adenocarcinoma cells
Da	Daltons
DMEM	Dulbecco's MEM
EDTA	Ethylenediaminetetraacetic acid
Efa-1	EHEC factor for adherence
EHEC	Enterohemorrhagic <i>Escherichia coli</i>
EPEC	Enteropathogenic <i>Escherichia coli</i>
Esp	<i>E. coli</i> secreted protein
FBS	Fetal bovine serum
GABA	glutamate/ γ aminobutyric acid
Gb-3	Glycolipid globotriaosylceramide-3 receptor
GI	Gastrointestinal tract
HC	Hemorrhagic colitis
HUS	Hemolytic-uremic syndrome
Iha	IrgA homologue adhesin
LB	Luria-Bertani
LEE	Locus of enterocytes effacement LEE
Ler	LEE-encoded regulator
MEM	Minimal essential media
ORF	Open reading frame
PBS	Phosphate buffered saline
pSK	pBlueScriptSK+ plasmid
RT – PCR	Real Time PCR
STEC	Shiga toxin producing <i>E. coli</i>
Stx	Shiga toxin
Tir	Translocated intimin receptor
TTP	Thrombocytopenic purpura
TTSS	Type III secretion system
UA	Unadapted/acid stressed cells
UPEC	Uropathogenic <i>Escherichia coli</i>
UU	Unadapted/unstressed cells
VT	Verotoxin

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1. Introduction

1.1 Enterohemorrhagic *Escherichia coli* O157:H7

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 is one of six pathotypes of pathogenic *E. coli* that expresses the 157th somatic (O) and 7th flagellar (H) antigen (1). Like commensal *E. coli*, EHEC is a facultative anaerobe that has adapted the ability to inhabit the human gastrointestinal tract (GI); however, it perturbs the resident flora that live in the GI tract and cause a variety of diseases including acute gastroenteritis, diarrhea, fever, nausea, vomiting and abdominal cramps (2, 3). During infection, EHEC colonizes the human bowel within 1-3 days of ingestion and can persist for a few days to a few weeks (3, 4). EHEC infections can lead to further life threatening diseases including thrombocytopenic purpura (TTP), acute renal failure, hemorrhagic colitis (HC) and haemolytic uremic syndrome (HUS) (2). In fact, EHEC is responsible for 90% of HUS infections worldwide (4) (**Figure 1.1**).

Morphologically, EHEC exists as a Gram-negative, rod shaped bacteria that ranges from 1-5 μm in size and 0.3-0.4 μm in thickness (5). Genomic comparison between EHEC and K12 strains reveals that almost half of EHEC-specific DNA is made of prophage DNA (6). Like other pathogenic bacteria, EHEC possesses several virulence factors including those encoded on pathogenicity islands: the locus of enterocyte effacement (LEE), the O157 plasmid, as well as the phage encoded Shiga-toxin (*stx*) genes (2). Most of these pathogenic regions are believed to have been horizontally acquired and have allowed EHEC to evolve into a deadly intestinal pathogen.

Figure 1.1 The epidemiology of EHEC-related infection. EHEC is responsible for over 90% of all HUS disease worldwide. Adapted from (1)

1.2 Significance of EHEC O157:H7

EHEC infections have been reported in at least 30 countries worldwide (7). Because healthy adults are naturally able to recover from these infections, cases of EHEC infections remain grossly underreported. Thus, EHEC O157:H7 infections only account for approximately 75,000 human related gastrointestinal infections across USA (8). Interestingly, the incidence of EHEC related disease is lower in third world countries that have no access to drinking water. This observation is often attributed to the high prevalence of enteropathogenic *E. coli* (EPEC) infections in developing countries that seems to provide cross-protection from EHEC among its victims (8).

Complications from EHEC disease can lead to a high mortality rate among very young children, immunocompromised individuals and the elderly. EHEC infections can cause hemorrhagic colitis, often described as “all blood, no stool” diarrhea, and can progress to HUS and death. While hemolytic uremic syndrome occurs in only 2-7% of infected victims, the lifelong complications that range from long-term renal failure to severe brain damage, cause high morbidity among EHEC infection survivors (2).

1.3 Transmission

EHEC can be transmitted through ingestion of contaminated food and water sources, through direct contact from one infected person to the other, and occasionally through occupational exposure (1). As little as 10^2 EHEC cells are needed to cause disease among its victims (9). Healthy cattle ruminant remains the number one reservoir of this deadly bacteria. As many as 30% of all cattle in the United States are asymptomatic carriers of the EHEC (10). In spite of the severe diseases EHEC is responsible for in humans, it is not able to cause illness in cattle and therefore causes major problems for the transmission of these bacteria. Approximately

75% of O157:H7 outbreaks, including EHEC, are linked to contaminated bovine-derived products including consumption of contaminated ground beef and raw milk (5). Manure contaminated runoff is also another source of EHEC contamination in crop irrigation systems as colonized cattle can shed EHEC bacteria yearlong, contaminating drinking water (10). Other food and waterborne outbreaks can be traced to contaminated fruits and vegetables, unpasteurized apple juice, unchlorinated drinking water. EHEC can also be transmitted through direct contact; outbreaks have been observed in day-care centers, among incarcerated individuals in prisons, visits to petting zoos and dairy farms (reviewed in 11). Occupational exposures to the bacteria among nurses, farmers, microbiologists can also lead to EHEC infections.

1.4 EHEC Pathogenesis

EHEC pathogenesis is a multi-step process that involves different interactions between bacteria effectors and host cell receptors. As mentioned above, EHEC virulence factors are contained within pathogenicity islands within the bacteria's genome. These virulence factors include the pO157 plasmid, the locus of enterocyte effacement and phage-encoded Shiga-toxin genes (**Figure 1.2**)

Figure 1.2 The virulence factors of EHEC are housed in three pathogenic sites: the LEE pathogenicity island, the Shiga-toxin pathogenicity island and the plasmid pO157. These virulence factors contribute to EHEC's virulent epidemiology. Adapted from (2).

Despite extensive research on the pO157 plasmid, its role in EHEC pathogenicity has yet to be fully elucidated. It is believed that pO157 encodes a hemolysin that allows EHEC to use blood released in the intestinal tract as a source of iron (12). Several studies have indicated the importance of pO157 in the expression of fimbrial adhesins in EHEC (16). Molecular studies reveal that pO157 is approximately a 92kb F-like plasmid including 7 insertion sequence elements, all of which are located near virulence genes, and 100 open reading frames (ORF) (16). Only 19 of these ORF's have been characterized including the type II secretory system (*etpC-epiO*), enterohemolysin (*hlyA-D*), catalase-peroxidase (*katP*), serine protease (*espP*), lymphocyte inhibitory factor (*lifA/efa*), the putative adhesin ToxB (*toxB*), and the C1 esterase inhibitor metalloprotease (*StcE*) (5). This plasmid has a heterogenous mosaic structure and can be found in almost all EHEC isolates in humans (5).

The virulence factors encoded on the locus of enterocyte effacement (LEE) are responsible for the distinct attaching and effacing (A/E) lesions observed on host epithelium during EHEC infection. These lesions are characterized by intimate adherence of bacteria to enterocytes, thinning and shortening of microvilli, degeneration of brush borders, and formation of cuplike pedestals due to actin polymerization on infected host cells (13). A/E lesions result from the intimate attachment of the bacteria to host epithelial that causes cytoskeletal restructuring, leading to the extensive histological damage of the brush border cells at the site of bacteria attachment (14). During *in vivo* experiments, investigators have found that the A/E lesions were mostly localized around the follicle-associated epithelium in bovine animal models (15).

Genomic sequence of the LEE locus reveals that approximately 7.5kb of this locus encodes putative prophage genes (16). The LEE pathogenicity island is represented in a variety

of intestinal pathogens including EHEC, related gastrointestinal pathogen enteropathogenic *E. coli* (EPEC) and *Citrobacter rodentium*. Transformation of K12 strains with the EHEC LEE island does not induce A/E lesions in *in vitro* experiments; in contrast, transformation of K12 MG1655 with EPEC LEE induces A/E phenotypes *in vitro*. This suggests that the molecular mechanisms that regulate LEE differ significantly in EHEC and EPEC (17).

The LEE region contains 5 operons that encode genes crucial for EHEC pathogenicity. LEE1, LEE2 and LEE3 encode the type III secretion system (TTSS), a molecular syringe-like system that injects virulent effector proteins into the cellular host (18). LEE4 encodes for the *E. coli* secreted proteins (Esp) genes, *espA*, *B* and *D*. The EspABD has been found to work in concert with the TTSS to inject EHEC proteins into the host (19). Finally, LEE5 encodes the translocated intimin receptor (*tir*), which in conjunction with the LEE encoded protein intimin, allows for the intimate attachment between EHEC and host epithelial cells (described later).

Adhesion of EHEC to host tissue is the first and most crucial step to cause disease. Adhesins encoded in the bacteria's genome mediate the crucial bacteria-host cell attachment that allow the bacteria to evade host immune response and proliferate while in the infected host. It is during attachment when EHEC causes characteristic attaching and effacing (A/E) lesions phenotype as a result of bacteria-host cell adhesion.

EHEC and other related Shiga-toxin producing *E. coli* (STEC) strains produce one or more phage-encoded Shiga-toxins (Stx) that are essential for the bacteria's virulence (20). These toxins are largely responsible for the development of hemorrhagic colitis and hemolytic uremic syndrome, the major hallmarks for EHEC pathogenesis (21). EHEC Stx are classified under two major groups: Stx1 (similar to the toxins produced by *Shigella dysenteriae* type 1) and Stx2 (20). Molecular studies suggest that bacteriophage were largely responsible for transferring the *stx*

genes from *Shigella* to EPEC, and eventually to EHEC eventually acquired during the course of its evolution (22, 23).

The Stx proteins belong to the AB₄ toxin family that bear one enzymatically active A subunit and identical pentamers of B subunits (24) (**Figure 1.3**). These toxins are composed of one A subunit that can be nicked and reduced to an enzymatically active A1 (27 kDa) and an A2 peptide (4 kDa) and a pentamer of B subunits (7 kDa/monomer) that binds to the eukaryotic cells (25).

Although Stx1 and Stx2 share very strong amino acid sequence homology, each toxin is antigenically distinct (26). As many as 3 Stx2 variants have been identified (Stx2c, Stx2d and Stx2e) that work in the same mechanism as Stx2 (27). Universally, Stx proteins bind to globotriaosylceramide (Gb3) or globotetra-osylceramide (Gb4) receptors expressed on a variety of epithelial and endothelial cells (28). Stx1 and all of Stx2 proteins with the exception of Stx2e bind to Gb3; Stx2e bind to Gb4 (28).

Welinder-Olsson *et al.*, show that Stx possesses the mechanism to cross the intestinal-epithelial barrier and induces neutrophil-rich inflammatory response in the gut (7). To actually cause disease, Stx must be able to reach the blood stream from the gut, though the underlying mechanism behind this phenomenon is poorly understood (5). During infection, EHEC Stx enter intestinal cells through a clathrin-dependent mechanism mediated by the binding of the B subunit to Gb3/Gb4 receptors (29); once the toxins are in the cytoplasm, the A subunit is cleaved by a calcium-sensitive serine protease called furin, resulting in a 27kDa N-terminal fragment (A1) and a 4kDa C-terminal peptide (A2) (30). The catalytically active A1 acts like a hydrolase and interacts with 60S ribosomal subunit and inhibits intracellular protein synthesis. The resulting

Figure 1.3 The Shiga-toxin protein in EHEC O157:H7 belongs to the AB4 family of toxins; the B subunit interacts with cellular receptor found on host cell membrane while the enzymatically active A subunit is responsible for the toxin's cytotoxic activity. Adapted from (3).

inhibition to protein synthesis leads to cell death. In addition, Stx proteins can also stimulate cell apoptosis through activation of a caspase cascade (31). Stx have been shown to induce the upregulation of proapoptotic *bax* gene, which activates caspase signaling events (32).

Ironically, antibiotic treatments do more harm than help in EHEC infections. Studies have demonstrated that commonly prescribed antibiotics cause lysis of EHEC cells and release of the deadly toxins in the intestinal epithelium (33).

Although Shiga-toxins are the most important virulence factor in EHEC epidemiology, *stx*- EHEC variants have been show to still cause diarrhea (34). This only suggests that there are other factors that are evidently important in EHEC pathogenesis.

1.5 Adhesins

Bacterial adherence has been established as an important virulence factor in microbial pathogenesis and is a prerequisite for establishing EHEC infections (35). Before a pathogen can invade, proliferate and cause disease in its targeted host, successful adhesion must be accomplished (36). Bacterial adhesins promote the initial attachment of the bacteria to host cells, allowing pathogen colonization (37). The specificity of adhesion to target tissue allows bacteria to determine and even differentiate sites for attachment and even invasion (38). For some pathogens, adhesins are also responsible for bacteria-cell contact and some are even important for toxin delivery (39). EHEC employs a number of both fimbrial and non-fimbrial factors for host-cell adhesion. These adhesins can be either filamentous or amorphous (39). Filamentous adhesins or fimbriae/pili are non-flagellar appendages that are projected throughout bacteria surface. The term fimbriae is used to designate this type adhesin, reserving the term pili isfor

sexual appendages (39). Amorphous adhesins are non-fimbrial or afimbriae adhesive structures. Both filamentous and amorphous adhesins require specific cell receptors for successful attachment.

1.5.1.2 Curli

1.5.1 Fimbrial Adhesins

As many as 16 fimbrial clusters distributed within *E. coli* O157:H7 strains have been identified and analyzed (40). Of these 16 clusters, 13 are believed to contain chaperone and usher genes, 4 are unique to O157:H7 serotypes and several share partial homology between non-pathogenic K12 and O157:H7 strains (40). Though EHEC encodes several of these fimbrial proteins including type 1 fimbriae, curli, long polar fimbriae-1 (LpF1) and long polar fimbriae-2 (LpF2), only LPF1/2 and curli clusters have been found to be functionally expressed in EHEC (Figure 1.4).

1.5.1.2 Type 1 Fimbriae

Although EHEC type 1 fimbriae is non-functional in EHEC, this fimbrial adhesin is found in almost 50-70% of *E. coli* (41). It is encoded by the *fim* gene clusters A to H located in the bacteria genome. Type 1 fimbriae is composed 500-3000 copies of the main structural subunit FimA, which forms the structural base that is around 7 nm thick and 0.1 and 2 μ m long. Multiple subunits of structure of FimF make up the filamentous part of the fimbriae rod and a 3 nm wide fibrillum at the end of the FimF structure contains FimG and the type 1 fimbriae adhesin tip FimH (42).

Type 1 fimbriae recognize specific mannose-containing oligosaccharides found on target tissue cells receptors (43). Receptors that highly express mannose sugars have very high affinity for the adhesin tip, FimH, as evident with the adhesin's selective binding with highly mannose

Figure 1.4 Known fimbrial operons in *E. coli* O157:H7 str. Sakai. Red filled arrows indicate major subunit within operon. Black filled arrows indicate unknown sequences. Schematic of long polar fimbria operon is based on *Lpf-1* in EHEC Sakai. Arrow sizes not drawn to scale. Adapted from (40).

glycosylated uroplakin UPIa over the structurally related but not as glycosylated uroplakin UPIb (44).

1.5.1.2 Curli

EHEC also produces long, proteinaceous, coiled, curli protein fibers. These curli proteins consists of repeating subunits CsgC that form non-branching β -sheet rich fibers that are approximately 100 amino acid residues long, which are not susceptible to protease or thermal degradation. Curli are assembled in the surface of most enteric microbes and form 6-12 nm in diameters with lengths that vary from 0.5 to 1.0 μ m (45). Curli mediates bacteria-host cell colonization by binding to the extracellular matrix protein, as well as fibronectin, laminin, plasminogen, human contact phase proteins located in the intestinal wall and the major histocompatibility complex class 1 molecules (46). Curli has also been shown to play a role in EPEC biofilm formation, bacteria-host invasion and immune system activation (47). The expression of curli is tightly linked to cellulose biosynthesis, resulting in the very tight host-bacteria cell and cell-surface interaction during pathogenesis (48).

1.5.1.3 Long Polar Fimbriae

Torres *et al.*, identified two loci in EHEC that showed strong homology to the long polar fimbriae in *Salmonella enterica* serovar Typhimurium (49). The six ORFs that were characterized in the first *lpf* operon (49) showed considerable similarity to *S. enterica lpf* gene cluster. The nomenclature of EHEC *lpf-1*, *lpfABCC'DE* is also similar to *S. enterica*.

Protein prediction analysis of the Lpf proteins suggests structural homology to the Fim proteins of type 1 fimbriae. The first gene in the operon, LpfA, shows high homology to FimA,

while proteins LpfB and LpfC/LpfC' are structurally similar to FimC and FimD, the usher-chaperone subunits of type 1 fimbriae (49).

Investigators examined the role of Lpf proteins in EHEC bacteria-host adhesion and found that the long polar fimbriae contributed significantly to the colonization of EHEC in *in vivo* studies (50). While they did not observe any changes in A/E lesions formed in either their single *lpf1* or double *lpf1 lpf2* mutant strains during microscopic studies, they did note that adhesion to human epithelial cells was significantly decreased in *lpf2* mutants after 3 h of infection during bacteria-host cell adhesion assay. To this, investigators concluded that the Lpf proteins may be required in initial bacteria adherence *in vitro* (50).

1.5.1.4 F9 fimbriae

F9 fimbriae is one of the 16 fimbrial clusters that is expressed *in vitro* (51). F9 fimbriae form short 1-2 μ M long fimbrial stalks that can be removed at 60 °C. The gene, chaperone and half of the usher of the F9 operon is deleted in *E. coli* K12 MG1655 strain; however, regions within this operon are 98% identical among *E. coli* O157:H7 and CFT073 strains (51). Cloning the F9 operon into the K12 strains increases bacteria-host cell adhesion. Adhesion of transformed K12 was 3.5 fold higher than control during bacteria-host cell adhesion assays (51). *In vivo* experiments show that a deletion in the major subunit of the F9 operon reduces the ability of *E. coli* O157:H7 strain EDL933 to colonize 1-2 week old calves (52, 53). This data strongly supports the importance of F9 operon in the successful adhesion of pathogenic *E. coli*.

1.5.1.5 *E. coli* Common Pilus

The *E. coli* Common Pilus (Ecp) found in a variety of *E. coli* O157:H7 serotypes, is composed of multiple subunits of the fimbriae's gene, *ecpA* (54). EcpA is revealed to be 60% hydrophobic and is assembled into a pili "MAT" (meningitis associated and temperature-regulated pili) (55, 56). Investigators demonstrate that ECP filaments are visible as early as 1.5 h after affection; *ecpA* (also know as *yagZ*) mutants also show deficiency in adherence onto human epithelial cells, indicating that it plays a role in EHEC bacteria-host cell adhesion (54).

1.5.2 Non-fimbrial Adhesins

In addition to fimbrial adhesins, non-fimbrial adhesins have also been implicated in EHEC O157:H7 bacteria-host cell adhesions. Several non-fimbriated proteins include intimin, Iha, Efa-a have been identified to be crucial to the bacteria's pathogenicity.

1.5.2.1 Intimin

The *eae* gene which encodes the non-fimbrial adhesion intimin is responsible for the intimate attachment of EHEC to host cells. Intimin binds to the translocated intimin receptor (Tir) which is injected into host epithelia using the type III secretion system. Intimin was predicted to be a 94 kDa membrane protein with several Ig-like domains (57). Its N-terminus bears a 30% sequence resemblance to an invasin protein and its C-terminus forms a C-type lectin domain that forms a rigid superdomain binding for Tir (58). Intimin receptor Tir is an effector molecule containing two transmembrane helices with an extracellular intimin-binding domain. Once injected into host cytoplasm, Tir is phosphorylated and becomes anchored in the membrane

and exposed for intimin binding (57). This extracellular domain forms two antiparallel α -helices joined by a β -hairpin. The intimin-Tir interaction mainly occurs around the residues that form the β -hairpin. Both of the Tir N and C-termini face the cytoplasm of the host cell and are involved in the cytoskeletal rearrangement due to downstream signalling events (58).

Molecular typing has identified several intimin classes (α , β , γ , δ and ϵ) though only β and γ have been implicated in EHEC O157:H7 pathogenesis (58). Although the binding distance between intimin and Tir is only 1334Å, it is enough to cause the damaged histological A/E lesions distinctive of EHEC colonization (57, 59).

1.5.2.2 Efa

Another non-fimbriated factor that is important for EHEC colonization is the *E. coli* factor for adherence (Efa) (60). EHEC O157:H7 contains a truncated *efa-1* gene that when mutated, significantly impairs the bacteria's adhesion onto human epithelial cells (62). Efa-1 was originally identified in Shiga-toxin producing *E. coli* (STEC) O111:H⁻ strains and conferred adherence onto Chinese hamster ovary cells (61). Genotypic analysis on Efa-1 in O111:H- strains reveals that it shares very strong amino acid identity with EPEC lymphostatin LifA, a lymphotoxin that contributes to EPEC adherence (59). Live animal studies also show that cattle infected with Δ *efa-1* in O111:H- strains showed decreased fecal shedding compared to their wild-type counterparts (61).

1.5.2.3 Iha

The IrgA homolog adhesin (Iha) is a 67 kDa protein identified in EHEC O157:H7. Transformation of non-pathogenic *E. coli* with *iha* confers an increased adhesion on human epithelial tissue *in vitro*, suggesting its role in adhesion. Molecular analyses show that the *iha* gene is present in 91% of all pathogenic EHEC strains, suggesting that Iha is a common adhesin used by a variety of virulent *E. coli* serotypes (63). Interestingly, *iha* expression is higher among *E. coli* strains found in bovine ruminant rather than humans. Iha was also shown to be important for bovine gut colonization of O157:H7 (64).

1.6 Regulation of Fimbrial Adhesin Genes

Over the last two decades, investigators have shown that pathogenic bacteria's microenvironment is crucial in the regulation and expression of fimbrial adhesin encoding genes and other virulence factors. Low *et al.*, surmise that EHEC carries an arsenal of fimbrial adhesin proteins that expressed only under very specific conditions and may confer the virulent phenotypes observed from this pathogen (40). Several of these microenvironments include suboptimal temperature and pH, osmolarity, bacteria cell density and ingestion stress.

Gene expression analyses have shown that expression of type 1 fimbrial protein gene *fimA* is temperature dependent. Expression of *fimA* transcripts appeared slightly higher at 30°C than at 37°C (65). It was determined that at 37°C, the invertible switch that regulates *fim* operon expression is at a neutral position and neither turns "on" nor "off" *fim* genes. However, at 30°C, the switch becomes biased to an "on" position, and thus results to slightly higher *fim* expression. This apparent environmental mode of regulation of *fim* suggests that the bacteria can easily adapt

to the conditions of its environment, which is a crucial attribute for its survival and pathogenicity (65).

Environmental pH also affects the transcription of *fim* genes. Neutral pH of 7 shows maximal expression of *fim*, whereas low pH (pH 5.5 or lower) negatively regulates the gene (66). Transcriptional analyses of *fimB* and *fimE* results in to a 2-fold decrease in expression when clinical UPEC was grown in pH 5.5. The expression of the invertible switch that regulates the *fim* promoter is also seen to decrease 4-fold when bacteria is grown at this pH, suggesting that the orientation of the switch is phased off (66).

Torres *et al.*, report that *lpf2* expression is environmentally regulated as well. Molecular work on *lpf2* reveal that its expression during lac fusion assays was observed highest when induced in media at pH 6.7 than in slightly acidic media at pH 5.5 (67). β -galactosidase assay also reveals that *lpf2* expression was modulated in media supplemented with NaCl. Expression of *lpf2* was reduced in half in media with high salt molarity when compared to controls (67).

Expression of *ecpA* is believed to be regulated by temperature and growth media. EHEC is able to produce ECP at temperatures below 37 °C, indicating its role in the bacteria's adhesion outside its host environment (54). ECP is also produced only when EHEC is grown in DMEM and not LB.

1.7 Acid Stress and EHEC O157:H7

The microenvironment that EHEC is exposed to during ingestion include a variety of stresses from gastric acid shock found in the stomach, bile-salt stress from the small intestine and short-chain fatty acid stress in the large intestine (68). To establish successful infection, EHEC must be able to survive in these ingestion stresses, especially the acute acid conditions of

stomach that can range between pH 1.4 to pH 2.0 among healthy individuals (69, 70). In addition, EHEC must also survive the acidic conditions commonly used for bacteria growth control in the food industry (71). Acid challenge such as organic acid spray washes are routinely used to destroy pathogens and other contaminants from beef carcasses in meat processing plants (71).

1.8 Acid Response Systems

In response to these acute acidic challenges, EHEC has developed a sophisticated acid tolerance response (ATR) mechanism. In the last decade, as many as four acid response (AR) systems have been identified (described below). These systems each illustrate a unique and independent acid stress mechanism, allowing EHEC to develop specific mechanisms for protection against acid challenged situations (72). These mechanisms of acid resistance contribute to the survival of even a few EHEC cells during gastric passage.

1.8.1 Oxidative Repressed Acid Resistance (AR1)

The oxidative repressed acid resistance (AR1) is the first acid response system identified and is also the one that is least understood. Stationary phase EHEC cells grown in LB media buffered at pH 5.5 exhibited significant resistance and protection against acute acid above pH 3.0 compared to the same cells grown at pH 8.0 (73). However, this acid tolerance response is repressed when stationary phase bacteria is grown in LB supplemented with glucose. Molecular studies reveal that the stationary phase alternative sigma factor, σ^S or RpoS, is required for these cells to induce and develop acid tolerance. σ^S is a global regulatory protein that works in conjunction with cyclic AMP (cAMP) and cAMP receptor protein (CRP) (74). Experimental

analyses have also shown that cells grown at pH 8.0 produce an inhibitory response against AR1 that prevents this acid response mechanism. Inhibition of AR1 is not induced below pH 5.0, thereby allowing the bacteria to survive acute pH conditions. Interestingly, supplementation of glutamate in the media even at pH 8.0 induces AR1 action, suggesting that environmental conditions play a role in EHEC acid tolerance response (75). While very little is known about AR1, investigators believe that it requires the putative glutamate/ γ aminobutyric acid (GABA) antiporter, GadC, and its mode of action overlaps with the other acid response systems that have been identified (72).

1.8.2 Glutamate-dependent Acid Resistance (AR2)

The most well- characterized acid resistance mechanism is the glutamate-decarboxylase dependent system. AR2 employs two decarboxylase isozymes encoded by the *gadA* and *gadB* genes, AraC-family regulatory protein products of *gadW*, *gadY* and *gadX* and the GABA antiporter GadC (76). Investigators believe that AR2 provides EHEC the best protection against acid stress, as it functions in acid-challenged media of pH 2.0 or less (73). The AR2 system maintains the internal cytoplasmic pH by converting glutamate into GABA and consumes a proton in the process (72, 74). When the external pH is at 2.0 or lower, the AR2 system maintains the cells' internal pH at 4.2 ± 0.1 (76). This mechanism of acid tolerance, however, is only induced if glutamate is supplemented in the acid challenged media.

Like AR1, AR2 induction occurs during stationary phase, although the *gad* genes are somewhat induced in log-phase cultures grown in minimal glucose media (72). Evidence show that AR2 partially depends on global regulator such as σ^S to function and to develop acid

resistance. However, it can also operate in an RpoS-independent fashion (77). Molecular mapping illustrates that both *gadB* and *gadC* are co-transcribed in one operon, while *gadA* is transcribed under a separate promoter. In natural conditions, these *gad* genes are repressed by CRP; however, after acid induction, a repressor protein, GadY, (also known as YhiX) stabilizes *gadX* mRNA that in turn activates *gadE*. GadE functions as an activator and turns on the transcription of *gadA* and *gadBC*, thereby allowing the AR2 system to function (75). Loss of function Δ *gadY* or Δ *crP* mutants results to a constitutive *gadA* and *gadBC* expression independent of pH. This suggests that AR2 is tightly regulated in nature, most likely to facilitate survival and pathogenesis of EHEC in response to a variety of environmental conditions (72).

Of the four AR systems, AR2 is believed to be most crucial for gastrointestinal survival of pathogenic *E. coli* (73). Interestingly, the GAD proteins are not active in log-phase cells, even if its gene transcripts are highly expressed. This strongly indicates that while the AR2 system is crucial for acid tolerance response, other systems are at work in the survival of acid-induced EHEC *in vivo* during logarithmic growth.

1.8.3 Arginine-dependent Acid Resistance (AR3)

The arginine decarboxylase-dependent system requires the addition of arginine in acid challenged minimal media for induction. AR3 functions in anaerobic conditions and only when cells are previously exposed to rich media (74). As with AR2, the mechanism of AR3 is simple; in the presence of acute acidity, arginine-inducible decarboxylase AdiA converts arginine to agmatine and in the process consumes a proton (78). The antiporter AdiC mediates the transfer of arginine across the membrane. Like AR2, AR3 increases cellular pH to 4.7 ± 0.1 allowing EHEC to survive low extracellular pH (76).

Mutational experiments demonstrate that a regulator protein, CysB, is involved in the control of AR3; *cysB* mutants fail to respond in an arginine-dependent fashion (71). Another AraC-family of protein regulators, AdiY, seem to activate the AR3 dependent *adiA* and *adiC* genes. Like AR2, σ^s seems to play a role in AR3 induction, but is not solely required for this acid tolerance response (74).

As with AR2, AR3 is crucial for the maintenance of internal pH in acid challenged media. Richard and Foster describe a sophisticated arginine-dependent acid resistance model, wherein excess protons that enter the cell are immediately consumed during decarboxylation (70). The consumption of a proton allows the conversion of arginine into agmatine and drives the internal pH to 4.0-5.0. Richard and Foster argue that protons in the form of HCl easily diffuse into EHEC cells in low pH environments. HCl species dissociate in the cell as H⁺ and Cl⁻. Antiporter, AriC, consumes a proton during decarboxylation. The negatively charged Cl⁻ ions are left in the cell and are ultimately discharged through chloride transport pumps to prevent hyperpolarization when cells are returned to neutral media (76, 79).

1.8.4 Lysine-dependent Acid Resistance (AR4)

The lysine-dependent decarboxylation system was first identified in non-pathogenic *E. coli* strains but its presence in O157:H7 pathotypes has been confirmed. AR4 protein products are encoded by the *cadBA* genes and like with AR2 and AR3, consumes a proton for every decarboxylation of lysine (80, 81). The lysine-dependent decarboxylation provides some degree of protection against mild acid shock, but is not sufficient to respond to pH lower than 3.0 (80). The decarboxylase encoded by the gene, *cadA*, is fully induced in the presence of low pH, anaerobic conditions and high concentrations of lysine in the media (80). Currently, it is not

known whether or not σ^s partially regulates AR4 response as with the other decarboxylase-dependent systems (80). Investigators also believe that the degree of acid protection provided by AR4 is not as strong as AR2 and AR3 (81)

1.9 Acid Stress on EHEC O157:H7

1.9.1 Acid Tolerance

In addition, acid stress challenge appears to play a crucial role in EHEC survival and virulence. Habituation or adaptation of EHEC cells in sublethal pH conditions seems to increase its survival in acute acidic environments. Leyer *et al.*, report that acid adaptation of *E. coli* O157:H7 beef isolates can confer resistance to lactic acid (81). Tosun *et al.*, also show that clinical isolate O157:H7 932 tolerate acute acidic conditions as low as pH 3.0 if previously exposed to pH 4.5 (82).

Acid adapted EHEC cells are also able to retain their acid resistance even after prolonged periods in cold storage (83). In spite of the long interval between slaughter and human ingestion, EHEC pre-exposed to weak organic acids in the farming industry, still exhibit a strong degree of acid resistance and therefore are able to face subsequent exposure to gastric acid (84).

Acid-adapted EHEC are also able to tolerate other environmental stresses compared to their non-adapted counterparts. Cheng *et al.*, demonstrate that acid-adapted cells show increased thermal and sodium chloride tolerance (85). Habituation in mild acid also provides EHEC a degree of freeze-thaw resistance. The cellular membranes of acid adapted cells seem to be more adaptable and therefore less susceptible to freeze-thaw rupture (86).

Genome array analyses also reveal that acid shock promotes differential gene expression in EHEC O157:H7. As many as 21 genes, including periplasmic protein encoding gene, *marB*, are significantly upregulated when cells are shocked in acid for 15 min (87). While the function of *marB* is yet to be elucidated, the *mar* operon confers multiple drug resistance in *E. coli* and when induced, increases the bacteria's survival in toxic environments (88).

Our lab has also showed that exposure of EHEC to a variety of acid adaptation and acid shock conditions results in the significant upregulation of virulence genes that encode flagella, adhesins and type III secretion proteins (69). These two studies indicate that acid stress induces gene expression of numerous virulence genes that can contribute and alter EHEC's pathogenic abilities.

1.9.2 Adhesion

Recent work in our lab has shown that exposure to acute acid stress can significantly enhance EHEC adhesion to human epithelial cells for up to 150-480% (89). Bacteria pre-exposed to acid treatments are able to adhere significantly better than unstressed controls. Previous studies on the related diarrhaegenic pathogen EPEC also showed enhanced host cell adhesion of the bacteria after acid stress (68). The acid-induced host cell adhesion enhancement for both EPEC and EHEC can be eliminated if the bacteria are treated with subinhibitory concentrations of erythromycin, an antibiotic known to inhibit bacterial protein synthesis (62). This indicates that the adhesion enhancement requires protein synthesis and likely involves either or both increased expression of known or new adhesins.

Transcriptomic experiments were performed in order to identify possible adhesin encoding genes that are upregulated after EHEC exposure to acid stress and/or adaptation. Our

Table 1.1. List of putative fimbrial genes that showed increased expression after adaptation at pH 5 for 60' and shock at pH 3 for 30'. Adapted from (63)

Gene ID	Putative Function	Fold Expression AA30
Z0639	Adhesin/Invasin-Like Protein	3.47
ECS1417	Putative 2-Component Regulator, 2 nd Curli Operon, <i>csgD</i>	3.02
ECS3723	Type III Secretion Protein EpaR1	4.01
ECS0597	Putative Fimbrial Protein, FimZ	2.38
B0293	Putative Fimbrial Protein, YagZ	4.98
B0170	Putative Membrane Protein – HofB	5.45
B0136	Putative Fimbrial Adhesin YadK	4.94

Figure 1.5 Real time PCR analysis of *yadK*. Data shows that the gene is increased by 2.5 fold in acid adapted, acid stressed bacteria.

1.10 Putative fimbrial protein YadK

One of the genes that showed a 5-fold increase in gene expression after EHEC exposure to acid stress treatment was predicated to encode a putative fimbrial adhesion, YadK (**Table 1.1**). The same pattern of increased *yadK* expression after acid adaptation, acid stress was also confirmed by quantitative real-time reverse-transcriptase (RT) - PCR, strongly suggesting that this gene responded to acid stress treatment (**Figure 1.5**). The significant upregulation of *yadK* transcripts in acid-induced bacteria strongly correlates with the increased adhesion of acid-stressed EHEC during bacteria-host cell adhesion experiments (90). This suggested that YadK may play a role in the adhesion of acid-stressed EHEC cells *in vitro*.

Bioinformatic analysis reveal that *yadK* is a 591 base pair long gene that is predicted to encode a minor subunit in a fimbrial adhesin complex expressed in the outer membranes of EHEC cells (**Figure 1.6A**). The *yadK* gene is identical in both EHEC serotypes EDL933 and Sakai. The *yadK* gene product is predicted to be fimbrial adhesin that is 196 amino acid in length with a molecular weight of 21.2kDa and a pI of 5.24. YadK bears a domain that belongs to the cl01416 superfamily. Members of the cl01416 family all carry a conserved fimbrial structure universally shared among several bacterial adhesins including the *fim* genes that are assembled into type 1 fimbriae.

yadK is predicted to be a minor subunit of the locus 2 putative fimbrial operon that has been identified among EHEC Sakai strains. Interestingly, the original MG1655 fimbrial locus that contains *yadK* (*yadC*, *yadK*, *yadL*, *yadM*, *htrE*, *ecpD* and *yadN*) was discovered missing in the EHEC EDL933 and Sakai genome. The same locus was replaced by a different set of fimbrial genes (Z0146, Z0147, Z0148, Z0149, Z0150, Z0151 and Z0152 in EDL933; ECs0139,

Ecs0140, Ecs0141, Ecs0142, Esc0143, Esc0144 and Esc0145 in Sakai) that share partial homology to the commensal strains (91). While these fimbrial genes follow the same nomenclature as the genes found in MG1655 (ie. Z0147 is also called “*yadK*”) and is predicted to follow similar functions, investigators believe that they were once removed and re-inserted into the bacteria’s chromosome and therefore are considered virulence factors (91). Like *yadK*, our lab’s microarray data show that all the other genes in locus 2 appear to be upregulated by acid as well (74, **Figure 1.6B**).

Homologs of *YadK* are also found among other enteric pathogens including *Salmonella* and *Shigella*. The amino acid sequences of *YadK* in EHEC and in *Salmonella* bear a 63% homology ($3e^{-42}$), and a 61% homology to the *Shigella* *YadK* ($4e^{-34}$).

A.)

B.)

Figure 1.6 A. Predicted protein fold structure of YadK. PHYRE protein prediction folding software predicts the folding of YadK to be similar to fimbrial protein FimF. **B. The yadK gene is located in locus 2 in both EHEC O157:H7 strains EDL933 and Sakai.** The predicted protein fold of the YadK adhesin shows strong homology to type-1 fimbriae minor subunit FimF. Adapted from (41)

			Treatment (AA30)
			fold-dif
loc2	Gene Description	Gene ID	
ECS0139	putative fimbrial protein [yadC]	ECS0139	1.7695
ECS0141	putative fimbrial protein [yadL]	ECS0141	1.47087
Z0149	putative fimbrial protein [yadM] probable outer membrane porin protein involved in fimbrial assembly [ecpD]	Z0149	1.99078
Z0150		Z0150	1.69741
	putative chaperone; yfcS [B]	B2336	1.40444
Z0152	putative fimbrial-like protein [yadN]	Z0152	2.66352

Figure 1.7 Transcriptional Expression of other Genes in locus 2 after Acid Adaptation and Acid Stress. Microarray analysis indicate that other genes in locus 2 are expressed positively after acid stress. (74)

Chaperone-Usher Mediated Biochemical Synthesis of the locus 2 Operon

The biogenesis of most surface-exposed fimbriated adhesins is often mediated by the chaperone-usher pathway that is universally conserved among Gram-negative bacteria including EHEC (92). These gene clusters are usually genetically organized in the same arrangement and encode two key functional proteins: an usher and a chaperone. These proteins are especially important in the biogenesis and assembly of the final fimbrial structure (93). The mechanisms that follow chaperone-usher biogenesis are reviewed by Capitani, *et al.*, (93) (**Figure 1.8**); briefly, pre-fimbrial proteins bearing N-terminal signal peptides destined for fimbrial assembly are synthesized in the cytoplasm and transported to the periplasm. The proteins are translocated through a type II secretion system.

Once in the periplasm, chaperones assist in the proper folding of these pre-fimbrial proteins. Crystal structures of type 1 and P-fimbrial pilins strongly indicate that pre-fimbrial subunit bear an immunoglobulin-like (Ig) structure composed of anti-parallel β -sheet (β A to β F) strands that form a flat barrel, with β F at the C-terminal end and 10-18 residues called the N-terminal extension (92). The pre-fimbrial structure lacks a final β -strand (β G), creating a hydrophobic groove within the protein that renders its Ig-fold incomplete. Because of this, the pre-fimbrial subunit remains unstable without the folding assistant of the boomerang-shaped chaperone. Chaperone N-terminal domains contain G1- β donor strand that when in contact with pre-fimbrials, completes a stable Ig-fold. Chaperones also contain conserved residues that anchor the C-terminal of pre-fimbrial subunits to guarantee the proper orientation of the fimbrial proteins in the fimbrial stalk (92, 93).

During polymerization of the fimbrial stalk, the pre-fimbrial protein is pushed to the cell surface from the membrane porin, usher, through donor-strand exchange. Polymerization occurs

Figure 1.8 The chaperone-usher mechanism illustrates the biosynthesis of fimbrial adhesins like type-1 fimbriae. The key factors in this mechanism include a boomerang-shaped chaperone protein (green) that assist in the folding of pre-fimbrial subunits and a membrane porin usher (red) that guides the translocation and assembly of the pre-fimbrial subunits to the fimbrial stalk (yellow). Adapted from (88).

without the hydrolysis of ATP or electrochemical gradients (94). Instead, donor-strand exchange drives the assembly forward: once the pre-fimbrials are brought by the chaperone to the usher, the pre-pilins are released (95). The N-terminal extension of incoming pre-fimbrial fibers are inserted in the hydrophobic groove in an anti-parallel fashion to βF . The N-terminal extension replaces the G1-b donor and completes a stable Ig-fold. Donor strand exchange occurs at the outer membrane of the usher at a time-efficient manner and allows the continuous polymerization of the fimbrial stalk (96). The location of fimbrial subunit genes in their respective fimbrial clusters defines stoichiometric concentrations of the expressed fimbrial adhesins; Fimbrial genes closer to the promoter are expressed at higher concentrations than genes further downstream (97, 98).

Protein fold recognition server (PHYRE) reveals a 100% homology between the predicted tertiary structures of YadK to the NMR structure of *E. coli* type 1 fimbria subunit FimF (99). Though there is no empirical evidence of the YadK structure, protein prediction software strongly indicates that the protein is structurally composed of anti-parallel β -sheets. The predicted fold-structure of YadK also suggests that it bears a domain that likely interacts with the chaperone-usher proteins in a chaperone-usher mediated assembly pathway. BLASTP analysis also shows that YadK resembles known fimbrial proteins that follow the usher-chaperone pathway. The locus 2 gene cluster that *yadK* belongs to follows the same genetic organization as other chaperone-usher exported fimbriae (40) (**Figure 1.9**). *ecpD* and *htrE* are predicted to be the chaperone and an usher of this operon respectively. If YadK follows a chaperone-usher biosynthesis, then it is likely exported to the periplasm by EcpD and translocated across the membrane by passing through HtrE. Once in the cell wall, YadK is assembled along the fimbrial

shaft along with the other minor subunits, and either functions in cell adhesion or builds the fimbrial stalk.

2. Materials and Methods

Figure 1.9 The genetic organization of *yadK* in locus 2 is similar to the genetic organization of other fimbrial loci that follow the chaperone-usher biosynthetic pathway. The protein *EcpD* resembles a chaperone protein whereas *HtrE* is predicted to be an usher. The *YadK* fimbrial protein (yellow) is predicted to be a minor fimbrial subunit in the entire locus. White filled arrows denote fimbrial usher-chaperone proteins; Red filled arrows denote major subunit. Arrows not drawn to scale. Adapted from (41).

1.12 Purpose of Investigation

While the mechanisms of EHEC pathogenicity have been extremely studied, the impact of acidic stress of gastrointestinal passage on EHEC virulence has not been fully elucidated. Identifying putative fimbrial adhesins that may play a role in bacteria-host tissue cell adherence will also improve our understanding of *E. coli* O157:H7 pathogenicity. One of these factors, *yadK*, is a putative fimbrial protein encoding gene that appears to respond to acute acid-induced EHEC.

While there is no published data corroborating the effect of acid on *yadK* expression, our lab has shown that it is significantly upregulated in acid-induced EHEC cells and raises questions of its role in EHEC pathogenicity. Thus, the investigation of the role *YadK* plays in acid-induced adhesion of EHEC O157:H7 is important in improving our understanding of EHEC pathogenicity.

1.13 Thesis Objectives

I: To construction of a $\Delta yadK$ mutant in EHEC O157:H7.

In order to identify the role of the putative fimbrial protein gene *yadK* in EHEC pathogenesis, a *yadK* mutant in 85-170 background must be constructed.

II: To characterize the adhesive properties of $\Delta yadK$ mutant.

The adhesin properties of a $\Delta yadK$ mutant will be assessed during both unadapted/unstressed and acid adapted/acid stressed treatments.

2. Materials and Methods

2.1 Bacteria Strains and Growth Conditions

Enterohemorrhagic *E. coli* 85-170 was used as the wild-type strain. **Table 2.1** lists all bacterial strains used in this study. Glycerol stocks were stored at -80°C and routinely streaked onto Luria-Bertani (LB) agar plates (1% tryptone; 0.5% yeast extract; 0.5% sodium chloride; 1.5% agar) or tryptose phosphate (TP) agar plates (BD Diagnostics System; 1.5% tryptose phosphate broth; 1.0% agar; (Invitrogen) 0.1% sodium pyruvate) with or without antibiotics. EHEC 85-170 strains harboring a *yadK* deletion mutation ($\Delta yadK$) and carrying the λ -red vector pKD46 (85-170(pKD46)) were grown with 100 $\mu\text{g/mL}$ of kanamycin and 100 $\mu\text{g/mL}$ ampicillin,

respectively. Broth cultures for all the strains were prepared by inoculating one single isolated colony into 10 mL of LB broth or TP broth (with or without antibiotics) and incubated aerobically at 37°C for 16-18 h with agitation. Plate cultures were maintained at 4°C for 2-4 weeks.

2.2 Cell Culture

Human colonic adenocarcinoma cells (CaCo2) were obtained from the American Type Culture Collection (ATCC) and used in various adhesion assays. ATCC growth and cell maintenance guidelines were adapted for the preservation of the human cells. Frozen stocks of CaCo2 cells were grown in Minimal Essential Media 1x Eagle's (MEM) (Wisent; with Earle's salts and L-glutamine) supplemented with gentamycin (20 $\mu\text{g/mL}$) and 20% fetal bovine serum (FBS). CaCo2 cells were grown at 37°C in air with 5% carbon dioxide (CO_2) and routinely fed with fresh media every 4 days. Passage of the cells was completed every 7 days.

Table 2.1 List of strains used in this study.

Strains	Description	Kan ^R	Amp ^R	pSK	Source
85-170	EHEC O157:H7 Derivative of 84-289 that spontaneously mutated in laboratory	—	—	—	Tzipori <i>et al.</i> , 1987
85-170(pKD46)	EHEC transformed with λ -red plasmid pKD46	—	+	—	This Study
$\Delta yadK$	<i>yadK</i> Deletion Mutant	+	—	—	This Study
85-170(pSK)	EHEC O157:H7 Clinical Isolate with pBlueScript SK +	—	+	+	This Study
$\Delta yadK$ (pSK)	<i>yadK</i> Deletion Mutant with pBlueScript SK +	+	+	+	This Study
$\Delta yadK$ (pSK- <i>yadK</i>)	Complemented <i>yadK</i> Deletion Mutant	+	+	+	This Study

2.3 Growth Assays

A growth curve analysis was performed to assess the growth kinetics of each strain prior to the acid experiments. An overnight broth culture of each strain was prepared and grown at 37 °C, with agitation. Twenty microliters of the overnight cultures were diluted into fresh 350 µL media of either LB or Dulbecco's Minimal Media (DMEM) (Wisent; 4.5g/L glucose, L-glutamine, sodium pyruvate, pH7.4). All of the diluted strains were loaded on a 96-well plate in triplicates and the growth kinetic experiment was performed in a microbiology workstation (Oy Growthcurves Ab Ltd.). The growth reading parameters included static incubation at 37°C with pre-shaking for 20 sec before readings, and reading measurements for every 20 min. The experiment was performed for a period of 16 h and statistically analyzed afterwards.

2.4 PCR Analysis

Polymerase chain reaction (PCR) was routinely used to amplify amplicons for Lambda Red Recombination and to screen for deletion mutants. Unless otherwise noted, all PCR reactions were performed using an HP Taq DNA Polymerase (UBI Bioinformatics), including the following reagents: 2.5 µL 10x Taq reaction buffer, 5 µL of 20 mM MgSO₄, 0.25 µL forward primer (final concentration of 10 mM), 0.25 µL reverse primer (final concentration of 10 mM), 2 µL 10 mM dNTP mix (Deoxynucleotide, UBI Bioinformatics Inc.) 0.5 µL template, 0.5 µL of 5u/µL Taq, and 16.5 µL of sterile nuclease free water (Gibco). All PCR reactions were performed in a 96-well Biorad PCR machine. PCR products (10 uL) were resolved on 1% agarose gels and visualized after ethidium bromide-staining under UV illumination. The primers used for this study are listed on **Table 2.2**.

Table 2.2 Primers used for PCR in this study.

Primer Name	Sequence (5' – 3')	Tm °C	Target Gene
<i>yadK</i> _F I-Red Recombination	5'CTGTCAGGTTGAAGTCAGTAATAATGGCGTTG TCGATCTCGTGTAGGCTGGAGCTGCTTC 3'	77.1	<i>kan^R</i> cassette pKD4/85-170 <i>yadK</i>
<i>yadK</i> _R λ-Red Recombination	5'GGTGCTACCGAATAAAATTGCTGTTGAGGAACC ATCCGTTTCATGGGAATTAGCCATGGTCC 3'	76.4	<i>kan^R</i> cassette pKD4/85-170 <i>yadK</i>
<i>yadK</i> -forward	5'GGCATCATAAAATCGTAC 3'	45.6	85-170 <i>yadK</i>
<i>yadK</i> -reverse	5'AACAATAACCTGGCTTCT 3'	45.6	85-170 <i>yadK</i>
<i>kan</i> -forward	5'TGTAGGCTGGAGCTGCTTC 3'	55.3	<i>kan^R</i> cassette pKD4
<i>kan</i> -reverse	5'ATGGGAATTAGCCATGGTCC 3'	53.8	<i>kan^R</i> cassette pKD4
<i>yadK</i> -forward SacII	5'AACCGCGGAAGGATATTAGAATGCTATGC AGGCATCTAA 3'	66.9	85-170 <i>yadK</i> SacII RE Site
<i>yadK</i> -reverse BamHI	5'TTGGATCCTATTATTCGTAAGATACGTAT 3'	56.2	85-170 <i>yadK</i> / BamHI RE Site
<i>yadC</i> -F	5'TTAGTCTGGCGTCACAAATG 3'	58.0	RT-PCR 85-170 <i>yadC</i>
<i>yadC</i> -R	5'TCTGGGTGCTTGGTATGAAG 3'	60.0	RT-PCR 85-170 <i>yadC</i>
<i>yadK</i> -F-RT-PCR	5'TGGTCTCTTGCGATAACATTC 3'	60.0	RT-PCR 85-170 <i>yadK</i>
<i>yadK</i> -R-RT-PCR	5'TGAGGGAACAGCATTGCCAG 3'	62	RT-PCR 85-170 <i>yadK</i>
<i>gapA</i> _F-jk1	5'AAAGGCGCTAACTTCGACCA 3'	51.8	RT PCR 85-170 <i>gapA</i>
<i>gapA</i> _R-jk2	5'GCAGCTTTTCCAGACGAAC 3'	53.8	RT PCR 85-170 <i>gapA</i>

2.5 Preparation of Chemically Competent Cells

A single isolated colony of 85-170 strain was inoculated in to fresh LB broth and grown overnight at 37 °C with agitation. The overnight culture was diluted 100 fold into fresh LB and re-cultured at 37 °C with agitation until optical density (OD₆₀₀) reached approximately 0.4. The cells were briefly chilled on ice for 10 min and harvested at 4,000 x g for 5 min at 4 °C. The cells were resuspended with 1:4 volume 100 mM iced-cold MgCl₂ and chilled on ice for 5 min. The cells were harvested as above and resuspended in 1:4 volume of iced-cold 100 mM CaCl₂. The cells were chilled on ice for 20 min, harvested as above and finally resuspended in 1:40 volume iced-cold 100 mM CaCl₂ + 15% glycerol. The cells were divided into 50 µL aliquots in pre-chilled 1.5 mL microcentrifuge tubes (Axygen) and freeze-snapped in liquid nitrogen before being stored at -80 °C.

2.6 Transformation of Chemically Competent 85-170

Transformation of chemically competent 85-170 cells with the plasmid pKD46 encoding the Lambda-Red Recombination proteins was adapted from One Shot Top10 Cloning protocol (Invitrogen). A 5 µL aliquot of pKD46 was added to 50 µL of chemically competent 85-170 cells. The mixture was cold shocked on ice for 30 min, heat shocked at 42 °C for 30 s and re-suspended with 150 µL fresh LB. The cells were incubated for 1.5 h at room temperature, with agitation, and plated on LB agar supplemented with 100 µg/mL ampicillin for overnight growth at 30 °C. Successful transformants were screened through replica-patch plating on fresh LB plates supplemented with 100 µg/mL ampicillin. The presence of pKD46 from clones that grew

from the ampicillin plates was confirmed by plasmid extraction using a HiYield Plasmid Mini Kit (UBI Bioinformatics, Inc.).

2.7 Preparation of Electrocompetent Cells

Lambda (λ) red recombination system is a novel molecular tool that aid in the partial or full inactivation of DNA fragments from bacteria chromosome through homologous recombination. Previous work on *E. coli* K-12 and O157:H7 strains demonstrate the efficacy of using λ Red as a genetic device to knock out gene elements (100, 101). The λ Red Recombination system encodes its own recombination functions *exo* (*red α*), *bet* (*red β*) and *gam* that can be conveniently housed in a plasmid, temperature sensitive plasmid pKD46 (102). λ *exo* functions to degrade one end of double stranded DNA at a 5' to 3' direction. It chews off mononucleotides from the 5' strand leaving behind a 3' overhang. This overhang provides a plausible space for single stranded, foreign DNA to base pair with the nicked genomic DNA upon recombination. The λ beta recombinase stabilizes single stranded, foreign DNA and prevents its degradation. Beta also promotes the base pairing of single stranded, foreign DNA to its complementary genomic overhang. Finally, λ *gam* binds to host RecBCD and inhibits host control over the recombination event (102).

Preparation of electrocompetent EHEC 85-170/pDK46 was adapted from Datsenko *et al.*, and from the Molecular Cloning: A Laboratory Manual (100, 103). A single isolated colony of the bacteria was inoculated in to fresh 10 mL LB broth containing 100 μ g/mL ampicillin and grown overnight at 30 °C with agitation. Following overnight growth, the culture was diluted 100 fold and re-grown into fresh LB broth with 100 μ g/mL ampicillin at 30 °C with agitation. λ Red system expression was induced with 1 mM final concentration of L-arabinose and the

bacteria was grown until an OD₆₀₀ of 0.400 was reached. The cells were briefly chilled on ice for 15 min and harvested at 4000 x g for 15 min at 4°C. The cell pellet was carefully resuspended in ½ volume iced-cold sterile water and once again harvested by centrifugation. The pelleted cells were washed with 1/50 volume of ice-cold sterile water and harvested as above. The cells were finally resuspended in 1/100 volume of ice-cold 10% glycerol and aliquoted into 50 µL-volume in pre-chilled, sterile microcentrifuge tubes and kept at -80 °C until used.

2.8 Construction of the *yadK* Deletion Mutant

The basic strategy is to replace a chromosomal sequence (gene to be inactivated) with a selectable antibiotic resistance cassette that is generated by PCR (**Figure 2.1**). In this procedure, recombination requires the phage λ red recombinase, which is synthesized under the control of an inducible promoter on the easily curable low copy plasmid pKD46 (100, 101). A kanamycin resistance cassette was PCR amplified using primers with extensions that are homologous to the gene to be inactivated (H1, H2). The resulting amplicon was introduced into competent EHEC carrying the λ Red system and homologous recombination is used to facilitate gene replacement.

For this study, a kanamycin resistance cassette flanked by 20 nt of homologous sequence to *yadK* gene was constructed by PCR using forward primer *yadK*_Fλ-Red Recombination and reverse primer *yadK*_R-λ Red Recombination (Table 2.2, **Figure 2.1**). The PCR cycle consisted of 1 min denaturation at 95 °C, followed by 30 cycles of 30 min at 95 °C, 45 s at 76 °C and 2.5 min at 72 °C with a final 7 min extension step at 72 °C. PCR products (10 uL) were resolved on 1% agarose gels and visualized after ethidium bromide-staining under UV illumination. The products were also purified using an Agarose Gel Extraction Kit (UBI Bioinformatics, Inc.),

following manufacturer's instructions. The purified product was further precipitated at -80 °C overnight. After precipitation the product centrifuged at 13000 rpm for 15 min at 4 °C and was washed twice with 1 mL 70% EtOH before being resuspended in a final of 2 µL sterile, nuclease free ddH₂O. Fifty microliters of electrocompetent EHEC 85-170 cells carrying pKD46 were transformed by electroporation (25 µF, 200 Ω) with 1 µL of purified PCR product.

One milliliter of SOC (2% tryptone; 0.5% yeast extract; 0.05% sodium chloride; 1.0% 250 mM KCl; 0.5% (v/v) 2 M MgCl₂; 2% (v/v) 20 mM glucose) was added to the cells following electroporation. The cells were recovered at 37 °C, 220 with agitation for 3 h and plated on LB agar plates supplemented with 100 µg/mL kanamycin for overnight incubation. Colonies that grew from the kanamycin agar plates were screened by re-patching onto fresh 100 µg/mL kanamycin plates and re-grown at 37 °C, overnight. Successful mutants were verified for the correct insertion of the kanamycin resistance cassette in the *yadK* locus through PCR using *yadK* primers. Bands showing the correct insertion of kanamycin within the *yadK* locus were gel purified (UBI Bioinformatica, Inc.) and confirmed through DNA sequencing (ACGT Company, Toronto, ON).

Fig 2.1 Schematic of Lambda-Red promoted PCR-mediated recombination (96, 97). A kanamycin resistance cassette was PCR amplified with primers flanked by 20 nt homologous to regions within *yadK* (H1, H2). The resulting amplicon was introduced into 85-170 carrying pKD46. Red recombination was induced with L-arabinose and successful mutants were selected, screened and verified.

2.9 Acid Stress Treatment

The acid treatment used for this study was adapted from DeJesus and coll. (68). A single isolated colony of 85-170 or 85-170 Δ *yadK* was inoculated into 10 mL LB broth (with or without 100 μ g/mL kanamycin) at 37 °C with agitation, and grown overnight. The culture was diluted 10 fold in DMEM (pH 7.4) and subcultured statically at 37 °C with 5% CO₂, until OD600 of 0.4-0.6 was reached. Two milliliters of the subculture was transferred to three different sterile 15 mL flat cap centrifuge tubes (VWR) and harvested by centrifugation. The cell pellets were resuspended with 10 mL DMEM + 25 mM MES (pH 5) and acid adapted statically at 37 °C with 5% CO₂ for 1 h. After adaptation, the bacteria cells were harvested by centrifugation, resuspended in 10 mL DMEM (pH 3) and acid shocked for 30 min at 37 °C with 5% CO₂. Unadapted, unshocked controls were also performed alongside acid stress treatment using 10 mL DMEM with 25 mM MOPS (pH 7.4) (for adaptation control) and 10 mL DMEM + 25 mM MOPS (pH 7.4), for acid shock control. **Figure 2.2** provides a flow diagram of the acid stress treatment.

Modified Chromosome

Fig 2.2 Scheme for acid treatment protocol. *Overnight cultures of EHEC were prepared and re-cultured to mid-log phase. Both the acid stress treatment and control experiments were performed in triplicates.*

2.10 Viability Assay Experiment

The survival of 85-170 wild-type or $\Delta yadK$ after acid stress treatment was assessed after each acid stress treatment experiment. Following acid treatment, 100 μL of acid-induced bacteria was loaded into 900 μL 1 x PBS (PBS, Wisent without calcium and magnesium) and serially diluted. The dilutions 10^{-3} to 10^{-5} were plated in triplicates onto pre-warmed TP agar supplemented with 0.1% sodium pyruvate and incubated at 37 °C overnight. Following overnight incubation, colonies between 30-300 counts were enumerated and used to calculate the percent survival of EHEC cells before and after acid stress treatments

2.11 Bacteria-Cell Adhesion Assay

Adhesion of 85-170 wild-type and $\Delta yadK$ was assessed using a bacteria-host-cell adhesion protocol described earlier (68). A week before the acid stress treatment experiment, CaCo2 cells were grown to 75-80% confluency in 25 cm^2 flask. The day before acid stress treatment, CaCo2 cells were enumerated using a haemocytometer: briefly, CaCo2 cells were washed twice with 1x PBS and detached after the addition of 1 mL trypsin-EDTA (Wisent ; 0.25% trypsin; 1mM EDTA·4Na) and incubation at 37 °C with 5% CO_2 for 15 min. The total cells in the flask were enumerated using a haemocytometer before being diluted into MEM (no additives) to reach a cell count of 2.5×10^5 cells (approximately 80% confluency). The cells were loaded into a 24-well flat bottom plates (BD Falcon) using 500 μL aliquots per well. The cells are grown overnight at 37 °C with 5% CO_2 .

On the day of the acid stress experiment, CaCo2 cells were again enumerated and averaged to determine the total cell count. The averaged epithelial cell count was used in

conjunction with the bacteria OD600 reading to determine the volume of bacteria to be loaded during infection. A multiplicity of infection (MOI) of 50-100 bacteria : 1 CaCo2 cell was used for all experiments.

Media from each Caco2 cell well was carefully removed and cells were washed 3x with 1x PBS. The appropriate volume of bacteria cells was added to each well and MEM (without additives) was used to bring up each well volume to 500 μ L. The plates were incubated at 37 °C and 5% CO₂ for a 3 h infection.

After infection, non-adherent bacteria were removed by aspiration. Each well was washed five times with 1x PBS. The CaCo2 cells with the adherent bacteria were detached from the bottom of the well with 250 μ L of trypsin-EDTA after incubation at 37 °C with 5% CO₂ for 15 min. A 50- μ L volume of FBS was mixed to each well to stop the trypsin process and 700 μ L of iced-cold sterile water was added to each well to lyse the remaining CaCo2 cells. The remaining bacteria cells were transferred to fresh 1.5 mL microcentrifuge tubes.

Serial dilution was performed to quantify the adherent bacteria from the experiment. The dilutions were plated on TP agar containing 0.1% sodium pyruvate and incubated at 37 °C overnight. Following overnight growth, the plates were quantified and colonies between 30-300 counts were used for statistical analyses.

2.12 *ΔyadK* Mutant Complementation Experiment

The full-length coding *yadK* gene was amplified by PCR with primers flanked by restriction enzymes BamHI and SacII (Table 2.1). The PRC reaction time included 1 min denaturation at 95 °C, 30 cycles of 45 s at 95 °C, 45 s at 60 °C, 1 min at 72 °C, a final

extension of 7 min at 72 °C with a hold time at 4 °C. PCR products (10 uL) were resolved on 1% agarose gel and visualized after ethidium bromide-staining under UV illumination.

The amplicon was then cloned into pCR2.1 and was used to transform chemically competent *E. coli* Topo10 cells using a TOPO TA Cloning Kit, following manufacturer's instructions (Invitrogen). Briefly, 4 µL of fresh PCR product was mixed with 1 µL salt solution, and 1 uL of TOPO vector. The reaction mix was incubated at 22 °C for 5 minutes. Chemically competent TOP 10 cells were transformed with reaction mix following manufacturer's instruction (see above for protocol).

Following electroporation, the cells were plated on LB agar plates with 100 µg/mL ampicillin and 50 µL of 20 mg/mL bromo-chloro-indolyl-galactopyranoside (X-Gal, Fermentas) for blue/white colony screening assay. White colonies were selected for patch plating and the plasmid carried by successful recombinant clones were purified using a HiYield Plasmid Mini Extraction Kit (UBI Bioinformatics, Inc.), following manufacturer's instructions.

A total of 26 µL of plasmid pCR2.1 carrying the cloned *yadK* gene was digested with 1.5 µL 4000u of BamHI and 3 µL 1200u of SacII (Fermentas) in a reaction mixture that included 3.4 µL buffer G and 0.6 µL sterile, nuclease free ddH₂O at 37°C for 1.5 hours. PCR products (10 uL) were resolved on 1% agarose gels and visualized after ethidium bromide-staining under UV illumination. A 30 µL aliquot of the isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible expression vector pBlueScript SK+ (58) was also digested in this manner.

A Rapid DNA Ligation Kit (Fermentas) was used to complete the complementation experiment. A total of 10 µL of the digested *yadK* insert was mixed with 1.35 µL of digested pBlueScript SK+, 4 µL of 5x rapid ligation buffer, 1 µL of 5u/µL T4 DNA ligase and 3.65 µL sterile, nuclease-free ddH₂O and the entire mixture was incubated at 22 °C for 5 min. A total of

2 μ L of the ligation mix was used for chemical transformation (see above for protocol). Successful clones were screened using restriction enzyme digestion with BamHI and SacII and confirmed by DNA sequencing (ACGT Co., Toronto, ON).

2.13 Induction of the complemented *yadK* in pBlueScript SK+

The expression of any genes cloned into pBlueScript SK+ is controlled by T7 promoter induced by IPTG (58). During the bacteria-cell host adhesion experiments, *yadK* expression in the complemented strain was induced with the addition of 1 mM IPTG (Invitrogen) during the subculturing step.

For transcriptional analyses experiments (described below), the induction of *yadK* in the complemented strain was achieved by inoculating 1 mL of overnight culture into 8 mL of fresh DMEM with 1 mM IPTG. The subculture was grown aerobically at 37 °C with agitation until an OD600 of 0.4-0.6 was reached.

2.14 Total RNA Isolation

Total RNA from 85-170(pSK), $\Delta yadK$ (pSK) and $\Delta yadK$ (pSK-*yadK*) strains were extracted to determine the expression of *yadK* and the downstream gene, *yadC*. Following IPTG induction (see above), bacterial strains were harvested by centrifugation and the pelleted cells were snap-frozen in liquid nitrogen and stored at -80 °C until needed for the RNA extraction.

The frozen pellets were slowly thawed on ice and resuspended with 1 mL Trizol (Gibco, Life Technologies Inc.). The thawed bacteria-Trizol mixtures were quickly loaded onto cold lysis matrix B tubes (MP Biomedicals). The tubes were vigorously shaken in the Fast-Prep FP 120 cell disruptor for 45 s and chilled on ice for 5 min. A 350- μ L volume of chloroform:isoamyl

alcohol (24:1) was added to each tube to precipitate any proteins from the disrupted bacteria. The tubes were gently inverted for 15 s, incubated at room temperature for 2 min and then centrifuged at 10,000 x g at 4°C for 15 min. The upper aqueous layer of each tube was transferred to a new 1.5 mL microcentrifuge tube. Another volume of 350 µL of chloroform:isoamyl alcohol (24:1) was added to the clear, aqueous layer. The tube was inverted gently and incubated at room temperature for 2 min. Following centrifugation at 10,000 x g at 4 °C for 15 min, the upper aqueous layer of each tube was transferred to a new 1.5 mL microcentrifuge tube.

The remaining nucleic acid in each tube was precipitated with the addition of 350 µL High Salt Solution (Molecular Research Centre) and 250 µL of room temperature isopropanol 100% and incubated at -20 °C from 15 min to overnight. After the precipitation step, all tubes were inverted several times and then centrifuged 10,000 x g at 4 °C for 15 min. The supernatant was aspirated and the pellets centrifuged for 5 min, to remove any remaining liquid. The pellets were air dried for 10-15 min and mixed with 75 µL of 1 x RNA-secure (Ambion). The tubes were incubated at 65°C for 10 min, with constant shaking to fully dissolve the RNA. The RNA concentration and integrity was measured spectrophotometrically and assessed with gel electrophoresis. The isolated RNA was either stored in -80 °C or used immediately for further experiments.

2.15 DNase Treatment

DNase treatment of the purified RNA samples was performed using a TURBO DNA Free Kit (Ambion). A total of 44 µL of purified RNA was incubated with 1 µL of 2u/µL DNase and

5 μ L of 10x Turbo DNase buffer for 30 minutes at 37°C. A 5 μ L volume of the DNase Inactivation Reagent was added to the mixture to stop the reaction. The reaction mixture was centrifuged at 10,000 x g for 15 min and the treated RNA was transferred to a clean 1.5mL microcentrifuge tube.

2.16 cDNA Synthesis

A qScript Flex Synthesis Kit (Ambion) was used to synthesize cDNA from purified RNA extracted from acid stress treated 85-170(pSK), $\Delta yadK$ (pSK) and $\Delta yadK$ (pSK-*yadK*) cells. Depending on the RNA concentration used, variable amount of DNase treated RNA was mixed with a variable volume of nuclease free water and 2 μ L of 10x random primer to complete a 15 μ L volume reaction. The reaction mixture was mixed by tapping, centrifuged for 5 min at 10,000 x g at room temperature and incubated at 65 °C for 5 minutes. After incubation, the reaction was briefly chilled on ice.

Four microliters of 5x qScript Flex Reaction Mix and 1 μ L of qScript Flex Reverse Transcriptase were added to the chilled reaction mixture. The mixture was left to stand on ice for a few minutes before being vortexed and collected through centrifugation. cDNA synthesis was completed by incubating the mixture for 10 min at 25 °C, 45 min at 42 °C, 5 min at 85 °C and a final hold at 4 °C.

2.17 Semi-quantitative Reverse Transcriptase PCR (RT-PCR)

The expression of *yadK* and *yadC* in the complemented $\Delta yadK$ (pSK-*yadK*) background was confirmed through semi-quantitative reverse transcriptase PCR (RT-PCR). Forward *yadK*-F and reverse *yadK*-R primers were used to amplify *yadK* while forward *yadC*-F and reverse *yadC*-

R primers were used to amplify *yadC*. A 25 μ L PCR reaction mixture consisting of 2.5 μ L 10 x PCR Buffer, 0.75 μ L 50 mM $MgCl_2$, 0.5 μ L 10 mM dNTPs, 0.15 μ L 100 μ M forward primer, 0.15 μ L 100 μ M reverse primer, 0.1 μ L AccuStart Taq polymerase (Quanta) and 20.85 μ L nuclease free ddH₂O. The reaction was incubated for 4 min at 94 °C, followed by 30 cycles of 15 s at 94 °C, 30 s at 55 °C, 30 s at 72 °C and a final hold at 4 °C. The housekeeping D-glyceraldehyde-3-phosphate dehydrogenase encoding GapA was used as a loading control for both reactions.

3. Results

3.1 Successful construction of the *yadK* insertional mutant using the λ -Red recombination system

A *yadK* deletion mutant ($\Delta yadK$) was constructed using the Lambda Red Recombination system. Several hundred clones were screened for the insertion of a kanamycin resistance cassette into the *yadK* locus by colony PCR. Two sets of primers including one pair that targets the *yadK* gene and another against the kanamycin resistance gene were used to determine the correct integration of kanamycin in *yadK* locus. One successful recombinant was obtained (Figure 3.1). The $\Delta yadK$ mutant (lanes 1, 11) produced an amplicon of 0.5 kb using the kanamycin resistance primers, indicating the successful insertion of kanamycin resistance cassette into the genome (lane 1). The same clone also produced an amplicon of 1.5 kb in size, confirming the integration of the antibiotic marker into the *yadK* locus (lane 11).

Figure 3.1 Screening of recombinant clones by PCR. PCR products amplified by kanamycin resistance cassette primers are 1.0 kb in size. PCR products amplified by *yadK* primers are 0.5 kb in size. The successful *yadK* mutant showed a PCR amplified product of 1.5 kb.

3.2 Sequence confirmation of $\Delta yadK$ mutant

The successful insertion of the kanamycin-resistance cassette into the *yadK* locus of EHEC 85-170 was confirmed by DNA sequencing (Figure 3.2).

```

yadK      ATGCTATGCAAGCATCATAAAAACGTAACCTTTT AGGGCTGGCAACA GCCTTATCACG 60
yadK/kan  ATGCTATGCAAGCATCATAAAAACGTAACCTTTT AGGGCTGGCAACA GCCTTATCACG 60
          *****
yadK      CCTTTTGCCTATTCGCGCCAGGATGTGGATCTCACCGCAAAGATCGTGCCAGCACCTGT 120
yadK/kan  CCTTTTGCCTATTCGCGCCAGGATGTGGATCTCACCGCAAAGATCGTGCCAGCACCTGT 120
          *****
FRT       GAAATTCTCTATACTTTCTAGAGAACTAGGAACTTCGGAATAGGAAC TTC 48
yadK/kan  GAAATTCTCTATACTTTCTAGAGAACTAGGAACTTCGGAATAGGAAC TTC 48
          *****
kan       ATGATTGAACAAAGATGGATTGCAACGCA GGTTCCTCCGGCCGCTTGGGTGGA GAGGCTATTC 60
yadK/kan  ATGATTGAACAAAGATGGATTGCAACGCA GGTTCCTCCGGCCGCTTGGGTGGA GAGGCTATTC 60
          *****
kan       GGCTATGACTGGGCAACAACAGACAAATCGCTGCTCTGATGCGCCGCTGTTCCGCTGTCA 120
yadK/kan  GGCTATGACTGGGCAACAACAGACAAATCGCTGCTCTGATGCGCCGCTGTTCCGCTGTCA 120
          *****
kan       GCGCAGGGGCGCCCGGTCTTTTTCGCAAGACCGA CCTGTCCGCTGCCCTGAA TGA ---- 176
yadK/kan  GCGCAGGGGCGCCCGGTCTTTTTCGCAAGACCGA CCTGTCCGCTGCCCTGAA TGA TGC 180
          *****
kan       -----ACTGCAAGACGAGGCGCGGCTATCGTGGCTGGCCAGCAGGGGCTTCTCT 228
yadK/kan  CTGAATGAACCTGCA GGAAGCGAGGCGCGGCTATCGTGGCTGGCCAGCAGGGGCTTCTCT 240
          *****
kan       TGGCGAGCTGTGCTCGACGTTGTCTACTGAA GCGGGAAGGGACTGGCTGCTATTGGGCGAA 288
yadK/kan  TGGCGAGCTGTGCTCGACGTTGTCTACTGAA GCGGGAAGGGACTGGCTGCTATTGGGCGAA 300
          *****
kan       GTGCGGGGCGAGGATCTCTGTCTCATCTCACCTTGTCTGTCGCGAGAAAGATATCCATCATG 348
yadK/kan  GTGCGGGGCGAGGATCTCTGTCTCATCTCACCTTGTCTGTCGCGAGAAAGATATCCATCATG 360
          *****
kan       GCTGATGCAATGCGCGGCTGCTATACGCTTGA TCCGGCTACCTGCCATTGCA CCA CCA 408
yadK/kan  GCTGATGCAATGCGCGGCTGCTATACGCTTGA TCCGGCTACCTGCCATTGCA CCA CCA 420
          *****
kan       GCGAAA CATCGCATCGACGAGCACGTACTCGATGG AAGCCGCTCTTGTGATCA GGAAT 468
yadK/kan  GCGAAA CATCGCATCGACGAGCACGTACTCGATGG AAGCCGCTCTTGTGATCA GGAAT 480
          *****
kan       GATCTGACGAAAGCATTCAGGGGCTCGGCGCCAGCCGAACTGTTGCGCAGGCTCAA GGCG 528
yadK/kan  GATCTGACGAAAGCATTCAGGGGCTCGGCGCCAGCCGAACTGTTGCGCAGGCTCAA GGCG 540
          *****
kan       CGCATGCCCAGGCGCA GGAATCTGTCGTGACCATGCGCATGCTGCTTGGCGAA TATC 588
yadK/kan  CGCATGCCCAGGCGCA GGAATCTGTCGTGACCATGCGCATGCTGCTTGGCGAA TATC 600
          *****
kan       ATGGTGGAAGATGGCCGCTTTCTGGAATTCATCGA CTGTGCGCGCTGGGTGTGGCGGAC 648
yadK/kan  ATGGTGGAAGATGGCCGCTTTCTGGAATTCATCGA CTGTGCGCGCTGGGTGTGGCGGAC 660
          *****
kan       CGCTATCAGGACATAGCGTTGGCTACCGCTGATATGCTGAAGAGCTTGGCGGCAATGG 708
yadK/kan  CGCTATCAGGACATAGCGTTGGCTACCGCTGATATGCTGAAGAGCTTGGCGGCAATGG 720
          *****
kan       GCTGACGCTTCTCTGCTTACGCTATCGCGCTCCGATTGCGAGCGCATCGCTTTC 768
yadK/kan  GCTGACGCTTCTCTGCTTACGCTATCGCGCTCCGATTGCGAGCGCATCGCTTTC 780
          *****
kan       TATCGCCTTCTTGA CCA GTTCTCTCTGA 795
yadK/kan  TATCGCCTTCTTGA CCA GTTCTCTCTGA 807
          *****
yadK      GCGGGA 6
yadK/kan  GCGGGA 6
          *****

```

Figure 3.2 The sequence confirmation of the $\Delta yadK$ disrupted locus. Sequence has been modified to only include the disrupted *yadK* gene, the upstream FRT site and the inserted *kan* resistance cassette. *yadK* = *yadK* gene; *kan* = *kan* resistance cassette; *yadK/kan* = sequence from successful mutant. Arrows represent the insertion of the kanamycin resistance cassette. Sequence alignment by performed using ChustalW (104).

3.3 Growth kinetic analyses of 85-170 wild-type and $\Delta yadK$ strains

A growth kinetics analysis was performed on the 85-170 wild-type and $\Delta yadK$ mutant strains to establish that the insertion of the kanamycin resistance cassette did not affect the $\Delta yadK$ mutant's growth rate. Both the 85-170 and $\Delta yadK$ doubled comparably for 180 min (Figure 3.2). Thus, any changes in adhesion levels observed from both strains are likely due to the mutation at the *yadK* locus and not because of incomparable growth ability due to the insertion of the kanamycin resistance cassette.

Figure 3.3 Growth kinetics of EHEC 85-170 (—) and $\Delta yadK$ (---) in LB broth at 37 °C. OD600 readings were taken every 20 min to analyze growth rates of wild-type and *yadK* mutant strains.

3.4 Inactivation of *yadK* gene does not affect the survival of EHEC 85-170 under acid stress

There was no significant difference between the viability of 85-170 wild-type strain and its $\Delta yadK$ mutant during unadapted/unstressed (UU) acid stress conditions. Similarly, there was no significant difference between the viability of 85-170 wild-type strain and its $\Delta yadK$ mutant after acid stress treatment (AA30). This strongly demonstrates that the disruption of *yadK* locus did not affect the ability of EHEC 85-170 strain to survive acid stress.

Figure 3.4 The viability of 85-170 wild-type and its $\Delta yadK$ mutant during unadapted, unstressed and during acid adapted, acid stressed conditions. The data represents the average of three independent trials ($n=3$). WT = wild-type, $\Delta yadK$ = $\Delta yadK$ mutant; UU = unadapted/unstressed; AA = acid adapted/acid stressed.

3.5 Putative fimbrial protein YadK is not involved in adhesion of unadapted/unstressed bacteria

The adhesion of 85-170 wild-type and $\Delta yadK$ mutant to CaCo2-cells were similar under unadapted/unstressed conditions. This suggested that the YadK protein does not appear to play a major role in bacterial-host cell adhesion in the absence of acid stress.

In contrast, the adhesion of the $\Delta yadK$ mutant cells was significantly decreased compared to wild-type cells following acid stress treatment. There was a 10-fold difference in adhesion levels between the acid treated 85-170 wild-type and the acid treated $\Delta yadK$ mutant cells, suggesting that the putative YadK fimbrial protein does play a role in the acid-induced adhesion of 85-170 cells to human epithelial cells *in vitro*. The acid stressed $\Delta yadK$ mutant also appears lower than the unstressed wild-type. This is likely due to the effect of the acid on the cells during the stresses and not because of the mutation at the *yadK* locus.

Figure 3.5 Adhesion was assessed and compared during a bacteria-host cell adhesion experiment. The data represents the mean of three independent trials $\pm$ standard deviation ($n=3$) with an MOI of 50-100. The adhesion to human epithelial cells has been normalized to the unadapted, unstressed treated wild-type. WT = wild-type; $\Delta yadK$ = $\Delta yadK$ mutant; UU = unadapted/unstressed; AA = acid adapted/acid stressed. 100% represents adhesion of 1.8 bacteria/Caco2 cell. * $P<0.05$.

3.6 Complementation experiment restores functional *yadK* gene in $\Delta yadK$ mutant

A complementation experiment was performed to restore the full-length coding *yadK* gene into the $\Delta yadK$ deletion mutant. The full-length coding *yadK* gene was amplified by PCR and cloned into pBlueScript SK+ inducible expression vector. pBlue Script SK+ plasmid carrying the successfully cloned *yadK* in the complemented $\Delta yadK$ strain ($\Delta yadK$ (pSK-*yadK*)) were purified and RE digested to confirm correct orientation. The full-length coding *yadK* gene cloned into pSK was confirmed through DNA sequencing. Its complete sequence was compared against the established *yadK* gene sequence found in EHEC EDL933 genome using ClustalW sequence alignment program (104).

comp	ATGCTATGCAGGCATCATAAAATCGTACACTTTTTAGGGCTGGCAACAGCCCTTATCACG	60
yadK	ATGCTATGCAGGCATCATAAAATCGTACACTTTTTAGGGCTGGCAACAGCCCTTATCACG	60
comp	CCTTTTGCCATTCGCGCCAGGATGTGGATCTCACCGCAAAGATCGTGCCAGCACCTGT	120
yadK	CCTTTTGCCATTCGCGCCAGGATGTGGATCTCACCGCAAAGATCGTGCCAGCACCTGT	120
comp	CAGGTTGAAGTCAGTAATAATGGCGTTGTGCGATCTCGGCACAGTGACGCTGGACTATTTT	180
yadK	CAGGTTGAAGTCAGTAATAATGGCGTTGTGCGATCTCGGCACAGTGACGCTGGACTATTTT	180
comp	GCCGACAAATGTAACCCCGACAACGGATTATGCGGGTGGCAAAACGTTTAAATGTGAATGTG	240
yadK	GCCGACAAATGTAACCCCGACAACGGATTATGCGGGTGGCAAAACGTTTAAATGTGAATGTG	240
comp	GTCTCTTGCGATAACATTCAAACGACACAGTCGAAATGAAATTAGATTTTCAGCCGACG	300
yadK	GTCTCTTGCGATAACATTCAAACGACACAGTCGAAATGAAATTAGATTTTCAGCCGACG	300
comp	GCGGGATCGCTTGACAGGCGAATAATCAAAATTTTAGTAATGAATATGAACAGCAGGCT	360
yadK	GCGGGATCGCTTGACAGGCGAATAATCAAAATTTTAGTAATGAATATGAACAGCAGGCT	360
comp	ACCGGGGCGAAAAACGTCGGCATTGTGATTTTTTCGGCCCAACCGAATCAGCAGACATTT	420
yadK	ACCGGGGCGAAAAACGTCGGCATTGTGATTTTTTCGGCCCAACCGAATCAGCAGACATTT	420
comp	AACGTGCGAGGAACGGATGGTTCCTCAACAGCAATTTATTCGGTAGCACCTGGCAATGCT	480
yadK	AACGTGCGAGGAACGGATGGTTCCTCAACAGCAATTTATTCGGTAGCACCTGGCAATGCT	480
comp	GTTCCCTCAACCTGGACCTTTTATTCCTCGATGACGCGGTGAATAATGCCCTTCCTCCT	540
yadK	GTTCCCTCAACCTGGACCTTTTATTCCTCGATGACGCGGTGAATAATGCCCTTCCTCCT	540
comp	GAAATCGGGAATGGTGAGAAGCCAGGTTATTGTTAACGTATCTTACGAATAATAGGATCCC	600
yadK	GAAATCGGGAATGGTGAGAAGCCAGGTTATTGTTAACGTATCTTACGAATAATAGGATCCC	591

Figure 3.6 Sequence alignment between the wild-type *yadK* gene in EDL933 strain and the *yadK* gene cloned into the pBlueScript SK+ inducible expression vector.

3.7 *yadK* expression is confirmed in the $\Delta yadK$ complemented strain, $\Delta yadK(pSK-yadK)$

The expression of *yadK* in the complemented $\Delta yadK(pSK-yadK)$ strain was confirmed by semi-quantitative RT-PCR analysis. A very faint band in the wild-type was also seen, confirming the expression of *yadK* in this strain. The *yadK* transcript was absent from the $\Delta yadK$ mutant, confirming the disruption of the *yadK* locus.

The reading frame of the target gene and that of the kanamycin resistance gene (antibiotic selection marker) are aligned, preserving the original downstream reading frames in order to avoid polar effects.

Figure 3.7 (A) The genetic organization of the locus 2 cluster showing putative fimbrial protein encoding gene *yadC* downstream from *yadK*. (B) RT-PCR gene expression profiles of *yadK* and *yadC* genes. 1) WT(pSK); 2) $\Delta yadK(pSK)$; 3) $\Delta yadK(pSK-yadK)$. The constitutively expressed *gapA* gene served as loading control.

3.8 Growth kinetic analyses 85-170(pSK) and $\Delta yadK$ complemented strains

The growth kinetics of 85-170(pSK) and $\Delta yadK$ (pSK) and complemented $\Delta yadK$ (pSK-*yadK*) strains were measured to confirm that all the strains grew comparably during the infection section of the study (180 min total). As before, all strains grew comparatively after 180 min of incubation, indicating that transformation with plasmid pSK+ did not affect growth yield under the experimental conditions tested.

Figure 3.8 Growth kinetics of EHEC 85-170(pSK) (—), $\Delta yadK$ (pSK) (----) and $\Delta yadK$ (pSK- *yadK*) (---) in LB broth at 37 °C. OD600 readings were taken every 20 min to analyze growth rates of 85-170(pSK), $\Delta yadK$ (pSK) and $\Delta yadK$ (pSK- *yadK*) strains.

3.9 Complemented strain behaves restores adhesion of acid-induced EHEC

The adhesion of the 85-170(pSK) and $\Delta yadK$ (pSK) and complemented $\Delta yadK$ (pSK-*yadK*) strains to CaCo-2 human epithelial cells were similar under unadapted/unstressed conditions as in Figure 3.9. The adhesion of the unadapted/unstressed 85-170(pSK) and acid adapted/acid stressed $\Delta yadK$ (pSK) to CaCo-2 human epithelial cells were significantly different, as expected. An increase in acid-induced adhesion was observed among the acid stressed 85-170(pSK). This phenotype was absent in acid induced $\Delta yadK$ (pSK) cells. Adhesion to CaCo-2 human epithelial cells was restored in the complemented $\Delta yadK$ (pSK-*yadK*). Once again, this reiterates that *YadK* plays a crucial role in the acid-induced adhesion of 85-170 bacteria cells to human epithelial cells *in vitro*. The restored adhesion observed in the $\Delta yadK$ (pSK-*yadK*), indicated that the absence of adhesion in the $\Delta yadK$ (pSK) mutant was due to the mutated *yadK* locus. Although the data shows similar patterns of adhesion as in Figure 3.5, it should be noted that the adhesion level of the strains transformed with pSK is slightly lower than normal. Reasons for this difference are discussed in section 4.0.

Figure 3.9 Adhesion was assessed and compared during a bacteria-host cell adhesion experiment. The data represents the mean of three independent trials $\pm$ standard deviation ($n=3$) with an MOI of 50-100. The adhesion to human epithelial cells has been normalized to the unadapted, unstressed treated wild-type. 85-170(pSK) = 85-170 transformed with pSK; $\Delta yadK$ (pSK) = $\Delta yadK$ mutant transformed with pSK; $\Delta yadK$ (pSK-*yadK*) = complemented $\Delta yadK$ mutant; UU = unadapted/unstressed; AA = acid adapted/acid stressed. 100% represents adhesion of 0.1 bacteria/Caco2 cell. * $P < 0.05$.

4. Discussion

Acidity is an important host barrier protection against disease-causing bacteria. Gastrointestinal pathogens must overcome the acute acid conditions of the stomach to be able to successfully colonize and cause illness in their victims. Several of these microorganisms including the major human pathogen enterohemorrhagic *Escherichia coli* O157:H7, have developed ways to adapt and survive against acid stress. EHEC possesses several sophisticated acid-response systems that allows it to tolerate acid-challenged media *in vitro* and *in vivo* (72, 73, 74). Whole genome transcriptional analyses also reveal that certain virulent genes are highly expressed in acid induced EHEC cells (69). These genes promote the bacteria's pathogenicity, including its adherence to host tissue cells.

This study identifies a putative fimbrial adhesin gene, *yadK*, that appears to play a crucial role in EHEC pathogenesis. Transcriptional studies on *yadK* show that the gene is significantly upregulated in acid-induced EHEC strain 85-170 (69). The upregulated expression of *yadK* corresponds to the increased adhesion of EHEC to human epithelial cells *in vitro*, we asked whether or not this gene may play a role in the acid-induced adhesion of EHEC cells.

In summary, a *yadK* ($\Delta yadK$) mutant was constructed using the λ -Red recombination system. Its level of adhesion to human epithelial cells was compared to 85-170 wild-type cells after either unadapted/unstressed and acid adapted/acid stressed conditions. The adhesion of wild-type and $\Delta yadK$ mutant did not differ significantly in the absence of acid stress. However, the adhesion of the $\Delta yadK$ cells significantly differed from wild-type after acid challenge. While acid adapted/acid stressed $\Delta yadK$ showed no increase in adhesion to CaCo2 cells, its acid challenged wild-type cells counterpart adhered 2.5-folds higher.

To determine whether or not the decreased adhesion in the $\Delta yadK$ was due to the disruption of the *yadK* locus, the $\Delta yadK$ was complemented with the full-length, coding *yadK* gene. The adhesion phenotype of the 85-170(pSK), $\Delta yadK$ (pSK) and $\Delta yadK$ (pSK-*yadK*) show no significant difference under unadapted/unstressed conditions. As expected, an acid induced increased in adhesion on human epithelial cells was observed in 85-170(pSK) cells compared to $\Delta yadK$ (pSK) after acid stress. Restoration of adhesion in $\Delta yadK$ (pSK-*yadK*) cells were also observed during acid adapted/acid stressed experiments.

The complementation experiment revealed that the levels of adhesion exhibited by the 85-170(pSK), $\Delta yadK$ (pSK) and $\Delta yadK$ (pSK-*yadK*) strains differed from those of the 85-170 wild-type and $\Delta yadK$ mutant after acid challenge (**Figure 3.9**). While 85-170(pSK) showed increased adhesion after acid adaptation/acid stress compared to acid adapted/acid stressed $\Delta yadK$ (pSK) and even the unadapted/unstressed control, its levels of increased adhesion did not correspond to the original 2.5-fold adhesion increase noted in wild-type 85-170 cells.

The complemented $\Delta yadK$ (pSK-*yadK*) cells showed restored adhesion function with levels almost similar to 85-170(pSK). When compared to acid adapted/acid stressed $\Delta yadK$ (pSK) cells, the complemented $\Delta yadK$ (pSK-*yadK*) showed a significant increase in adhesion to CaCo2 cells. Acid adapted/acid stressed $\Delta yadK$ (pSK-*yadK*) appeared to adhere almost 2-folds higher than acid adapted/acid stressed $\Delta yadK$ (pSK). However, the acid induced increase in adhesion observed in 85-170(pSK) and complemented $\Delta yadK$ (pSK-*yadK*) were not as high as the acid induced adhesion observed in acid challenged wild-type and mutant cells, as observed in Figure 3.5.

It is plausible that the inclusion of the cloning vector pBluescript SK+ in the strains during the complementation experiments affected the bacteria at molecular level. Gallant *et al.*,

recently documented that common β -lactamases such as ampicillin affected proper cellular wall formation in *Pseudomonas aeruginosa* strains complemented with vectors containing β -lactamase resistance cassettes (105). β -lactamase works to prevent the degradation of the bacteria peptidoclycan wall from β -lactam class antibiotics such as ampicillin. One of its mode of action includes preventing the degradation of NAM and NAG linkage. In this study, investigators found that complementing *Pseudomonas aeruginosa* mutants with pUCP20, which encoded an ampicillin resistance marker TEM-1, disrupted the bacteria's ability to twitch, adhere to other bacteria and form biofilm communities. It is probable that the β -lactam resistant peptidoclycan may actually have an altered cellular organization therefore disrupt normal cell wall related activity including biofilm formation (105). The investigators propose that horizontal acquisition of β -lactam resistance including plasmid vectors may have some physical cost in the bacteria's survival including impaired cell wall function.

While the introduction of pSK+ in the strains does not appear to affect the bacteria's growth rate or survival, it may influence the bacteria's adhesive properties. Since YadK is predicted to be part of a fimbrial operon that is presumably located in the cellular wall of EHEC, it is possible that the production of the β -lactamase from pSK+ influences the adhesion of the cells *in vitro*. It is likely that cloning the full-coding *yadK* gene into a different expression vector carrying a different antibiotic resistance cassette may alleviate this problem and show the same level of increased acid induced adhesion as observed in acid challenged wild-type and mutant strains. While there is no molecular or empirical proof to support this presumption, the adhesion of all three 85-170(pSK), $\Delta yadK$ (pSK) and $\Delta yadK$ (pSK- *yadK*) did appear much lower compared to 85-170 wild-type and $\Delta yadK$ cells during the experiments during both unadapted/unstressed treatment (result not shown).

YadK is predicted to be a fimbrial adhesin protein that is part of a fimbrial operon, locus 2 (40). The genetic organization of *yadK* in locus 2 resembles other chaperone-usher mediated fimbrial operons. The gene *yadK* has also been suggested as a virulent factor in pathogenic *E. coli*, as its insertion in EHEC genome differs from the location in commensal *E. coli*. However, in spite of the current knowledge on this gene, *yadK* or its adhesin product has never been identified before nor has it been implicated in bacteria-host cell adhesion. Regardless, the knowledge that a putative fimbrial protein in pathogenic *E. coli* can respond positively towards acid stimuli considerably broadens our understanding of both environmental and genetic factors that promote its virulence.

The pH 6 antigen encoding gene (*psa*) in pneumonic causing plague, *Yersinia pestis*, has been strongly investigated as an acid-induced virulence factor that promotes adhesion of *Y. pestis* on human respiratory tract epithelial cells *in vitro* (106). Investigators identified this chromosomally encoded fimbrial surface protein during transcriptional studies performed on several *Yersinia* serotypes (reviewed in 107). The gene encoding PsA is expressed maximally in acidic environments, particularly during the acidification of phagolysosomes. *psa* transcripts were observed strongest after cultures were grown in pH 6 medium (108).

Although it is likely that a multitude of proteins and other virulence factors are required to for the pathogenesis of *Y. pestis* infection, the discovery of PsA identifies an acid inducible virulence factor that promotes the pathogenesis of the pneumonic plague (108). In the same manner, while there are likely many other acid-induced virulence factor candidates that are involved in *E. coli* O157:H7 pathogenesis, this study is the first to identify a putative fimbrial protein that is likely induced because by acute acid stress.

Previous work has shown that the increased expression of *yadK* correlates with increased adhesion of EHEC 85-170 cells on tissue epithelial cells in vitro (95). This study shows that disruption of the *yadK* locus eliminates the increased adhesion observed among acid-stressed EHEC cells. The results of this study demonstrate that a putative adhesin may likely play an important role in the adhesion of acid stressed pathogenic enterohemorrhagic *E. coli* O157:H7.

This study also shows that acid has a considerable effect on the pathogenesis of *E. coli* O157:H7 bacteria. Acid has often been used as a means to control bacteria growth in the food industry. Similarly, acid is also one of the defense barriers that humans employ against pathogenic microorganisms during colonization. Over the past decade, studies have established that EHEC is able to survive and adapt in acute acid conditions. EHEC challenged in moderate acid media are able to tolerate very acidic conditions than non-acid adapted cells (71). Food scientists have also shown that acid-adapted EHEC cells are able to tolerate heat conditions, freeze-thaw practices (84, 86). Recent studies have shown that acid also increases virulence factors in EHEC cells (88). Whole genome profiling reveals that acid-induced EHEC show significantly upregulated levels of flagella, adhesins, and other virulent effectors (69, 88).

Base on the current knowledge of how acid affects EHEC pathogenicity and findings on *yadK*, as a novel gene that appears to plays a role in acid-induced EHEC cells, acid appears to induce virulence factors in EHEC O157:H7. These results strongly suggest that acid stress, which is a part of the host's natural assault to resist invasion by foreign organisms, may actually regulate factors responsible for enhanced bacteria-host attachment, resulting in increased EHEC virulence. From these experiments, one can easily speculate that acid promotes virulence expression in EHEC O157:H7 and increases its pathogenicity and ability to cause disease (Figure 4.1)

Furthermore, the modulation of *yadK* adhesin expression by environmental stress such as acid that results in specific host-cell adhesion appears to be a direct response to the bacteria's localized microenvironment. This strongly indicates that pathogens like EHEC O157:H7 may be primed by host environmental conditions it encounters. Food sanitation practices involving the use of acid against pathogenic microorganisms may actually promote expression of virulence factors in these pathogens and contribute to their pathogenicity. The acid in the human stomach may actually serve to induce expression of these virulence genes.

Finally, the investigation of a putative fimbrial protein, YadK that appears to play a role in acid-induced adhesion of EHEC O157:H7 cells significantly contribute to our growing understanding of pathogenic *E. coli* virulence. Knowing that acid may actually encourage increased virulence in EHEC, including the upregulation of novel fimbrial adhesins like YadK, may influence our current food sanitation practices. Antimicrobial strategies that can neutralize the effects of acid on EHEC or use novel adhesins like YadK as therapy targets may reduce the colonization of this deadly pathogen. In general, the knowledge garnered from this study provides us with new insights on EHEC O157:H7 that can be used as both preventive and therapeutic measures in the future.

Figure 4.1 The effect of acid on the virulence of EHEC O157:H7. Acid, which is once thought of as a substantial means of controlling pathogen growth, appears to increase the virulence of pathogenic *E. coli* O157:H7. Exposure of EHEC cells to acid appears to promote the induction and regulation of virulent genes including the increased expression of a putative fimbrial adhesin gene, *yadK*. As a result, acid stressed EHEC adheres to human epithelial cells much better than their no acid stressed counterparts.

5. Future Directions

(1) There is more work to be done in both understanding EHEC O157:H7 virulence and characterizing the adhesin gene, *yadK*. While this study provides meaningful insights on the *yadK* gene, including the possible environmental factors that regulate it, nothing is known about the adhesin protein. Further work must be done to examine YadK and determine how much of the protein product is translated, processed and assembled after acid stress, where it localizes in the cell and whether it binds directly on human epithelial cells.

(2) While this study suggests that *yadK* is significantly upregulated in the presence of acid, the molecular factors that promote and disable its expression are still unknown. It would be noteworthy to investigate the molecular regulation of *yadK in vivo* and shed light to how the locus 2 operon functions. What other environmental conditions promote the expression of the genes in this operon? What prevents locus 2 expression?

(3) The role of YadK as a putative virulence factor also needs to be further examined. Our data suggests that YadK does play a role in adhesion of acid-stressed bacteria; however, it is not enough to establish that it is a novel putative fimbrial protein that promotes virulence in EHEC.

(4) Recent characterization of *yadK* suggests that it responds to quorum sensing signal, auto-inducer 2 (AI-2) (109, 110, 111). Recent developments in quorum sensing among commensal *E. coli* demonstrate that *yadK* is induced by AI-2 stimulation during *in vitro* studies. The process of quorum sensing allows bacteria to regulate important physiologically processes such as bioluminescence, antibiotic production, virulence, and plasmid conjugal transfer in a diverse community (109). Bacteria express low molecular weight signalling pheromones, referred to as autoinducers, as means of cell-to-cell communication. Several classes of

autoinducers have been identified including AI-1, AI-2, AI-3 and AI-peptides (110). Of these, AI-2 expression has been observed specifically among commensal and pathogenic *E. coli*, and is believed to be released when bacteria reaches stationary phase.

Two gene array studies have shown that the gene *yadK* is strongly induced by AI-2 concentration in media. DeLisa, *et al.*, identify *yadK* as one of the genes that responded to differential AI-2 concentration (109). Both *yadK* and locus 2 major subunit *yadN* show a significant 3.8 and 3.5-fold increase among *E. coli* strains resuspended in media with high AI-2 concentration. Mutation of LsrK, a cytoplasmic kinase that inhibits the repressor of AI-2 receptor LuxS, reveals a significant 2.4-fold decrease of *yadK* expression. As a result, investigators suggest that the presence of AI-2 in the environment strongly regulates the expression of this gene (111). Finally, Soni *et al.*, reports that the presence of AI-2 in culture media influences the expression of *yadK* in EHEC O157:H7 strains (110).

Investigation on *yadK* reveals that it responds to quorum sensing signals and may be implicated in *E. coli* biofilm formation. Biofilm formation is a pathogenic mechanism that hinders the eradication of organisms. Work will be performed to test the ability of EHEC O157:H7 and its *YadK* deficient mutant to form biofilms under acid stress conditions.

6. References

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