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The impact of acid stress on escherichia coli O157:H7 virulence

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**THE IMPACT OF ACID STRESS ON *ESCHERICHIA COLI*
O157:H7 VIRULENCE**

by

Belinda House
Bachelor of Science, Ryerson University, 2003

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

Toronto, Ontario, Canada, 2006

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THE IMPACT OF ACID STRESS ON *ESCHERICHIA COLI* O157:H7 VIRULENCE

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Master of Applied Science
Environmental Applied Science and Management, Ryerson University

Abstract

Escherichia coli O157:H7 infection is a leading cause of hemorrhagic colitis, and hemolytic uremic syndrome. Many opportunities for acid stress exposure exist for this food and waterborne pathogen, including gastric acid shock. Yet little is known how this affects *E. coli* O157:H7 virulence. The effect of various acid stress protocols on *E. coli* O157:H7 survival, verotoxin production, and the ability to adhere to host epithelial cells was examined. Brief acid shock alone at pH 3.0 decreased the host cell adhesion capability by a factor of 4.3-4.8, yet when the acid shock was preceded by adaptation at pH 5.0, a 1.6-3.2 fold enhanced adhesion of surviving organisms to epithelial cells relative to unstressed organisms was observed. However, acid stress did not affect verotoxin production. Pretreatment of acid stressed bacteria with erythromycin eliminated the acid-induced adhesion enhancement, suggesting that protein synthesis is a requirement for the enhanced adhesion observed with acid-adapted acid-shocked *E. coli* O157:H7. Real time PCR analysis of locus for enterocyte effacement (LEE)-encoded virulence factors, intimin and EspA, revealed no significant upregulation for the acid stress treatments associated with the increased host cell adhesion. On the contrary, elevated mRNA levels for both intimin and EspA were observed for bacteria subjected to brief acid shock alone even though the host-cell adhesion was significantly decreased with these treatments. These results suggest that complex regulation mechanisms for LEE encoded virulence factors exists and that *E. coli* O157:H7 virulence can be enhanced after acid stress through increased adhesion to host epithelial cells.

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1.0 Introduction

1.1 General Introduction

Enterohemorrhagic *Escherichia coli*, (EHEC) are enteric pathogens of worldwide importance and have been implicated as the causative agents in many food and water-borne outbreaks. They are responsible for a range of illnesses including, nonbloody diarrhea, but the most serious manifestation of EHEC-induced disease is the development of hemorrhagic colitis (HC), i.e. bloody diarrhea which can progress to several life threatening diseases such as hemolytic uremic syndrome (HUS) and thrombic thrombocytopenic purpura (Buchanan and Doyle, 1997; Paton and Paton, 1998; Law, 2000; Stevens *et al.*, 2004; Caprioli *et al.*, 2005). HUS has been reported to be a major cause of acute renal failure in children with approximately 5% of cases resulting in death (Boerlin *et al.*, 1999; Dahan *et al.*, 2004; Tarr *et al.*, 2005).

The most well known serotype, *E. coli* O157:H7 (designated by its somatic, O, and flagellar, H, antigens), was first documented in 1982 in several food-borne outbreaks associated with raw/undercooked hamburger (Buchanan and Doyle, 1997; Law, 2000). In Canada, from 1995 to 1999, more than 1100 cases of *E. coli* O157:H7 infection were reported each year and it is estimated that more than 13 000 food-borne *E. coli* O157:H7 infections occur yearly in Canada, but many go unreported (Laboratory Centre for Disease Control, Public Health Agency of Canada: Canada Communicable Disease Report, 2000, 2003). In the USA, *E. coli* O157:H7 causes approximately 40, 000 infections and 250 deaths each year (Russell *et al.*, 2000). Currently, antibiotic therapy has proven to be ineffective against this organism and, in some cases, has worsened the illness (Russell *et al.*, 2000; Tarr *et al.*, 2005).

EHEC/STEC (Shiga toxin producing *E. coli*) have a zoonotic origin and reside in the anorectum of ruminants, including cattle (Buchanan and Doyle, 1997; Russell *et al.*, 2000; Caprioli *et al.*, 2005; Ceponis *et al.*, 2005). Major routes of transmission include fecal contaminated meats, fruits, vegetables, and water supplies. Outbreaks have occurred among persons visiting farms and petting zoos, and person-to-person transmission has

been reported in hospital and daycare settings (Caprioli *et al.*, 2005; Ceponis *et al.*, 2005).

EHEC are highly infectious; outbreaks have resulted from infective doses less than 100. The pathogenic and host factors that contribute to EHEC-induced illness and disease are not fully understood and subject to intense research (Caprioli *et al.*, 2005). The production of Shiga toxins (Stx) or verotoxins is one of the major defining characteristics of *E. coli* O157:H7 since Stx production is a prerequisite for HC and HUS (Law, 2000, Caprioli *et al.*, 2005). These potent cytotoxins act on the glomerular and brain microvascular endothelial cells and activate prothrombotic and proinflammatory cascades, which eventually lead to HUS and complications of the central nervous system (Caprioli *et al.*, 2005). Besides Stx production, colonization of the host intestinal mucosa is another major virulence determinant. An important early step in the colonization of the large intestine is the initial attachment of *E. coli* O157:H7 to host cells, which prevents removal of this pathogen by peristaltic flow (Law, 2000; Torres *et al.*, 2005). Following initial adherence, *E. coli* O157:H7 is able to cause disease by injecting various virulence factors into the host cell using a type III secretion system. Intimate attachment of the bacteria to host cells is mediated through the binding of the bacterial outer membrane adhesin, intimin to a bacterial protein translocated into the host cell and inserted in the host cell membrane. Translocated proteins are responsible for attaching and effacing (A/E) lesion formation and activating and inhibiting signal transduction pathways in host cells which can ultimately lead to disease (Frankel *et al.*, 1998).

E. coli O157:H7 are food-borne pathogens and many outbreaks have been associated with food consumption, particularly acidic foods. Furthermore, as with all enteric bacteria, survival through the acidic gastrointestinal tract has been correlated with acid adaptive responses.

The majority of *E. coli* O157:H7 research is centered around two important virulence aspects of this organism, which focus on the mechanisms of environmental stress responses, including acid stress, and the pathogenic mechanisms involved in

EHEC-induced illness and disease. However, to date very limited research has been conducted on the interaction of these two significant virulence factors. Does acid stress influence the pathogenesis and virulence of this organism? The implications of acid stress are very serious since many opportunities exist for acid exposure, including food, food decontamination procedures, and importantly passage through the host gastrointestinal tract. My research therefore focuses on the effect of acid stress on the virulence of this gastrointestinal pathogen. The remainder of this introduction first details the mechanisms of virulence and different virulence factors implicated in EHEC-induced disease, and is followed by a review of the various *E. coli* acid stress responses and important environmental situations that may provide acid stress exposure. Finally, the effect of acid stress on EHEC virulence will be reviewed along with the effect of stress, including acid stress, on the virulence of other related gastrointestinal pathogens.

1.2 EHEC Virulence Factors

To date, EHEC virulence has been attributed to several factors and the main genes involved in EHEC pathogenesis are shown in Figure 1.1. The major virulence factors are the production of Shiga toxins, which are designated into two primary classes, Stx1 and Stx2 (Elhanafi *et al.*, 2004). Other virulence factors required for human illness and disease include various genes necessary for A/E lesion formation and signal transduction, including the *E. coli* attaching and effacing gene (*eae*). These genes are located on a 43-kb locus for enterocyte effacement, (LEE), pathogenicity island. In addition, several other genes encoded on the pO157 plasmid have been implicated in EHEC pathogenesis (Elhanafi *et al.*, 2004).

Figure 1.1: EHEC Genes Involved in Pathogenesis (Natero and Kaper, 1998).

1.2.1 Shiga Toxins/Verotoxins

One of the most important virulence factors of EHEC infection is the production of Shiga toxins or verotoxins (VT) which act to inhibit the synthesis of host cell proteins and contribute to EHEC-induced renal disease and vascular complications of hemorrhagic colitis.

These toxins are encoded on lysogenic bacteriophages and are expressed under conditions similar to that of the intestinal tract (Hoey *et al.*, 2003). The Shiga toxin family is comprised of two principal immunologically non-cross reactive groups, Stx1 and Stx2. Stx1 is highly homologous to Shiga toxin of *Shigella dysenteriae* type 1 toxin and is conserved in all strains, while there are numerous variants of Stx2, thus contributing to the complexity in understanding the role these toxins play in disease (Law, 2000; Hoey *et al.*, 2003). These phage-encoded toxins are approximately 70, 000 daltons and consist of an enzymatically active A subunit combined with five identical B subunits.

Most EHEC O157:H7 isolates produce Stx2, and some have been reported to produce both, however Shiga toxin 2-producing strains tend to be implicated more frequently in severe disease conditions including HC and HUS. HUS causes thrombotic microangiopathy (TMA) in affected organs, including the kidneys, brain and gastrointestinal tract (Matussek *et al.*, 2003). Interestingly, research completed by Boerlin *et al.*, (1999) on the associations between STEC virulence factors and disease has provided evidence of synergism between the adhesin intimin and Shiga toxin 2. Using univariate analysis and multivariate logistic regression analysis, these researchers examined the distribution of virulence factors using an international collection of STEC isolates representing a broad range of STEC serotypes from various sources. According to their results, only *stx2* and *eae* virulence factors were significantly associated with serotypes found in humans and the association was even more significant with isolates from serotypes implicated in severe human disease. Their results suggest a possible association of these factors with the severity of human disease.

E. coli O157:H7 and other EHEC strains are non-invasive and it is proposed that these potent toxins first pass through the gut lumen to reach the systematic vasculature and subsequently cause damage to endothelial cells of the target organs. Recent research has suggested that Stx toxins may play an important role in inducing a multifaceted host inflammatory response. After passage through the intestinal epithelium, Shiga toxins reach endothelial cells lining small blood vessels lining the gut, kidney etc. In addition to

aggravating the intestinal damage associated with infection, these toxins act on glomerular and brain microvascular endothelial cells and activate prothrombic and proinflammatory cascades, which lead to HUS and complications of the central nervous system (Caprioli *et al.*, 2005).

The B subunit (7.7 kDal) binds to specific host cell surface glycolipid receptors, specifically to the terminal galactose moiety of globotriaosylceramide (Gb₃), also referred to as CD77 (Hoey *et al.*, 2003). Endothelial cells are rich in Gb₃ receptors and hence the action of the B-subunits account for the affinity of the toxin for the renal glomeruli (Natero and Kaper, 1998; Law, 2000). Upon binding to CD77, the toxin is internalized into the host cell by receptor-mediated endocytosis and transferred via the trans-golgi network to the endoplasmic reticulum, nuclear envelope and nucleus. The A subunit (32 kDal) is responsible for the inhibition of host protein synthesis by an N-glycosidase activity which inactivates the 28S ribosome and also activates expression and secretion of chemokines such as interleukin 8, GRO α and GRO β , leading to activation of pro-inflammatory responses. Shiga toxins also induce a cascade of responses including elevated levels of pro-apoptotic proteins, mitochondrial dysfunction and activation of apoptosis caspase cascades (Hoey *et al.*, 2003).

Shiga toxins are cytotoxic to human renal endothelial cells. The characteristic renal histopathology associated with Stx includes swollen glomerular endothelial cells and platelet and fibrin deposition within the glomeruli. It is a decrease in the rate of glomerular filtration that causes the acute renal failure associated with HUS (Paton and Paton, 1998).

However, the production of toxin alone is most likely insufficient to cause disease. Shiga toxins (both Stx1 and Stx2) when added to colonic epithelial cells, were shown to move across the cell layer without any cytotoxic effects, however when STEC strains were incubated with the cell monolayer, toxin(s) were transported through the cells and at the same time the cell monolayer was destroyed. These observations

suggest that STEC strains may produce other potent toxic products and/or there are possibly other bacterial factors that enhance Shiga toxin activity (Law, 2000).

1.2.2 Adherence to Host Cells

Although EHEC-produced verotoxins have been implicated as the causative agents associated with the vascular complications of infection, it has been shown that adherence to host epithelial cells and the subsequent colonization of the distal ileum, cecum and colon are sufficient for the production of diarrhea (Tzipori *et al.*, 1987; Campellone and Leong, 2003).

E. coli O157:H7 adhesion is a complex process and it has been suggested that this pathogen uses several different mechanisms for attachment depending on the surface (human or cattle intestinal cells, abiotic surfaces and food) (Torres *et al.*, 2005). In general, bacterial adhesion first involves the primary docking stage, which is then followed by the secondary locking phase. The primary docking stage is reversible and occurs due to several physiochemical factors such as electrostatic and hydrophobic interactions, temperature, and hydrodynamic forces. However, the subsequent locking phase is irreversible and is molecularly mediated via the binding of specific adhesins to the surface (Torres *et al.*, 2005).

The ability of many enteric pathogens to adhere to epithelial cells is a key virulence factor as it allows the pathogens to transport toxins effectively to host organs, survive peristaltic clearance and obtain access to host-derived nutrients (Campellone and Leong, 2003). The mechanisms associated with the intimate adherence of EHEC to host epithelial cells represents one of the most studied features pertaining to their pathogenesis. This intimate adherence to epithelial cells, both *in vivo* and *in vitro*, results in the formation of attaching and effacing (A/E) lesions (Garmendia *et al.*, 2005). The A/E lesion is characterized by localized destruction of the brush border microvilli, intimate adherence of the bacteria adjacent to the host cell membrane and rearrangement of the host cytoskeleton to form actin pedestals on the apical membrane underneath the attached bacteria (Kaper, 1998; Campellone and Leong, 2003; Dahan *et al.*, 2004; Deng *et al.*,

2004; Garmendia *et al.*, 2005; Caprioli *et al.*, 2005). A model for the early and later events associated with EHEC adherence to host cells is presented in Figure 1.2.

In vitro, there is heterogeneity pertaining to EHEC adherence phenotypes, which is found to be dependent on the host epithelial cell lines used (Paton and Paton, 1998). Some strains, including *E. coli* O157:H7, have been reported to exhibit a 'diffuse' adherence to epithelial cells characterized with bacteria distributed evenly over the surface of the host cells. Other strains have been shown to exhibit a localized adherence phenotype where bacteria adhere in tight clusters and form microcolonies (MC) at limited sites on the epithelial cells (Paton and Paton, 1998). It has been recently suggested that *E. coli* O157:H7 adherence to epithelial cells occurs in two stages and requires both LEE and non-LEE encoded virulence factors. EHEC O157:H7 will initially show diffuse adherence followed by the MC-developing intimate adherence stage (Tatsuno *et al.*, 2000). *E. coli* O157:H7 strains have been reported to adhere to intestinal epithelial cells of rabbits, gnotobiotic piglets, chickens, mice, and humans (Sherman *et al.*, 1987; Nagano *et al.*, 2003). In general, EHEC adherence to epithelial cells in culture is variable and dependent on the strain and cell line used in the experiment (Sherman *et al.*, 1987; Windsor *et al.*, 1992).

Figure 1.2: A Model for the Early and Later Events in EHEC Adhesion to Host Cells (Torres *et al.*, 2005)

Adhesion schemes of EHEC. **A:** Early events in EHEC adhesion to host cells. EspA filaments of the TTSS and other possible adhesions such as LP fimbriae, mediate initial attachment of the bacteria to host cells. Various effector proteins (Esps), including Tir, are translocated into the host cell via the TTSS. Tir (transmembrane intimin receptor) localizes to host plasma membrane and takes on a hairpin loop. **B:** Later events in EHEC adhesion to host cells. Intimate adhesion of the bacteria to the host cell is mediated by the binding of the bacterial adhesin intimin to its receptor Tir. Intimin also binds to a host cell receptor, nucleolin. Translocated effector proteins, including Tir, trigger cytoskeleton rearrangements, disruption of the epithelial barrier, cytotoxicity and are responsible for attaching and effacing (A/E) lesion formation, activating and inhibiting signal transduction pathways in host cells which can ultimately lead to disease.

1.2.3: LEE Pathogenicity Island

The majority of genes required for the formation of A/E lesions are located on a 43 kb pathogenicity island in the O157:H7 chromosome termed the locus of enterocyte effacement (LEE) (Kaper, 1998; Campellone and Leong, 2003; Deng *et al.*, 2004; Caprioli *et al.*, 2005; Garmendia *et al.*, 2005). LEE positive strains of EHEC have been associated with severe human disease (Caprioli *et al.*, 2005). The LEE of enteropathogenic *E. coli* (EPEC) and *E. coli* O157:H7 are very similar in structure, organization and gene arrangement, although the EHEC LEE is slightly larger (Law, 2000). LEE is comprised of three functionally different modules; the 5' end consists of three operons (LEE1 to LEE3), which encodes a positive regulator, Ler and the type III secretion system (TTSS) responsible for the export of effector molecules. The central functional region of LEE is composed of an operon (LEE5), which encodes the outer membrane adhesin intimin and the translocated intimin receptor (Tir). The 3' end of LEE (LEE4) encodes secreted proteins EspA, B and D and other TTSS structural proteins, translocators and effectors (Dahan *et al.*, 2004; Caprioli *et al.*, 2005).

Regulation of LEE gene expression is complex and is dependent on quorum sensing, environmental conditions and regulators (Garmendia *et al.*, 2005). LEE regulation is hierarchical; the positive global regulator *ler* is first induced which then activates the other operons (Lio and Syr, 2004). Lio and Syr (2004) have recently proposed a model for LEE regulation. Essentially in low temperatures or growth in M9 media, all LEE operons are repressed by the nucleoid-associated protein, H-NS and LOO44 (GrlR). Under proper environmental conditions (i.e. growth in DMEM) *E. coli* O157:H7 LEE-1 is activated and the expressed Ler activates the expression of the other LEE promoters. Activation of LEE1 by *ler* may possibly be indirect. Currently, BipA, FIS, IHF, GrlA and QseA have all been implicated in the activation of LEE1 (Lio and Syr, 2004).

Recent studies have shown that the LEE-encoded TTSS is being used to translocate other effector proteins not located in the LEE pathogenicity island but at other sites in the genome and these proteins have been implicated to have an important role in infection and disease (Garmendia *et al.*, 2005).

1.2.4: The Filamentous TTSS

Pathogens, including *E. coli* O157:H7 utilize the TTSS to translocate virulence factors in a single step from the bacteria to the host cells. This multicomponent apparatus is assembled from approximately 20 genes and is illustrated in Figure 1.3 (Garmendia *et al.*, 2005). The basal body is composed of the secretin EscC, and the inner membrane proteins EscR, EscS, EscT, EscU and EscV. The lipoprotein EscJ connects the inner and outer membrane ring structures and EscF comprises the needle structure. Polymerization of the EspA subunits form the EspA filament. A translocon pore in the host cell plasma membrane is mediated by EspD and EspB and connects the bacteria to the host cell via EspA filaments. SepD and SepL are cytoplasmic components of this system, and it has been suggested that these proteins may play a role in “switching” the type of proteins secreted through the TTSS, from translocator proteins to effector proteins. Cytoplasmic ATPases from the bacterial F₀F₁ ATPase provides energy to the system and promotes protein secretion (Garmendia *et al.*, 2005).

Figure 1.3: The Filamentous Type III Secretion Apparatus (Garmendia *et al.*, 2005).

1.2.5: Other LEE-encoded Virulence Factors

EspA filaments are significant adhesion factors as mature filaments establish a transient between the pathogen and host and allow translocation of effector proteins (Garmendia *et al.*, 2005). Once in the host cytosol these effector proteins trigger: (1) cytoskeleton rearrangements (Tir, EspH, EspF and EspG), (2) disruption of the epithelial barrier (EspF and Map), (3) cytotoxicity (EspF and Cif) and (4) responses from the host cells that will ultimately cause watery diarrhea (Dahan *et al.*, 2004; Garmendia *et al.*, 2005). Once effector proteins have been translocated, EspA filaments and the needle-like complex are removed from the bacterial surface to allow intimate attachment of the bacteria and host. Once intimately attached, down regulation of LEE encoded genes occur. Interestingly, recent research shows that EHEC EspA filaments are able to bind to RBC membranes and induce hemolysis (Dahan *et al.*, 2004).

Intimin, encoded by the *eae* gene, is a 94-97 kDa outer membrane protein which mediates the intimate attachment of EHEC to the host cell. It is exported to the periplasm via the general secretory pathway and is inserted in the outer membrane. There are two functional regions of this protein; a well conserved N-terminal region and a highly variable C-terminal region, which defines several intimin types. Intimin types ϵ and γ are found in EHEC strains, whereas type α is mainly found in EPEC. EHEC serotype O157:H7 produce γ intimin and binding of EHEC with this type of intimin is restricted to follicle-associated epithelium of the Peyers patches (Caprioli *et al.*, 2005). The C-terminal region of intimin extends from the bacteria and interacts with receptors already present in the host plasma membrane (Garmendia *et al.*, 2005). In addition to binding to Tir, γ intimin is able to bind to nucleolin, a receptor encoded by the host cells (Sinclair and O'Brian, 2002). Although the physiological relevance of this interaction remains unclear it has been suggested that it may promote initial adherence to the host cells (Campellone and Leong, 2003). To date, only intimin has been shown to play a role in intimate adherence of *E. coli* O157:H7 and is clearly associated with the later stages of adherence (Torres and Kaper, 2003).

The LEE-encoded effector molecule, Tir (transmembrane intimin receptor), once translocated, localizes to the host cell plasma membrane and using its two membrane-spanning transmembrane domains and takes on a hairpin-loop structure with both of its C and N termini located within the host cell. The central extracellular domain of this structure, exposed at the surface of the host cell allows the binding of intimin (Garmendia *et al.*, 2005). The amino- and carboxyterminal portions of Tir, located in the host cell cytoplasm, interact with several focal adhesion and cytoskeleton proteins thereby joining the extracellular bacterium to the host cytoskeleton, which eventually leads to actin pedestal formation beneath the adherent bacteria (Campellone and Leong, 2003; Garmendia *et al.*, 2005).

The effector protein, TccP (Tir-cytoskeleton coupling protein) is located within the prophage CP-933U carried by EHEC strains and is translocated through the TTSS and has recently been reported to be required for EHEC-induced actin polymerization and

A/E lesion formation (Garmendia *et al.*, 2004). Once translocated, TccP associates indirectly with Tir and then binds to neuronal Wiskott-Aldrich syndrome protein (N-WASP) and is able to then stimulate Nck-independent actin polymerization leading to pedestal formation (Garmendia *et al.*, 2004).

1.2.6: pO157:H7 Plasmid

E. coli O157:H7 possesses a 93-104 kB plasmid termed pO157, which has been suggested to be important in the organism's pathogenesis (Kaper, 1998). The plasmid encodes several virulence associated genes such as *toxB*, *katP*, *espP*, *tagA*, *etcC-O*, and *hlyA-D* (Tatsuno *et al.*, 2001). EspP, a serine protease, is responsible for the cleavage of the human coagulation factor, V. It has been suggested that the degradation of factor V may contribute to mucosal hemorrhage, which is affiliated with hemorrhagic colitis (Law, 2000). Recent research suggests that the *toxB* gene present on this plasmid may play a role in host-cell adhesion. *E. coli* O157:H7 strains harbouring derivatives of this plasmid lacking the *toxB* gene, demonstrated reduced adhesion to cultured epithelial cells (Stevens *et al.*, 2004). Furthermore, a type II secretion system has been associated with this plasmid, yet the secreted targets pertaining to this system have not been identified (Tatsuno *et al.*, 2001; Caprioli *et al.*, 2005). Recent studies by Grys *et al.* (2005) have found that the StcE protease, also located on this virulence plasmid, may contribute to the intimate adherence of *E. coli* O157:H7. The *stcE* gene, upregulated by Ler, is able to degrade the layer of mucins and glycoproteins on the host cells and thereby aid the bacterium by subverting the host mucosal defenses.

1.2.7: EHEC Adhesins (Other than intimin)

To date, other than intimin, a number of adhesins both fimbrial and non-fimbrial have been suggested to play a role in EHEC host cell adhesion. However, despite extensive research efforts, very little is known about the identities of fimbrial proteins implicated in the binding of EHEC to host intestinal cells (Torres *et al.*, 2005). Complete genomic sequence analysis of two EHEC O157:H7 strains has indicated that a minimum of 12 regions encodes putative adhesins (both fimbrial and non-fimbrial). Two of these regions contained genes very similar to the long polar (LP) fimbria (*lpf*) operon of

Salmonella enterica serovar Typhimurium and recent studies have suggested that LP fimbriae 2 may contribute to the early stages of EHEC O157:H7 adhesion to cultured epithelial cells and contribute to the colonization and persistence in swine and sheep (Torres *et al.*, 2004).

A nonfimbrial factor encoded outside of the LEE pathogenicity island, *efa-1* (EHEC factor for adherence) has recently been implicated in adherence to Chinese hamster ovary (CHO) cells (Torres *et al.*, 2005). Serotype O157:H7 lacks the full length of this gene, however a truncated version of *efa-1* exists for two fully sequenced strains, EDL933 and O157Sakai (Torres *et al.*, 2005). It has been proposed that this gene has a role in bacterial adhesion and intestinal colonization. Interestingly, this gene shares significant homology to the *toxB* gene located on the pO157 virulence plasmid, which has been associated with adherence to epithelial cells. Stevens *et al.* (2004) has shown that a deletion in the *E. coli* O157:H7 *toxB* gene resulted in decreased expression and secretion of LEE-4 type III secreted proteins and decreased microcolony (MC) formation on epithelial cells. They also reported that a mutation in the truncated *efa-1* impaired MC formation on HeLa cells. Interestingly, deletions of both of these genes resulted in extensive reduction in MC formation suggesting a possible synergistic effect.

Various other reports have been published implicating EHEC proteins as mediators of adherence with no apparent pathogenic role. Outer membrane protein A (OmpA) was recently shown to act as an adhesin in *E. coli* O157:H7 adherence to HeLa and Caco-2 cells (Torres and Kaper, 2003). The *Vibrio cholerae* IrgA homologue adhesin, Iha has also been implicated as an EHEC O157:H7 adhesin to HeLa cells (Tarr *et al.*, 2000).

1.2.8: Regulation of Type III Secretion (TTS)

The molecular mechanisms for the hierarchy control of both translocator and effector protein secretion in A/E pathogens is poorly understood (Deng *et al.*, 2005). Some researchers have proposed that direct bacterial contact with host cells triggers type III secretion, however the nature of this contact remains unclear. Also, many

environmental factors are thought to induce secretion of effectors into culture media (Roe *et al.*, 2003; Deng *et al.*, 2005). There is evidence that LEE-encoded virulence factors may be regulated at the posttranscriptional level (Roe *et al.*, 2003; Stevens *et al.*, 2004; Deng *et al.*, 2005). Deng *et al.*, (2005) have proposed that translocator and effector secretion in growth media of EHEC and EPEC occurs most likely at the posttranscriptional level. They suggest that calcium and other environmental signals may play a role in this regulation. Additionally, Stevens *et al.* (2004) report that the putative virulence factors Efa1 and ToxB affect the expression of LEE-encoded type III secreted proteins posttranscriptionally, however the mechanism for this remains to be elucidated.

According to a model proposed by Roe *et al.* (2003b) (see Figure 1.4 below), LEE4 expression occurs posttranscriptionally in *E. coli* O157:H7 and is dependent on secretion phenotype and culture conditions. Following LEE4 transcription, the *espADB* mRNA transcript in low secretors is turned over without translation until cell contact, and in high secretors translation of the transcript is triggered by growth in HEPES buffered minimal essential media or cell contact. This model also suggests that translation and secretion of *E. coli* secreted proteins (EspS) is coupled at the type III secretion system.

Figure 1.4: Model of LEE4 Expression and Esp Protein Secretion in EHEC O157:H7 (Roe *et al.*, 2003b)

(1) Transcription of *LEE4* occurs from a promoter in front of *sepL* which produces a 5.6 kb mRNA containing *espADB*. (2) This is immediately processed to produce at least three mRNA species including a 1.2 kb transcript that encodes *sepL* and a 2.8 kb transcript that encodes *espADB*. (3) Processing of the 2.8 kb transcript is a key step in determining whether or not secretion occurs. (3a) Under appropriate environmental conditions, such as growth in Hepes-buffered minimal essential medium for high secretors or cell contact for low secretors, the transcript is translated at the type III secretion apparatus (TTSS). (3b) When secretion is not triggered, for example low secretors grown in Hepes-buffered minimal essential medium, the mRNA is still produced, but is not efficiently translated at the TTSS. It is proposed that a pool of this mRNA is present in the cell and allows immediate secretion of EspA, B and D under the right conditions, such as cell contact. (4) The mRNA may either associate directly with the TTSS (4a) or be trafficked to the TTSS via a chaperone (4b). (5) The final step is the coupled translation and secretion of the Esps at the TTSS. The effective export of at least EspD requires a chaperone, CesD [72], also located in the membrane, presumably within the apparatus.

1.2.9: Host Cell Signaling Responses

The classic intestinal histopathology associated with *E. coli* O157:H7 infection consists of hemorrhage and edema in the lamina propria and colonic biopsy specimens from patients also show focal necrosis and infiltration of neutrophils. A/E histopathology has been seen with infant rabbits, gnotobiotic piglets and cultured epithelial cells (Natero and Kaper, 1998).

EHEC mucosal lesions are characterized with high concentrations of polymerized actin, increased levels of intracellular calcium and phosphatidylinositol-3-kinase (IP₃). The process of A/E lesion formation is a result of activation of the signaling molecules in the cytosol, phosphatidylinositol-3-kinase, phospholipase C and 5-lipoxygenase. *E. coli* O157:H7 infection causes increased host cell paracellular permeability mediated by stimulation of protein kinase C and myosin light chain kinase (Ceponis *et al.*, 2005). EHEC infection also triggers activation of MAP kinases and nuclear transcription factors (NF- κ B and AP-1) which result in secretion of interleukin-8, which is responsible for delivering neutrophils to the bacterial-host infection site. The interaction between bacterial flagellin and epithelial cells has been suggested to mediate this process (Berin *et al.*, 2002).

Watery diarrhea may be induced by loss of absorptive intestinal epithelial surface resulting from microvilli effacement, the inflammatory response to infection, and active chloride secretion resulting from signal transduction, whereas bloody diarrhea has been attributed to verotoxin damage to small blood vessels in the large intestine (Law, 2000; Gyles, 2004 unpublished).

In addition to causing cytotoxicity, Shiga-like toxins have also been implicated to induce programmed cell death or apoptosis. HEP-2 cells exposed to the toxins for 24-48 hours have been shown to show classic morphologic features associated with apoptosis such as cell shrinkage, membrane blebbing, and nuclear chromatin condensation (Ceponis *et al.*, 2005). Shiga toxins interact with host CD77 surface receptors (gangliosylceramide, Gb₃) of the plasma membrane and induce host programmed

cell death by releasing cytochrome c from the mitochondria, activating caspases and degrading poly-ADP-ribose polymerase (PARP) deoxyribonucleic acid repair enzymes (Ceponis *et al.*, 2003).

Recently, it has been shown that *E. coli* O157:H7 inhibits the signaling of Stat-1, which mediates a host pro-inflammatory response, which is dependent on viable bacteria bound to the cell surface (Ceponis *et al.*, 2003).

1.3: Acid Stress and *Escherichia coli*

In the face of constant changing environments, many bacteria, including *Escherichia coli*, are exposed to many different stresses, and their ability to sense and respond to these environmental stresses has long been appreciated (Merrell and Camilli, 2002). One such stress associated with gastrointestinal (G.I.) pathogens is their survival to extremely lethal pH found in the stomach, with pH values as low as 1.5-2.5 (Foster, 2004). Many G.I. pathogens are able to cause disease by breaching this host defense, including *Vibrio cholerae*, *Helicobacter pylori*, *Listeria monocytogenes*, and *Escherichia coli* (Merrell and Camilli, 2002; Foster, 2004). Some pathogens employ strategies like *Vibrio cholerae*, where extremely large numbers of bacteria infect the host and only a few actually survive and subsequently cause disease. However, other gastrointestinal pathogens such as *H. pylori* and *E. coli* use a proactive approach and expend energy on a counterdefence, which allows survival in extreme acid environments. As such, the infectious dose (ID) of enteric pathogens correlates to their respective acid tolerance. *V. cholerae*, non-typhi *Salmonella*, spp., and *Shigella flexneri* have oral IDs of approximately 10^9 , 10^5 , and 10^2 , respectively (Lin *et al.*, 1996; Audia *et al.*, 2001). The oral ID of enterohemorrhagic *E. coli* (EHEC) strains is thought to be similar to that of *Shigella flexneri*, which has an oral ID of 10^2 (Lin *et al.*, 1996).

E. coli, an intestinal microbe, actually prefers neutral pH for growth. Both pathogenic and nonpathogenic strains of *E. coli* are capable of surviving acid stress, although research has shown that pathogenic strains are better able to cope with acid and other environmental stresses (Yuk and Marshall, 2004).

After passage and survival through the stomach, which harbours a pH of 2, the pathogens encounter the less acidic pH of the small intestine. The small intestinal pH is approximately 4-6 and includes the presence of weak volatile fatty acids as a result of fermentation from normal intestinal flora. The exposure of enteric pathogens to these weak intestinal acids increases the acid stress to potentially lethal levels and those that survive will subsequently cause disease. Organisms with inducible mechanisms of acid resistance will thus be better able to survive and hence cause disease (Lin *et al.*, 1996).

At present, four distinct acid resistance systems in *E. coli* have been identified, and these systems appear to be dependant on the growth media, acid shock media, and the different experimental pH and time values used (Diez-Gonzalez and Karaibrahimoglu, 2004; Foster, 2004). They are referred to as AR system 1 (oxidative or glucose repressed), AR system 2 (glutamate-dependent), AR system 3 (arginine-dependent) and AR system 4 (lysine dependent) (Diez-Gonzalez and Karaibrahimoglu, 2004; Foster, 2004).

1.3.1: AR System 1: Oxidative or Glucose Repressed

The oxidative AR system 1 is the least understood and is induced when cells are grown to stationary phase in rich media such as Luria-Bertani broth (LB) (Audia *et al.*, 2001; Foster, 2004). This system is repressed in LB media with glucose, hence the name glucose repressed. AR system 1 allows cells to survive pH levels as low as 2.5 and this system is dependant on cAMP (cyclic AMP), CRP (cAMP receptor protein) and the alternate sigma factor, σ^s , encoded by the gene *rpoS* (Foster, 2004). Also, the F_0/F_1 proton translocating ATPase is thought to be involved, but its role either as a component of the system, or to produce ATP to fuel the system remains unclear (Foster, 2004). Once induced, how exactly this AR system protects cells exposed to lethal acid remains to be elucidated (Cui *et al.*, 2001; Foster, 2004). Interestingly, only cells that have been grown in LB broth buffered to pH 5.5 can survive transfer to minimal media at pH 2.5, whereas cells previously grown in LB pH 8.0 cannot. This is a result of the culture producing an inhibitor at high pH that blocks the system. However once the cells are washed and

glutamate or glutamine is added, AR system 1 can be recovered (Audia *et al.*, 2001). This recovery requires the GadC glutamate/GABA antiporter and suggests that there might be overlap between AR system 1 and AR system 2 (Audia *et al.*, 2001).

1.3.2: AR System 2: Glutamate-dependent acid resistance

Of the four AR systems, the glutamate-dependent AR system has been reported to be the most effective and allows cells to survive pH challenges of 2.0 for several hours in the presence of glutamate (Audia *et al.*, 2001; Diez-Gonzalez and Karaibrahimoglu, 2004; Giangrossi *et al.*, 2005). This system is induced either by entry to stationary phase or log phase growth in minimal media (Bhagwat, 2003; Richard and Foster, 2004; Seputiene *et al.*, 2006). This system, dependent on glutamate, is composed of at least three genes, *gadA* and *gadB* which encode the two glutamate decarboxylase isoforms, and *gadC*, which encodes the putative glutamate/ γ -aminobutyrate (GABA) antiporter. The isoenzymes, GadA and GadB are pyridoxal-phosphate enzymes that replace the α -carboxyl group of glutamate using cytoplasmic protons to produce GABA and CO₂. GABA is then expelled by the antiporter in exchange for new substrates.

This AR system is complex and subject to multiple levels of control. At present at least 11 regulatory proteins are known to be involved: σ^{70} (RpoD) and σ^s (RpoS) sigma factors, CPR, the cAMP receptor protein, H-NS, the histone-like protein, the EvgAS and TorRS two component systems, the TrmE GTPase, the AraC-like regulators YdeO, GadX, GadW, and the LuxR-related activator GadE (Foster, 2004; Giangrossi *et al.*, 2005). GadE (formerly known as YhiE) is the central activator of this system and binds to a 20-bp sequence called the *gad* box, located 63-bp upstream from the *gadA* and *gadB* transcriptional start sites. Expression of both the *gadA* and *gadB* loci, hence acid resistance, is dependent on both the *gad* box and the GadE activator, whereas the requirements/roles of the other 10 regulators is dependant on the growth phase and the media used (Foster, 2004). In total the 11 regulators comprise reiterative control circuits, which are used largely to control the GadE activator. Recent microarray analyses suggest that the location of several acid-regulated genes support the existence of a fitness island for acid adaptation on the *E. coli* chromosome (Hommais *et al.*, 2004). As a result of

these complex regulatory GadE activation circuits, this AR system can be induced under a variety of environmental conditions, including growth in stationary phase or log phase growth in minimal media (Bhagwat, 2003; Richard and Foster, 2004; Foster, 2004; Seputiene *et al.*, 2006). In fact, one *gadE* activation circuit functions in minimal glucose media whereas at least two or three are needed for growth in LB. Regardless of the induction conditions, cells can survive pH as low as 2.0. However, a more robust survival is seen with stationary phase cells (Foster, 2004).

1.3.3: AR System 3: Arginine-dependent acid resistance

This system is induced following growth in LB-Glucose and requires the amino acid arginine to be present in the challenge media. This AR system is not as effective as the glutamate dependent system and tends to provide cells with a more modest level of protection at pH 2.5 (Audia *et al.*, 2001). The mechanism of this AR system is comparable to the glutamate dependent system. The enzyme, arginine decarboxylase, encoded by the *adiA* gene, converts arginine to agmatine and consumes a proton in the process. Like GABA, the cognate AdiC antiporter expels the decarboxylation products in exchange for new amino acid (arginine) substrates (Foster, 2004).

Although this acid resistance system is less studied than the glutamate dependent system, its regulation is no less complex. In between the arginine decarboxylase gene (*adiA*) and the arginine/agmatine antiporter (*adiC*), lies the *adiY* gene, which encodes an AraC-like regulator, AdiY. Over expression of AdiY has been shown to activate both *adiA* and *adiC*, however mutations in chromosomal *adiY* did not appear to have an effect on the transcription of these genes, suggesting that perhaps the environmental conditions required for AdiY has yet to be identified (Foster, 2004). Transcription of the *adiA/C* genes occur only under anaerobic conditions in low pH rich media and it has been suggested that the CysB, a regulatory protein, may activate these genes under these conditions (Foster, 2004). RpoS, the alternative sigma factor has also been implicated to have a role in this AR system, however it is not required for transcription (Foster, 2004).

1.3.4: AR System 4: Lysine-dependent acid resistance

Like AR systems 2 and 3, this system as well involves amino acid decarboxylation. Lysine decarboxylases consume intracellular protons while removing CO₂ from lysine and these decarboxylation products, CO₂ and cadaverine are removed from the cell by the CadB antiporter in exchange for new substrates (Gong *et al.*, 2004). This AR system is less effective than both the glutamate and arginine dependant systems (Diez-Gonzalez and Karaibrahimoglu, 2004; Richard and Foster, 2004).

The induction of lysine decarboxylase, encoded by *cadA* requires low pH, anaerobic conditions and media containing high concentrations of lysine (Diez-Gonzalez and Karaibrahimoglu, 2004). It appears that each of these conditions is regulated independently, but it is unclear if one of these factors plays a more important role.

1.3.5: Inducible Amino Acid Resistance Systems: pH homeostasis and Electrical Membrane Potential

It was previously thought that acid protection by these amino acid decarboxylase mechanisms was conferred by the removal of intracellular protons, thereby allowing internal pH to increase to levels that would allow cell survival. However, it has been recently shown that amino acid-dependent AR systems not only increase internal pH required for pH homeostasis, but also reverse transmembrane potential, mimicking the strategies used by acidophiles (Richard and Foster, 2004).

AR systems 2 and 3 (glutamate and arginine dependent) are able to contribute to pH homeostasis by allowing the internal pH of *E. coli* to be higher in the presence of these amino acids than in the absence. The internal pH values generated by these systems correlate to the pH optima of the two decarboxylases, approximately pH 4 for glutamate decarboxylase and pH 5 for arginine decarboxylase (Foster, 2004). The most recent working model for these systems proposes that a balance exists between proton influx and decarboxylase reactions. This balance prevents the internal pH of acid stressed cells (at pH 2.5) from increasing to pH values above the pH optimum of the decarboxylase (Foster, 2004). Decarboxylases with higher pH optima, for example, lysine offer little or

no protection to cells at pH 2.5 because the initial proton influx will result in an internal pH which is too low below the working optima of the decarboxylase, hence the reason that the effectiveness of this AR system is very low (Foster, 2004).

In addition to threshold internal pH, this model also proposes that reverse transmembrane potential may be a more important acid resistance strategy in cell survival. Essentially under extremely acidic conditions, with the aid of CIC H^+/Cl^- antiporters, and decarboxylase reactions, *E. coli* may convert membrane potential ($\Delta\Psi$) from negative inside to positive inside to repel protons and alleviate the excessive proton motive force that can form (Richard and Foster, 2004). It has been proposed that the reversal of the transmembrane charge is a result of both the production of positively charged decarboxylation products and proton influx (Foster, 2004).

1.3.6: Acid Adaptation of *Escherichia coli*

Studies has shown that after exposure to mildly acidic environments, microorganisms can acquire increased acid resistance and survive longer periods when transferred to a more acidic environment. This phenomenon is known as the acid adaptation response, acid tolerance response or acid habituation (Cheng *et al.*, 2002; Cheng *et al.*, 2003; Koutsoumanis and Sofos, 2004). To date it has been reported that several food-borne pathogens such as *Listeria*, *Salmonella* and *E. coli* O157:H7 have shown acid adaptation and increased acid resistance responses to subsequent acid stress (Cheng *et al.*, 2002). Acid tolerance response in bacteria is mediated in three different ways. The first strategy involves composition changes in the bacterial membrane, the second entails enzymatic or physiological maintenance of internal pH and the third mechanism is a repair and/or prevention strategy pertaining to damaged essential cellular components by the acidic pH (Choi *et al.*, 2000). Molecular and physiological changes that are associated with acid adaptation have shown that this response is complex and different systems are induced which are dependent on the organism, growth phase, medium, type of acid stress and other environmental factors (Koutsoumanis and Sofos, 2004). Paul and Hirshfield (2003) have reported that pre-exposure of exponential phase *E. coli* to pH 5.5 and 4.3 resulted in the production of various acid shock proteins. They

also report that different acid shock proteins are produced during adaptation and are dependent on the adaptation pH, which lead to differences in protection efficiency. Generally speaking, *de novo* protein synthesis and changes in the fatty acid content of cell membranes have been associated with protection of habituated cells to extreme acid stress (de Jonge *et al.*, 2003; Koutsoumanis and Sofos, 2004).

Research has shown that acid adaptation increases the acid tolerance of *Escherichia coli* O157:H7 strains and that these strains are more resistant than nonadapted, alkali-adapted or nonpathogenic *E. coli* (Yuk and Marshall, 2004). Also, the extent of increased acid tolerance of *E. coli* O157:H7 are dependent on the conditions of the subsequent challenge, the adaptation time and the different strains used (Cheng *et al.*, 2002).

Koutsoumanis and Sofos (2004) reported that increased acid resistance was found when stationary phase *E. coli* O157:H7 were acid adapted in the pH range of 4.0-5.5, with pH 5.0 conferring the maximum acid tolerance. However, Tosun (2005) find that pH 4.5 will induce the highest level of protection for *E. coli* O157:H7 cells subsequently stressed at pH 3.0. Supplementing the media with glucose can also generate acid-adapted *E. coli*. Buchanan and Edelson (1996) reported that in the presence of glucose EHEC is able to acidify the media through glucose fermentation and subsequently induce an acid adaptation response. Interesting, after 18 hours of growth in unbuffered TSB plus glucose (pH 7.0 $\pm$ 0.3), the final pH of the media was in the range of 4.6-5.2, corresponding to the optimal adaptation pH from previous published reports. It has been reported that pH ranges of 4.5 - 4.9 of exponential phase *E. coli* will induce the highest level of protection for pH 2.5 challenges (Seputiene *et al.*, 2006). However, Paul and Hirshfield (2003) have recently reported that protection against pH 3 for exponential phase cells are superior when cells have been adapted to pH 4.3 than at pH 5.5. Their research also indicates that *E. coli* pretreatment between pH 4 and 6 does not result in a single flat stress response and instead may be comparable to the biphasic ATR of *Salmonella typhimurium*. This organism requires several acid shock proteins which are

induced at two stages of adaptation, which prevent or repair cellular damage caused by the acid stress (Paul and Hirshfield, 2003; Seputiene *et al.*, 2006).

Interesting, phospholipids have recently been recognized to have a major role in the adaptation response of exponential phase *E. coli* (Canet *et al.*, 2003). It was shown that the survival of an exponential phase culture of *E. coli* adapted at pH 5 and subsequently stressed at pH 3 was abolished if either phosphatidylethanolamine (PE) or both phosphatidylglycerol (PG) and cardiolipin (CL) were missing, suggesting that phospholipids may either have a regulatory role or provide a membrane environment suitable for acid adaptation. Furthermore, non-adapted stationary phase cultures of *E. coli*, lacking PE were determined to be much more sensitive to acid shock at pH than the wild type parental strain (Canet *et al.*, 2003).

Once adapted, *E. coli* O157:H7 is able to survive lethal pH levels as low as 2.5 for 2 hours, representing an average situation in the human stomach (Gorden and Small, 1993). It has been recently shown that in an environment with changing pH (like the stomach), acid sensitive cultures can become acid resistant within 17 minutes (de Jonge *et al.*, 2003).

Thus, a vital characteristic of microbial pathogens that infect via the oral-fecal route is their survival rates after exposure to extreme and moderate acidic environments. This ability to survive acidic environments allows for survival in acidic foods, the gastrointestinal tract, animal feed, and food-processing conditions (Lin *et al.*, 1996).

1.3.7: Acid Adaptation in Food, Food Processing Conditions, and in the Bovine Gut

Shiga toxin producing *E. coli* (STEC) have been found in the gut of numerous animals including cattle, sheep, pigs, goats, dogs, birds and cats (Paton and Paton, 1998; Caprioli *et al.*, 2005). However, cattle and other ruminants are considered to be the principal reservoir for important human STEC pathogens, including serotype O157:H7 (Paton and Paton, 1998; Calloway *et al.*, 2003; Omisakin *et al.*, 2003; Caprioli *et al.*,

2005). EHEC O157:H7 are transient members in their normal gut micro flora and appear for the most part to be asymptomatic. Fecal shedding of serotype O157:H7 is sporadic and is influenced by many factors including the age, diet, stress, population density, geographical region, and season (Paton and Paton, 1998). It has recently been suggested that pathogenic *E. coli* residing in cattle may have evolved partially through the intensification of ruminant production and farm management processes, including various feeding regimes (Gilbert *et al.*, 2005). Nutrition and dietary stress has been proposed to have profound effects on the prevalence of *E. coli* in the gut of ruminants (Diez-Gonzalez *et al.*, 1998; Gibert *et al.*, 2005). It has been suggested that high grain diets may induce acid resistance in the rumen and select for *E. coli* O157:H7 and other acid resistant STEC (Diez-Gonzalez *et al.*, 1998; Gilbert *et al.*, 2005). A recent study by Gilbert *et al.* (2005) has proposed that diet plays an important role on *E. coli* numbers and concentration of EHEC virulence genes in the feces of cattle. Cattle fed high grain resulted in increased *E. coli* numbers and decreased fecal pH whereas roughage and roughage + molasses diets resulted in decreased *eae* (intimin), *stx₁* (Shiga toxin 1), and *ehxA* (enterohaemolysin) concentrations.

It has been suggested that environmental and food contamination of STEC is largely due to cattle husbandry since the majority of *E. coli* O157:H7 outbreaks have been associated with ground beef and/or cattle manure (Callaway *et al.*, 2003; Caprioli *et al.*, 2005). In addition to foods of bovine origin, acidic food products such as apple cider, yogurt, mayonnaise, most vegetables and fermented salami have been implicated as vehicles for *E. coli* O157:H7 transmission (Leyer *et al.*, 1995; Berry and Cutter, 2000; Yokoigawa *et al.*, 2003; Caprioli *et al.*, 2005). Processed foods contain organic acids and fermentation processes, which increase the production of these acids and lower pH, may render pathogens more resistant to gastric acidity (Duffy *et al.*, 2000; Yuk and Marshall, 2004). Another situation where acid adaptation might occur is in the rumen or feces where weak organic acids such as benzoic and phenylacetic acid are present (Leyer *et al.*, 1995; Berry and Cutter, 2000).

Recently, concerns have been raised about the decontamination efforts employed by the beef industry. In Canada, solutions of weak organic acids are used in the early stages of beef carcass processing in efforts to reduce the microbial load, however these practices may render surviving pathogens more acid resistant and even provide cross protection to other stresses (Berry and Cutter, 2000; Samelis *et al.*, 2001; Canadian Food Inspection Agency, 2006). Berry and Cutter (2000) have reported that acid adaptation of *E. coli* O157:H7 using a 2% acetic acid spray enhanced survival of this organism from prerigor beef carcass tissue.

In the majority of food proteins, glutamate has been reported to be the most abundant amino acid. Furthermore, free glutamate has also been found in significantly high concentrations in various foods, including beef (Diez-Gonzalez and Karaibrahimoglu, 2004). According to Diez-Gonzalez and Karaibrahimoglu (2004), the concentrations of glutamate in food would be sufficient in the protection of *E. coli* O157:H7 from the stomach gastric juices and from acid washed meat carcasses. As a result, in EHEC strains, the glutamate-dependent system is believed to be the most effective and relevant AR system in the gastrointestinal tract and in food matrices (Diez-Gonzalez and Karaibrahimoglu, 2004).

1.4: Stress and Pathogen Virulence

Stress conditions encountered by bacteria are found in minimally processed foods, food processing and decontamination conditions, storage, animal feed, and host defense systems, such as the stomach and gastrointestinal tract. It is well documented that pathogens, including *E. coli* O157:H7, exhibit responses to sub-lethal environmental stresses (Duffy *et al.*, 2000; Cheng *et al.*, 2002; Cheng *et al.*, 2003; Koutsoumanis and Sofos, 2004; Yuk and Marshall, 2004). These important stress responses allow survival under more harsh conditions, enhance virulence, and induce cross protection to other subsequent stresses (Wang and Dolye, 1998; Leenanon and Drake, 2001; Yuk and Marshall, 2003; Elhanafi *et al.*, 2004; DeJesus *et al.*, 2005). Wang and Dolye (1998) have reported that heat shock was shown to enhance the acid tolerance of *E. coli* O157:H7. Acid adaptation and starvation were shown to increase heat and freeze-thaw resistance of

both pathogenic *E. coli* O157:H7 and nonpathogenic strains (Leenanon and Drake, 2001). Cheng *et al.*, (2002) reported that the survival rates of acid adapted *E. coli* O157:H7 to various stresses were dependent on strain and the type of subsequent stress.

The effect of acid stress on EHEC virulence is poorly understood and given the opportunities for acid stress of this pathogen during both food processing and ingestion, it is an area of research which demands greater attention. There has been a recent report pertaining to the impact of certain stresses on specific EHEC virulence factor expression. Elhanafi *et al.* (2004) have indicated that acid adaptation of *E. coli* O157:H7 at pH 5.5 increased the expression of the virulence proteins, intimin and enterohemolysin. However, these researchers only investigated intimin expression after acid adaptation. Furthermore, they did not investigate the adherence phenotype of acid adapted EHEC to host cells, nor did they examine the effect of acid adaptation followed by acid stress on intimin production. This is critical since acid adapted EHEC from beef carcasses subjected to organic acid rinse procedures must still undergo severe acid shock during ingestion.

Controversial reports pertaining to the effect of acid stress on *E. coli* O157:H7 verotoxin secretion and production have been reported. According to Yuk and Marshall (2004), acid adapted *E. coli* O157:H7 strains produced less total verotoxin than non-acid adapted cells and those cells which were alkali-adapted. Similarly, Duffy *et al.* (2000) also reported decreased verotoxin secretion in acid adapted *E. coli* O157:H7. However, Elhanafi *et al.* (2004) examined Shiga toxin gene *stx₂* expression and Stx2 production of *E. coli* O157:H7 and found that growth pH or cold acid stress had no effect on post stress *stx₂* expression or Stx2 production. Further research is needed to fully understand the effect of acid stress on the production and secretion of verotoxin since these published reports have only examined the impact of acid adaptation. The impact of lethal acid stress exposure, mimicking the passage through the stomach, on *E. coli* O157:H7 virulence needs to be explored.

Recently, Tatsuno *et al.* (2003) reported increased adhesion of *E. coli* O157:H7 strains with a disruption in *yhiE* and *yhiF* genes to Caco-2 cells. These authors suggested that GadE, encoded by the *yhiE* gene, which is a positive regulator for the glutamate dependent AR system, might be a negative regulator for the LEE-encoded genes of the type III secretion system. However, Bhagwat *et al.* (2005) were unable to confirm these results. In their studies, EHEC strains naturally mutated in the *gadE* genes did not show increased adherence to Caco-2 cells, but in fact adhered slightly lower than the strains that had the *gadE* mutation restored. In fact, a mutated strain that had both *gadE* and *rpoS* restored resulted in significantly enhanced adhesion compared to the original mutant strain, suggesting a synergistic effect of the two acid resistance regulators.

Environmental stresses have recently been reported to enhance the virulence of a related pathogen, enteropathogenic *E. coli*. According to DeJesus *et al.* (2005), EPEC-host cell adhesion was significantly enhanced after bile salt stress and brief acid stress. Their studies also suggested that heat-shock protein 70 (Hsp70) might be involved in the enhanced host-cell adhesion of acid stressed EPEC (De Jesus *et al.*, 2005). Other gastrointestinal pathogens have also been reported to show enhanced virulence after acid stress. *Clostridium difficile* host-cell attachment was shown to be increased by various environmental factors including high sodium, acidic pH and iron starving (Waligora *et al.*, 1999). Induction of the acid stress response in *Vibrio cholerae* resulted in a competitive advantage in the pathogen's ability to colonize and survive within the host (Merrell and Camilli, 2002).

It has been suggested that 'hyperinfectivity' after host-passage may be more similar to the natural phenotype of the pathogen and may enhance various virulence factors and dramatically reduce the dose required to infect secondary individuals (Wiles *et al.*, 2005). For example, transcriptome analysis of uropathogenic *E. coli* from the urine of infected mice revealed that these pathogens were in a rapid growth state and expression of type 1 fimbriae, a virulence factor involved in adherence, was significantly highly upregulated *in vivo* (Snyder *et al.*, 2004).

Prior ingestion stress has been shown to play a role in host cell adhesion for several gastrointestinal pathogens including *Citrobacter rodentium*, *Vibrio cholerae*, and enterohemorrhagic *E. coli*. (Bauer and Welch, 1996; Merrell *et al.*, 2002; LaRocque *et al.*, 2005; Wiles *et al.*, 2005). After passage through the murine gastrointestinal tract, *C. rodentium* was shown to be in the ‘hyperinfectious’ state, which dramatically lowered the oral ID and influenced the tissue distribution of colonizing bacteria (Wiles *et al.*, 2005). A comparative study on EHEC-host cell adhesion revealed that clinical isolates adhered better to human colonic cells than did contaminated food isolates (Bauer and Welch, 1996). Transcriptional profiling of *V. cholerae* recovered directly from patient stools during both early and late stages of human infection has shown that the expression of *tcpA*, a major subunit of toxin-coregulated pilus (TCP) required for intestinal colonization, was significantly more highly expressed in samples from early stages of infection (LaRocque *et al.*, 2005). However, this ‘hyperinfectious’ state may only be temporary, *Citrobacter rodentium* returned to ‘culture’ infectivity with one overnight passage in rich medium (Wiles *et al.*, 2005). Together, these findings suggest that stresses encountered by gastrointestinal pathogens during infection, including acid stress, might induce this ‘hyperinfectious’ phenotype shared among host-passaged pathogens and may enhance the virulence of these organisms.

1.5: Purpose of Investigation and Hypothesis

The effect of acid on *E. coli* O157:H7 virulence is a new field of research with very limited reported findings. In fact, to my knowledge, Elhanafi *et al.*, (2004) were the first to report on the effect of acid on *E. coli* O157:H7 virulence factors associated with intimate adhesion to host cells. As mentioned previously, these researchers only reported significant differences of intimin expression after acid adaptation. Their research did not investigate the actual adherence phenotype of acid adapted *E. coli* O157:H7 to host cells, nor did their research examine the effect of acid adaptation followed by acid stress on intimin production. This is important since acid adaptation of *E. coli* O157:H7 can result from various food processing and decontaminating conditions, and can even occur in the bovine gastrointestinal tract and acid adapted *E. coli* O157:H7 must still undergo lethal acid shock due to the low pH of the stomach to survive and establish infection.

Furthermore, research on other virulence factors such as verotoxin production and secretion has also only involved acid adapted *E. coli* O157:H7. As such, this research thesis focused on the virulence of *E. coli* O157:H7 following acid stress, including acid adaptation followed by subsequent acid shock. The impact of several acid stress protocols was examined. The mRNA levels of two known virulence factors, intimin and EspA were assessed after acid stress.

It was hypothesized that acid stress would have a significant impact on the viability and virulence of *E. coli* O157:H7 to host cells.

2.0 Materials and Methods

2.1: Bacteria Culturing

E. coli frozen stock strains, 85170, CL56, 8624, GFP8624, and HB101, were stored at -80°C and were routinely streaked onto Luria-Bertani (LB) agar plates (tryptone, 1% (w/v); yeast, (0.5% (w/v); sodium chloride, 1% (w/v); agar 1.5% (w.v)). Strains were stored at 4°C and subcultured every three weeks (Riordan *et al.*, 2000). LB broth cultures were prepared by inoculating 1 colony from the LB plate cultures into 10 mL of LB broth and incubated at 37°C for 16-18 hours at 200 rpm. For strain GFP8624, final concentrations of 100 ug/mL of kanamycin and ampicillan were added to all plates and liquid media. Table 2.1 below details the bacterial strains utilized in this thesis project.

Table 2.1: Bacterial Strains Used

Strain	Serotype/Description	LEE	VT1	VT2	Reference or Source
85170	O157:H7; derivative of parent strain 84-289 that spontaneously mutated in the laboratory	+	—	—	Tzipori <i>et al.</i> , 1987
CL56	O157:H7	+	+	+	Philpott <i>et al.</i> , 1998
8624	Clinical Isolate O157:H7	+	—	+	Gansheroff <i>et al.</i> , 1999
GFP8624	8624 with plamid pJBA29 harbouring GFP; pJBA29 previously isolated from <i>E. coli</i> JB122	+	—	+	Kanetani, 2005
HB101	Nonpathogenic laboratory <i>E. coli</i> K-12 strain	—	—	—	Louie <i>et al.</i> , 1994

2.2: Tissue Cell Culturing

Human epithelial cell lines, Hep2 (laryngeal carcinoma cells), and CaCo2 (colonic adenocarcinoma cells) were obtained from the American Type Culture Collection (ATCC) and were used in various adhesion assays. Vero cells or African Green Monkey Kidney cells were kindly provided by Dr. Lingwood (Hospital of Sick

Children, Toronto) and were used in Vero Cell Cytotoxicity Assays (see Table 2.2 below).

All tissue cell culture conditions were adapted from ATCC guidelines and growth and cell maintenance conditions consisted of growing the cell lines in Minimal Essential Media 1x Eagle's (mod) (Wisent; with Earle's salts and L-glutamine) supplemented with gentamycin (20, 000 ug/mL) and 10% (v/v) fetal bovine serum (FBS) (Wisent) for Hep2 and Vero cell lines and 20% (v/v) for CaCo2 cells (MEM with additives). All cell lines were grown at 37 °C and 5% carbon dioxide (CO₂) (v/v). Maintenance of Vero and Hep2 cells were achieved by subculturing the cells into fresh pre-warmed media every 3-4 days. CaCo2 cell line maintenance required fresh media after the 4th day of incubation and on the 6-7th day, the cells were subcultured into fresh media.

Table 2.2: Cell Lines Used in This Study

<i>Cell Line</i>	<i>Morphology</i>	<i>Species</i>	<i>Tissue/Tumor</i>	<i>Source</i>
Hep-2- (CCL23)	Epithelial	Human	Larynx, carcinoma	ATCC
CaCo2 -(HTB37)	Epithelial	Human	Colon, adenocarcinoma	ATCC
Vero - (CCL 81)	Fibroblast	African Green Monkey	Kidney	Hospital for Sick Kids (Lingwood Lab)

2.3: Acid Stress of *E. coli*

Unless otherwise indicated, following overnight growth in LB, a 1/5 dilution of *E. coli* 85170, 8624, CL56, GFP8624, or HB101 was made into prewarmed Dulbecco's Modified Eagle Medium (DMEM) (Wisent; 4.5g/L glucose, L-glutamine, sodium pyruvate) and incubated statically for 2-2.5 hours at 37°C, 5% CO₂ to exponential growth phase.

Secretion of LEE-encoded virulence factors are known to be increased when EHEC strains are grown in DMEM (Abe *et al.*, 2002; Lio and Syr, 2004). In fact, preincubation of exponential *E. coli* O157:H7 in DMEM at 37°C 5% CO₂ for approximately 2 hours resulted in maximal adhesion to CaCo2 cells (Abe *et al.*, 2002). Also, environmental conditions similar to those in the intestine such as the presence of 5% CO₂ was shown to enhance secretion of pathogenic proteins and VT1 production in EHEC (Yoh *et al.*, 2003). Similarly, early log phase growth in DMEM with high glucose has also been reported to increase EPEC LEE-protein secretion and increase LA patterns to host epithelial cells (Rosenshine *et al.*, 1996; Kenny *et al.*, 1997; Vanmaele and Armstrong, 1997).

Unless otherwise indicated the following acid stress protocol was used, 2 mL of the stock bacteria solution was aliquoted to 14 mL falcon tubes (one for each treatment type) and centrifuged at 3300 rpm for 10 minutes at 18°C and the resulting supernatant was removed using an aspirator. The bacteria then were subjected to several different acid stress treatments, each outlined below:

Acid Stress Treatment 1: No Buffers and Adaptation at Room Temperature

After DMEM preinducement and centrifugation, strain(s) were resuspended in 10 mL of sterile filtered DMEM pH 5. The control treatment(s) (unshocked) were resuspended in 10 mL of sterile filtered DMEM pH 7. All bacteria were left at room temperature for 60 minutes (adaptation) and then centrifuged at 3300 rpm for 10 minutes at 18°C. The supernatant was removed from all tubes and the bacteria designated for acid shock treatment were resuspended in 10 mL sterile filtered DMEM pH 3, whereas the control was resuspended in sterile filtered DMEM pH 7. All bacteria treatments were left at room temperature for the designated shock times and then were centrifuged at 3300 rpm for 10 minutes at 18°C. The supernatant was removed and all treatment types were resuspended 2 mL of sterile filtered DMEM pH 7.

Acid Stress Treatment 2: 25 mM MES and Adaptation at 37°C, 5% CO₂

Treatment 2 is a modification of Treatment 1 with the following changes, (1) a 25 mM MES (2-Morpholinoethanesulfonic acid, monohydrate) buffer was added to DMEM pH 5 treatments and (2) acid adaptation was now carried out at 37°C, 5% CO₂ rather than at room temperature.

Acid Stress Treatment 3: 25 mM MES, 25 mM MOPS and Adaptation at 37°C, 5% CO₂

Treatment 3 is a modification of Treatment 2 with one minor change, a 25 mM MOPS (3-(N-Morpholino)-propanesulfonic acid) buffer was added to DMEM pH 7.4 treatments during acid adaptation, acid stress, and the final neutralization resuspensions.

Acid Stress Treatment 4: Bacterial Preparation for RNA extraction

Treatment 4 is a modified version of Treatment 3. Essentially, 40 mL of preinduced stock bacteria was aliquoted to 50 mL Falcon tubes (one for each treatment type) and centrifuged at 3300 rpm for 10 minutes at 18°C. The resulting supernatant was removed using an aspirator. All stress conditions were carried out exactly the same as Treatment 3 except that the stressing/shocking density used was 1:1 rather than 1:5.

Table 2.3 summarizes the various experimental acid stress treatments used in this study.

It is known that various environmental factors including nutrient level, growth phase, amino acid availability, temperature and pH can affect the acid resistance/adaptation in *E. coli* (Sun *et al.*, 2005). DMEM with high glucose and L-glutamate ensures induction of log phase cells utilizing the glutamate dependent AR system (Bhagwat, 2003; Seputiene *et al.*, 2006).

Recent studies on the acid response of exponential phase *E. coli* K-12 has shown that the highest level of protection against pH 2.5 challenges was obtained after adaptation at pH 4.5-4.9 for 60 minutes (Seputiene *et al.*, 2006).

Table 2.3: Acid Stress Experimental Treatments

<i>Experimental Treatment</i>	<i>Description</i>
UU	Unadapted, Unshocked
AU	Adapted, Unshocked
UU15	Unadapted, Unshocked for 15 minutes
UU30	Unadapted, Unshocked for 30 minutes
UA15	Unadapted, Shocked for 15 minutes
UA30	Unadapted, Shocked for 30 minutes
AA5	Adapted, Shocked for 5 minutes
AA15	Adapted, Shocked for 15 minutes
AA30	Adapted, Shocked for 30 minutes
Note:	Adapted pH 5 1 hr, Unadapted pH 7 1hr, Shocked pH 3 (various times), Unshocked pH 7 (various times)

Following acid stress, the experimental samples were ready for use in subsequent viability, adhesion, cytotoxicity and RNA extraction experiments.

2.4: Percent Survival of Preinduced *E. coli* O157:H7

Acid stressed bacteria were produced using one or more of the different Acid Stress Treatments as described above. A dilution series for each experimental treatment was made in 100 mM Tris-buffered Saline pH 7.4 (TBS) and the bacteria were plated out on tryptose phosphate agar containing 0.1% (w/v) sodium pyruvate. All plates were incubated at 37°C for 16-18 hours. TP agar with 1% sodium pyruvate has been used to culture acid stressed *E. coli* strains (including O157:H7) since it is a medium that has been found to improve the recovery of acid stressed cells (Leyer *et al.*, 1995; Brown *et al.*, 1997). In fact recent studies have shown that sodium pyruvate is able to resuscitate *E. coli* O157:H7 stressed cells from the viable non-culturable state, a state induced by response to various stresses (Asakura *et al.*, 2005). Colony counts for plates that contained 30-300 colony forming units (CFU) were completed and used in subsequent calculations to determine the percentage of surviving organisms after acid stress (as assessed by culturability).

2.5: Bacterial- Host Cell Adhesion Assay: Colony Plate Count Method

Adherence of various *E. coli* strains to either HEp2 or CaCo2 cells was determined by a colony plate count assay as described previously by De Jesus *et al.*, (2005). Using a haemocytometer (Hausser Scientific), epithelial cells grown in a 75 cm² vented flask (BD Falcon) were enumerated and added to either a 24-well or 6-well flat bottom plates (BD Falcon) so that the cell monolayer would be approximately 80-90% confluent on the day of the experiment ($\approx 2 \times 10^5$ (24-well) or 8×10^5 (6-well) cells/well). MEM with additives was added to each well to bring it up to either 0.5 mL (24-well) or 3 mL (6-well) and plate(s) were incubated overnight at 37°C, 5% CO₂. Overnight LB cultures of the appropriate strain(s) were prepared as previously described.

After overnight culture in LB, the bacteria were then preinduced and acid stressed using one of the previously described Acid Stress Treatments. Epithelial cell counts (cells/mL) from several wells were determined and averaged with the aid of a haemocytometer. This cells/mL value was used in conjunction with the bacterial OD₆₀₀ value to determine the approximate amount of bacterial solution to be added to each well such that a multiplicity of infection (MOI) of 100:1 was achieved. To confirm the MOI, serial dilutions of all experimental treatments were made in 100 mM TBS and plated out on TP agar. Only plates with 30-300 colonies were used in subsequent calculations.

Prior to bacterial infection, the MEM (with additives) of each well (either 6 or 24-well plates) was removed and washed three times with 1 x phosphate buffered saline, pH 7.4 (PBS, Wisent without calcium and magnesium). The appropriate amount of bacteria were added to their respective well so that the MOI of 100:1 was achieved and MEM (without additives) was added to each well to bring the volume up to either 0.5 mL (24 well plate) or 3 mL (6 well plate). The well plate(s) were incubated at 37°C, 5% CO₂ for three hours or six hours depending on the experiment.

For 6-hour adhesion assays, the media was removed from the wells after three hours and washed three times with PBS. Fresh MEM (without additives) was added to

each well and the plates were incubated at 37°C, 5% CO₂ for the remaining 3 hours of infection as previously described by Gansheroff *et al.*, (1999).

After the appropriate infection time elapsed, the media and non-adherent cells and bacteria were removed from the wells. The wells were washed five times with PBS pH 7.4 and the monolayer of cells were trypsinized by adding 250 uL trypsin-EDTA (0.25% trypsin; 1mM EDTA-4Na) and incubating the plate(s) at 37°C, 5% CO₂ for approximately two minutes. After epithelial cell removal from the wells, 50 uL of FBS was used to stop the trypsin reaction and the cells from each well were added to either a 1.5 mL eppendorf (24-well plates) or a 14 mL falcon tube (6-well plates). The wells were rinsed with 200 uL of cold sterile distilled deionized H₂O (ddH₂O) and the washings were added to the tubes. To lyse the epithelial cells, cold sterile ddH₂O was added to each tube (1 mL for 24 well plates; 2.5 mL for 6-well plates).

Serial dilutions using 100 mM TBS were performed for each treatment (with replicates) to determine the number of viable bacteria bound to adherent cells. The appropriate dilutions were plated on TP Agar and incubated between 16-18 hours at 37°C. Following incubation, the plates were counted and those that had colonies between 30-300 were used for further calculations and statistical analysis.

2.6: Erythromycin Bacterial- Host Cell Adhesion Assay: Colony Plate Count Method

The following method for incubation of the bacteria with erythromycin prior to adhesion assays is a modification of a previously described method by De Jesus *et al.* (2005). After overnight culture in LB, *E. coli* 85170 was preinduced and acid stressed using Acid Stress Treatment 1 as previously described. Three experimental treatments were subjected to the acid stress, UU, (unadapted, unshocked), AA15 (adapted, shocked for 15 minutes), and AA30 (adapted, shocked for 30 minutes). Following the acid stress, the 2 mL of neutralized bacteria suspension in DMEM pH 7 for each treatment was divided into two 1 mL aliquots and placed in 1.5 eppendorf tubes; 1 mL of each treatment

to be subjected to erythromycin treatment and the other without. All tubes were centrifuged at 3300 rpm for 10 minutes at 18°C and resuspended in 1 mL DMEM pH 7 with or without 0.06 mg/mL erythromycin (Sigma) depending on the treatment. All tubes were left at room temperature for thirty minutes. The effect of erythromycin on acid stressed bacterial adhesion to HEp2 cells was determined by a 6-well colony plate count assay as previously described.

2.7: Mannose Bacterial- Host Cell Adhesion Assay: Colony Plate Count Method

Bacterial adherence to HEp2 cells in the presence of mannose was assessed with modifications of previously described methods (Sherman *et al.*, 1987, DeJesus *et al.*, 2005). Overnight LB cultures of *E. coli* 85170 were preinduced and acid stressed using Acid Stress Treatment 3 as previously described. Three experimental treatments were subjected to the acid stress, UU30, (unadapted, unshocked for thirty minutes), UA30 (unadapted, shocked for 30 minutes), and AA30 (adapted, shocked for 30 minutes). Following the acid stress, the 2 mL of neutralized bacteria suspension in DMEM pH 7 for each treatment was divided into two 1 mL volumes and placed in 1.5 eppendorf tubes; 1 mL of each treatment to be subjected to mannose treatment and the other without. All tubes were centrifuged at 3300 rpm for 10 minutes at 18°C and resuspended in 1 mL DMEM pH 7.4 with or without 1% D(+)- mannose (Sigma) depending on the treatment. The effect of mannose on acid stressed bacterial adhesion to HEp2 cells was determined by a 24-well colony plate count assay as previously described.

2.8: Acid Stressed *E. coli* O157:H7 85170 Growth during 3 hour Adhesion Assays

This control experiment was performed to determine the effect of acid stress on the growth of *E. coli* 85170 throughout the infection time in a three-hour bacterial-host cell adhesion assay. Overnight LB cultures of *E. coli* 85170 were preinduced and acid stressed using Acid Stress Treatment 3 as previously described. Cells exposed to three experimental treatments were subjected to the acid stress, UU30, (unadapted, unshocked for thirty minutes), UA30 (unadapted, shocked for 30 minutes), and AA30 (adapted,

shocked for 30 minutes). After acid stress, the treatments were normalized using prior viability data to ensure that the initial inoculum was approximately the same. To validate the normalization, a dilution series was prepared in 100 mM TBS for each normalized treatment and plated on TP agar and incubated at 37°C for 16-18 hours. After normalization, 100 μ L of bacteria from each treatment was added to an empty (cell-free) well from a 24-well plate and MEM (without additives) was added to each well to bring the volume up to 0.5 mL. The well plate was incubated at 37°C, 5% CO₂ for three hours. After incubation, the media and bacteria were removed and added to 1.5 mL eppendorf tubes. Serial dilutions using 100 mM TBS were performed for each treatment to determine the effect of acid stress on bacterial growth during infection. The appropriate dilutions were plated on TP Agar and incubated between 16-18 hours at 37°C. Following incubation, the plates were counted and those that had colonies between 30-300 were used for further calculations and statistical analysis.

2.9: Effect of Buffers on Acid Production of *E. coli* O157:H7 85170

A control experiment was designed to assess the effect that the two different Acid Stress Treatments had on *E. coli* 85170 acid production. An overnight LB culture of 85170 that was preinduced in DMEM was used. Six experimental treatments, UU15, UA15, AA15, UU30, UA30 and AA30 were subjected to Acid Stress Treatments 1 and 3 as previously described with minor modifications. After acid adaptation, the bacteria were centrifuged at 3300 rpm for 10 minutes at 18°C and the supernatant for all experimental treatments for each of the two Acid Stress Treatments were sterile filtered using a 0.2 μ m Supor membrane (Pall) and a 10 mL Luer-lok syringe (BD Falcon) into a 14 mL white capped falcon tube. The remaining pellet was resuspended in the appropriate DMEM solution for shocking depending on both the experimental treatment and the Acid Stress Treatment. After acid shocking, the tubes were centrifuged at 3300 rpm for 10 minutes at 18°C and the supernatant for all experimental treatments for each of the two Acid Stress Treatments were sterile filtered using a 0.2 μ m acrodasic membrane filters and a 10 mL luer lock syringes into 14 mL white capped falcon tubes. All treatments were neutralized with the appropriate DMEM solution (depending on Acid

Stress Treatment) and allowed to stand at room temperature for thirty minutes. After centrifugation at 3300 rpm for 10 minutes at 18°C, the supernatant for each treatment was again sterile filtered. The pH of the supernatant after acid adaptation, acid shocking, and neutralization for thirty minutes for each experimental treatment acid stressed with both Acid Stress Treatments were read. The increase/decrease in pH in comparison to the original DMEM solution used for that particular treatment was recorded.

2.10: Qualitative Microscopic HEp2-GFP8624 Acid Adhesion Assay.

This procedure was adapted from Kanetani (2005) with minor modifications. Before the experimental day, two 6-well plates were seeded with HEp2 cells as previously described except for one modification, a 25 mm x 25 mm glass coverslip (VWR) was placed in each well prior to the addition of HEp2 cells and MEM with additives.

After overnight culture in LB, *E. coli* GFP8624 was preinduced and acid stressed using Acid Stress Treatment 2 as previously described. Four experimental treatments were subjected to the acid stress, UU, (unadapted, unshocked), AU (adapted, unshocked), AA15 (adapted, shocked for 15 minutes), and AA30 (adapted, shocked for 30 minutes). Epithelial cell counts (cells/mL) from several wells were determined and averaged with the aid of a haemocytometer. This value was used in conjunction with the bacterial OD₆₀₀ value to determine the approximate amount of bacterial solution to be added to each well such that a multiplicity of infection (MOI) of 100:1 was achieved. To confirm the MOI, serial dilutions of all experimental treatments were made in 100 mM TBS and plated out on TP agar. Only plates with 30-300 colonies were used in subsequent calculations.

Prior to bacterial infection, the MEM (with additives) was removed and each well was washed three times with 1 x PBS. The appropriate amount of bacteria were added to their respective well so that the MOI of 100:1 was achieved and MEM (without additives) was added to each well to bring the volume up to 3 mL. A negative control

containing only HEp2 cells was also prepared. The well plate(s) were incubated at 37°C, 5% CO₂ for four hours or six hours depending on experiment.

For 6-hour adhesion assays, the media was removed from the wells after three hours and washed three times with PBS. Fresh MEM (without additives) was added to each well and the plates were incubated at 37°C, 5% CO₂ for the remaining 3 hours of infection.

After the appropriate infection time elapsed, the media and non-adherent cells and bacteria were removed from the wells. The wells and coverslips were washed five times with PBS pH 7.4 and the cells were fixed by adding 2 mL of freshly made 4% paraformaldehyde (Sigma-Aldrich) in PBS and incubated at room temperature for thirty minutes. After incubation, the cells were washed two times with PBS and stored at 4°C until microscopic examination. For microscopic preparation, the coverslip from a well was carefully removed with forceps and placed face down on a glass slide (25 mm x 75mm, VWR) containing 1 drop of PBS. Excess PBS was removed and the coverslip was sealed and ready for examination.

A Carl Zeiss (Oberkochen, Germany) LSM 510 Scanning Confocal Microscope was used. Images were taken with an Argon Ion Laser 488 514 nm using two channels, Channel D, 543, 488 and Channel 1, 488. Image analysis and z-stacking was completed with LSM 510 software package.

2.11: Total RNA Isolation of Acid Stressed *E. coli* O157:H7 85170

Total RNA was isolated as described by Hanna *et al.*, (2001). An overnight LB culture of 85170 that was preinduced in DMEM was used. Six experimental treatments, UU15, UA15, AA15, UU30, UA30 and AA30 were subjected to Acid Stress Treatment 4 as previously described. Following acid stress the cells were centrifuged at 3300 rpm for 10 minutes at 18°C and the supernatants were aspirated. The acid stressed bacterial pellets were kept frozen at -20°C for no longer than 1 week prior to RNA extraction. All RNA work was completed on ice with gloves and appropriate RNase treatment supplies

and reagents. Each pellet was resuspended in 1 mL Trizol reagent (Gibco, Life Technologies Inc.) and transferred to cold Fast-Prep tubes and shaken with the Fast-Prep FP 120 cell disruptor for 45 sec. Treatments were chilled on ice for 2 minutes and then 350 uL of chloroform : isoamyl alcohol (24:1) was added to the tubes and shaken vigorously for 15 seconds. The tubes were incubated at room temperature for 2 minutes and then centrifuged at 13 000 rpm at 4°C for 15 minutes. The upper aqueous layer of each tube was transferred to a new 1.5 mL eppendorf tube. Once again, 350 uL of chloroform : isoamyl alcohol (24:1) was added and the tubes were shaken vigorously for 15 seconds and incubated at room temperature for 2 minutes. Following centrifugation at 13 000 rpm at 4°C for 15 minutes, the upper aqueous layer of each tube was transferred to a new 1.5 mL eppendorf tube. The RNA in each tube was precipitated by addition of 350 uL High Salt Solution (Molecular Research Centre) and 250 uL of isopropanol. All tubes were inverted several times and then centrifuged 13 000 rpm at 4°C for 15 minutes. The supernatant was removed and the RNA pellet was centrifuged again at 13 000 rpm at 4°C for 5 minutes. The supernatant was removed and the pellets were air dried for 10-15 minutes and then 75 uL of 1 x RNA-secure (Ambion) was added to each pellet and incubated at 65°C for 10 minutes. Following incubation, the RNA was pipetted to dissolve and mix the RNA. Prior to overnight storage at -80°C, the RNA concentration and integrity was measured spectrophotometrically and assessed with agarose gel electrophoresis

2.12: DNase Treatment of Acid Stressed *E. coli* O157:H7 85170

DNase treatment was performed as described by Hanna *et al.*, (2001). Using the concentration of RNA extracted from the A₂₆₀ OD readings, the appropriate volume to load 20 ug of RNA was added to 1.5 eppendorf tubes along with 30 uL of 10 x DNase buffer, 30 uL of DNase I (Promega, RQI DNase) and the calculated volume of DEPC H₂O to bring the total volume of the solution to 300 uL. All samples were incubated at 37°C for 45 minutes to 1 hour and extracted with 225 uL chilled Trizol reagent and 75 uL of chloroform : isoamyl alcohol (24:1). After incubation on ice for ten minutes the samples were centrifuged at 14 000 rpm at 4°C for five minutes and the upper aqueous

layer was transferred to new 1.5 mL eppendorf tubes. Once again, the RNA was extracted with 225 uL Trizol reagent and 75 uL of chloroform : isoamyl alcohol (24:1) and centrifuged at 14 000 rpm at 4°C for five minutes. The upper aqueous layer was transferred to a new tube and the RNA was precipitated with 40 uL of 2M ammonium acetate and 1 mL of cold 100% ethanol. All samples were inverted several times and the RNA was allowed to precipitate overnight at -80°C. Following overnight precipitation, all samples were centrifuged at 14 000 rpm at 4°C for thirty minutes. The supernatant was removed and the samples were centrifuged again for five minutes at 14 000 rpm at 4°C. The resulting RNA pellet was washed with 1 mL of cold 70% ethanol and centrifuged at 14 000 rpm at 4°C for ten minutes. The supernatant was removed and the samples were allowed to air dry for 15-20 minutes. The RNA was resuspended in 23 uL of diethyl pyrocarbonate (DEPC) H₂O (Sigma-Aldrich) and incubated at 55°C for ten minutes. Prior to overnight storage at -80°C, the RNA concentration and integrity was measured spectrophotometrically and assessed with agarose gel electrophoresis.

2.13: cDNA Synthesis of Acid Stressed *E. coli* O157:H7 85170

Synthesis of cDNA was completed as previously described by Korithoski *et al.* (2005). A 1st Strand cDNA Synthesis kit (Fermentas #K1612) was used according to the manufacturer's specifications. Using the concentration of DNase treated RNA obtained from the A₂₆₀ OD readings, the appropriate volume to load 1 ug of RNA was added to 0.5 mL eppendorf tubes along with 1 uL of random hexamers and the correct volume of DEPC H₂O to bring the total volume up to 11 uL. A master mix solution containing the following reagents were prepared for each reaction; 4 uL of 5 x buffer, 1 uL of RNase inhibitor (20 U/uL), and 2 uL of 10 mM dNTPs, for a total volume of 7 uL for each reaction. Using a PCR Block Cycler, the RNA solutions were first incubated at 70°C for five minutes. Following incubation, 7 uL of master mix was added to each reaction tube when the cycler temperature dropped to 4°C. The RNA solution and master mix solutions were incubated at 25°C for five minutes and the cDNA program was paused to allow addition of 2 uL of M-MULV reverse transcriptase (20 U/uL) for a total volume of 20 uL except for the "no RT" controls, which had a total volume of 18 uL. All reactions

were incubated at 25°C for 10 minutes, followed by a 37°C incubation of one hour, and a final 70°C incubation for 10 minutes. After cDNA synthesis, the Block Cyclor incubated all reactions at 4°C until removal. Select samples and their corresponding “no RT control” were electrophoresed to ensure cDNA synthesis. All samples were stored at -20°C until use in subsequent RT-PCR optimization and analysis experiments.

2.14: Genomic DNA Isolation of *E. coli* O157:H7 85170

This procedure, adapted from Hanna *et al.* (2001), was modified using a Puregene kit (Gentra Systems). An overnight culture of 85170 in 1 mL of LB broth was prepared. The next day the bacterial solution was centrifuged at 3300 rpm at 4°C for fifteen minutes. The supernatant was aspirated and the pellet was resuspended in 600 uL of Cell Suspension Solution. 100 uL of Lysozyme (Sigma #L7651) 10 mg/mL in 25 mM Tris pH 8.0 was added and the reaction tube was mixed and incubated at 37°C in a water bath for 2 to 3 hours. Cells were resuspended at 13 000 rpm at room temperature for three minutes and the resulting supernatant was discarded. The pellet was resuspended in 600 uL of Cell Lysis Solution and incubated at 80°C heating block for five minutes to complete cell lysis. Once the solution reached room temperature, 200 uL of Protein Precipitation Solution was added and vortexed vigorously and was centrifuged at 13000 rpm at room temperature for three minutes. The supernatant was transferred to a new 1.5 mL eppendorf tube and 600 uL of 100% isopropanol was added. The DNA was allowed to precipitate and DNA threads were removed into a prechilled 70% ethanol solution. The ethanol was drained and 50 uL of DNA hydration solution was added to resuspend the genomic DNA. Following this, 1 uL of RNase (10mg/mL) was added to the tube and the DNA was stored overnight at 4°C and then at -20°C. Prior to overnight storage at 4°C, the genomic DNA concentration and integrity was measured spectrophotometrically and assessed with agarose gel electrophoresis.

2.15: Primer Optimization for *E. coli* O157:H7 85170

All primers were designed with MacVector primer design software and obtained from Operon. The primers used in this study are shown in Table 2.4. Primers were

dissolved in the appropriate volume of DEPC H₂O to 50uM concentration and aliquoted in 1.5 mL eppendorf tubes and stored at -20°C until use. Since primers were designed to EHEC EDL933 and not 85170, a primer optimization protocol modified from Pfaffl *et al.*, (2002) was carried out to ensure that the correct gene/amplicon was amplified and to also determine the best annealing (T_A) and melting (T_m) temperatures. Gradient PCR was completed for each set of primers using genomic DNA as the template and a temperature range of +/- 5°C using the gradient PCR program on the PCR block cycler (iCycler). A Master mix solution was made with the following reagents for each reaction, 1uL of genomic DNA (600 ug/mL), 38.5 uL DEPC H₂O, 5 uL of 10x buffer, 4 uL 25 mM MgCl₂, 1 uL of 10 mM dNTPs, 0.5 uL of both forward and reverse primers (50 uM), 0.5 uL of Taq Polymerase. After initial denaturation for 10 minutes at 94°C, 35 cycles were run with the following conditions: 94°C for 1 minute (denaturation), variable T_A's for primer annealing (depending on primers) for 1 minute, and 72°C for 1 minute (elongation), followed by a 72°C 10 minute extension step. All samples and any negative controls were electrophoresed to ensure proper binding of primers and the best T_A was chosen for each primer.

Table 2.4: Primers Used in this Study

<i>Gene</i>	<i>Forward 5' to 3'</i>	<i>Reverse 5' to 3'</i>	<i>Amplicon Length</i>
Intimin – (<i>eae</i>)	“GCAACTCTTGGGGCAAATAGTC”	“GTTTTAGCAGACACGACGACCTC”	101 bp
GapA (<i>gapA</i>)	“AATCCGTGTTACCGCTGAACG”	“GTGATGTGTTTACGAGCAGTTTCG”	120 bp
EspA – (<i>espA</i>)	“TTGAGGAACTCGGTGTTTTTCAG”	“CGTGGTTGACGCTTTAGATGCC”	122 bp

2.16: Real Time RT-PCR of Acid Stressed *E. coli* O157:H7 85170

Real-time polymerase chain reaction (RT-PCR) was carried as previously described by Korithoski *et al.* (2005) with modifications. Quantitect SYBR Green PCR kit (Qiagen) was used for amplification and detection of RT-PCR products. Each reaction contained 2 uL of diluted cDNA (diluted 1:25 – 1:100), 12.5 uL of 2x SYBR Green

mixture (SYBR Green, deoxynucleoside triphosphates, MgCl₂, and Hot Start Taq Polymerase), 0.25 uL 50 mM forward primers, 0.25 uL 50 mM reverse primers, and 10 uL of DNase free H₂O (Gibco). Using the LightCycler Cooling Block and centrifuge adaptors (Roche Scientific), all reagents were pipetted into 20 uL LightCycler Capillary tubes (Roche Scientific), capped and centrifuged for 10 seconds at 3000 rpm. Real time PCR was carried out with the LightCycler 2.0 System (Roche Scientific) with the following programs: (1) Preincubation: 95°C for 10 minutes, (2) Amplification: 30-40 cycles (depending on target gene) with the following conditions, 95°C for 30 seconds, variable T_A depending on primers for 30 seconds, followed by 72°C for 30 seconds, (3) Melting Curve Analysis: A continuous cycle with the following conditions, 95°C for 0 seconds, variable T_m depending on the primers for 15 seconds, 95°C for 0 seconds, (4) Cooling program which dropped the temperature to 40°C for 30 seconds. Triplicates were done for each sample (unless otherwise stated) including the blank or “no template control” (NTC). Using LightCycler Software 4.0 (Roche Molecular Biochemicals) the Second Derivative Maximum Method was used for Cp (crossing point) determination.

2.17: RT-PCR : Data Analysis

Calibrator normalized relative quantification with efficiency correction was completed using LightCycler Relative Quantification Software 4.0. The equation used, which yields the same results as the Pfaffl normalization method is as follows (Pfaffl *et al.*, 2002):

$$\text{Ratio} = \frac{(E_{\text{reference}})^{\text{Cp Sample}}}{(E_{\text{target}})^{\text{Cp Sample}}} \quad / \quad \frac{(E_{\text{reference}})^{\text{Cp Calibrator}}}{(E_{\text{target}})^{\text{Cp Calibrator}}}$$

where :

- E_{target} is the real-time efficiency of target gene
- $E_{\text{reference}}$ is the real-time efficiency of a reference/ housekeeping gene
- Cp Sample is the crossing point of all experimental treatments for both the target and reference genes
- Cp Calibrator gene is the crossing point of the control genes (in this case unadapted and unshocked treatments)

Calibrator normalized ratios (unadapted, unshocked treatments) are designated 1.0 and all other experimental treatments are expressed as n-fold differences to these treatments.

2.18: RT-PCR Optimization (Validation of *gapA* Housekeeping Gene, cDNA Contamination and Standard Curves)

Validation of Glyceraldehyde 3-P Dehydrogenase A (*gapA*) Housekeeping Gene

A modified method outlined in the Roche Applied Science Technical Note No LC15/2002 was used to validate *gapA* as a suitable housekeeping gene for Acid Stress Treatment #4. Essentially the *gapA* Cp values from five different cDNA pools were obtained and compared across all experimental treatments.

Real Time cDNA Optimization

To ensure that all cDNA samples were free from contamination, RT-PCR runs for all undiluted cDNA negative controls (“No RT controls”) were completed using *gapA* primers as previously outlined.

Standard Curves

Real-time PCR standard curves for all genes (housekeeping and target genes) were constructed using genomic DNA to obtain amplification efficiencies for both target and reference genes (housekeeping genes). Serial dilutions of genomic DNA ranging from 200 ng to 0.001 ng were prepared for all genes and RT-PCR runs for each standard and NTC (no template control) were run in duplicate as previously outlined. LightCycler Software 4.0 was used to construct the standard curves and calculate PCR efficiencies.

2.19: RNA/DNA Spectrophotometric Measurements

A 1:50 dilution of either RNA or DNA (2 uL nucleic acid and 98 uL of DEPC H₂O) was made for each treatment to measure the concentration and integrity of the nucleic acid after each treatment (RNA extraction, genomic DNA extraction, DNase treated RNA). A 100 uL quartz cell was used to take a UV-VIS scan of the nucleic acid.

From the scans, the absorbance at 260 nm (A_{260}) was used to determine the concentration and the ratio A_{260}/A_{280} was used to ascertain the purity of the sample (Pfaffl *et al.*, 2002).

2.20: RNA/DNA Agarose Gel Electrophoresis

To ensure RNA/DNA integrity and optimal primer annealing temperatures agarose gel electrophoresis was used throughout RT-PCR sample preparation and optimization and the protocol was adapted from Pfaffl *et al.*, (2002). To prepare the gels, 0.5 g of agarose was added to 50 mL 1 x TAE buffer and microwaved at full power for 1 minute. Approximately 3-5 uL of ethidium bromide was added to the heated agarose and swirled to mix. After the agarose solution cooled down somewhat, it was poured into an appropriate agarose gel tray, capped at both ends with a comb inserted. After approximately thirty minutes, the gel had solidified and was loaded into the electrophoresis apparatus and filled 1 x TAE running buffer. About 1-2 uL of nucleic acid was mixed with the appropriate volume of 1x tracking dye for a total volume of 7 uL. The samples were loaded onto the gel along with 3 uL of 1 kB ladder. The gel apparatus was hooked up to a powerpak set at 100 volts and electrophoresed for approximately forty five minutes or until the loading dye reached $\frac{3}{4}$ down the gel. After electrophoresis the gel was photographed with GENESNAP Gel Imaging Software.

2.21: Vero Cell Cytotoxicity Assays.

The vero cell cytotoxicity assay was a modification of several procedures (Karmali *et al.*, 1985; Khine *et al.*, 2004).

One day prior to the experiment confluent (80-90%) 75 cm² flasks of vero cells were trypsinized with 2 mL of trypsin-EDTA and incubated for approximately 2 minutes at 37°C, 5% CO₂. Following trypsinization, 58 mL of MEM with additives was added to each flask and thoroughly mixed. Seven 96-well plates (BD Falcon), one for each experimental treatment type (UU15, UA15, AA15, UU30, UA30, AA30) and one for the control were seeded with 200 uL of the vero cell solution using a multichannel pipettor. The plates were incubated overnight at 37°C, 5% CO₂.

An overnight LB culture of 8624 that was preinduced in DMEM was subjected to Acid Stress Treatment 3 as previously described. After acid stress, the treatments were normalized using prior viability data to ensure that the initial inoculum was approximately the same. To validate the normalization, a dilution series was prepared in 100 mM TBS for each normalized treatment and plated on TP agar and incubated at 37°C for 16-18 hours. For verotoxin production, 1 mL of each normalized bacteria treatment and 14 mL of Penassay broth (antibiotic medium no. 3; Difco Laboratories) was added to 15 mL falcon tubes and incubated overnight at 37°C and 200 rpm. Following overnight incubation, 2 mL of each treatment were used to obtain OD₆₀₀ readings and/or serial dilutions in 100 mM TBS were completed and plated on TP agar and incubated at 37°C for 16-18 hours. All treatments were centrifuged at 3300 rpm for 10 minutes at 18°C and 10 mL of the supernatant was sterile filtered into 15 mL falcon tubes using 0.2 µm membrane filters and 10 cc Leur-Lok syringes (BD Falcon) and the resulting supernatant filtrates were stored at -20°C for no longer than a week until use. The remaining supernatant was aspirated and bacterial pellet was resuspended in 1.5 mL of 0.1 mg/mL colymycin (Parker Davis) and incubated statically at 37°C for 30 minutes. The treatments were then centrifuged at 3300 rpm for 10 minutes at 18°C and the periplasmic extracts were sterile filtered into 1.5 mL eppendorf tubes using 0.2 µm membrane filters and 3 cc leur-lok syringes (BD Falcon). The resulting periplasmic extracts were stored at -20°C for no longer than a week until use.

As a positive control, a verotoxin 1 (VT1) stock standard of 1.1 µg/mL was used which was kindly provided by Dr. Lingwood (Hospital for Sick Children, Toronto). Yoh *et al.* (1997) has shown that the cytotoxicity of VT1 and VT2 on vero cells were similar, hence the use of a VT1 standard even though the bacterial strain used in this study produces only VT2. Nine serial dilutions ranging from 1.1×10^{-1} ng/mL to 1.1×10^{-9} ng/mL of this stock standard were made fresh in MEM (no additives) on the day of the experiment and 50 µL of each standard (with replicates) was added to the control 96 well plate so that final VT1 standard concentrations in the wells ranged from 2.2×10^{-1} ng/mL to 2.2×10^{-7} ng/mL. On the day of the experiment, frozen experimental extracts and filtrates were thawed and dilutions of the samples; 1/1, 1/4, 1/8, 1/32, 1/128, 1/512,

1/1024, and 1/2048 were made in MEM (no additives) and 50 uL of each dilution for each experimental treatment (both periplasmic extracts and supernatant filtrates) were added with replicates to the wells. For the control wells, 50 uL of MEM (no additives) were added. All plates were incubated for 2-3 days at 37°C, 5% CO₂.

After incubation the solutions in the wells were removed and the remaining live cells in each well were fixed with 65 uL of a 2% formalin solution (0.05% of a 40% formaldehyde (v/v), 95% PBS (v/v)). The plates were incubated at room temperature for approximately 1-2 minutes and the formalin solution was removed from the wells. To stain the cells, 65 uL of Crystal Violet Tissue Culture stain (0.26% of 0.5% Crystal Violet Stock Solution (v/v), 0.05% absolute ethanol (v/v), 0.05% of 40% formaldehyde (v/v), 0.64% of PBS (v/v)) was added to each well and incubated at room temperature for a minimum of twenty minutes. The crystal violet stain was removed and the wells were washed four times with ddH₂O and left to air dry. To quantitate the results, 100 uL of 10% acetic acid solution (Fisher Scientific) was added and plates were read at 570 nm in an ELISA plate reader.

2.22: Statistical Analysis

The mean and standard deviations of all quantitative adhesion and cytotoxicity assay results were calculated and analysis of variance (ANOVA) was used to determine if the results for acid stressed *E. coli* O157:H7 strains were significantly different from the control unstressed cells. Depending on the ANOVA results, post-hoc Tukey and Kramer tests were completed using Lumenaut Statistics Version 3 (an Excel Add-in software tool). Both parametric (t-tests) and nonparametric statistical analyses were conducted for real time RT-PCR results. The nonparametric statistical analysis was completed using Relative Expression Software Tool – Multiple Condition Solver or REST-MCS© (Pfallel *et al.*, 2002).

3.0 Results

3.1: Percent Survival (as assessed by culturability) of *Escherichia coli* O157:H7 After Acid Stress

The survival of log phase *E. coli* O157:H7 strains 85170, CL56 and GFP8624 were examined after acid stress exposure and normalized to their corresponding unadapted and unshocked controls. Various acid shocking protocols were used, and in all cases, acid adaptation followed by acid shock significantly lowered the percent survival of cells compared to the controls ($p \leq 0.05$). Table 3.1 below summarizes these results.

Table 3.1: Summary of Surviving *E. coli* O157:H7 after Acid Stress (as assessed by culturability)

Treatment	85170 (Acid Treatment 1)	85170 (Acid Treatment 3)	CL56 (Acid Treatment 2)	GFP8624 (Acid Treatment 2)
UU15		100.0 $\pm$ 8.7		
UU30		100.0 $\pm$ 5.9		
UU	100 $\pm$ 7.9		100.0 $\pm$ 11.4	100.0 $\pm$ 19.6
AU				84.2 $\pm$ 6.3
UA15		80.9 $\pm$ 12.9		
UA30		71.2 $\pm$ 10.1		
AA5			69.6 $\pm$ 20.9	
AA15	46.5 $\pm$ 17.5	54.5 $\pm$ 9.5	51.8 $\pm$ 22.9	56.0 $\pm$ 3.2
AA30	37.7 $\pm$ 19.1	52.2 $\pm$ 16.3	47.2 $\pm$ 28.5	58.4 $\pm$ 2.5

Results are expressed as means $\pm$ standard deviations. Normalized results for 85170, CL56 and GFP8624 are from 4,3 and 1 independent experiment(s) respectively with a minimum of 2 replicates for each treatment. [UU15, UU30: Unadapted, unshocked for 15 and 30 minutes (controls); UU: Unadapted, unshocked (control); AU: Acid adapted, unshocked; UA15, UA30: Unadapted, acid shocked for 15 and 30 minutes; AA5, AA15, AA30: Acid adapted, acid shocked for 5, 15, and 30 minutes.]

3.1.1: Percent Survival of *Escherichia coli* O157:H7 85170 After Acid Stress

Two different acid treatments were used to assess the normalized percent survival of *E. coli* O157:H7 85170. Bacteria subjected to Acid Treatment 1 were adapted at room temperature in either pH 5.0 (acid adapted) or pH 7.0 (unadapted) DMEM without any buffering agents. However, DMEM was supplemented with 25 mM MES (pH 5.0) and 25 mM MOPS (pH 7.4) in Acid Treatment 3 and the adaptation step was carried out at

37°C and 5% CO₂. Acid shocking was done at pH 3.0 (unbuffered) at room temperature for both treatments. Regardless of the acid protocol used, the percentage of surviving bacteria that were acid adapted-acid shocked after 15 or 30 minutes was significantly lower by 45-62% compared to the unstressed treatments ($p \leq 0.05$).

The effect of acid shocking alone on the percent survival of *E. coli* O157:H7 85170 using Acid Treatment 3 was also determined. The percent survival of both unadapted-acid shocked *E. coli* O157:H7 85170 after 15 and 30 minutes was significantly lower (by 19-29%) than the unadapted-unshocked controls ($p \leq 0.05$). However the experimental results indicate that acid adaptation followed by acid stress was more detrimental to the survival of *E. coli* O157:H7 85170 than brief acid stress alone. The percent survival of both unadapted-acid shocked cells after 15 or 30 minutes was significantly higher than their acid adapted-acid stressed counterparts by 19- 26 % ($p \leq 0.05$). The results of acid stress on the percent survival of *E. coli* O157:H7 85170 (as assessed by culturability) is shown in Figure 3.1.1.

Figure 3.1.1: Percent Survival of *E. coli* O157:H7 85170 after Acid Stress

Effect of acid adaptation plus acid shock or acid shock alone on the survival of *E. coli* O157:H7 85170 was assessed by plate count assay ($n \geq 2$). This is the result of 4 independent experiments and the values are means $\pm$ standard deviations. Acid Shocked Treatment 1: No buffering of all media and adaptation at room temperature. Acid Shock Treatment 3: 25 mM MES and MOPS and adaptation at 37 °C and 5% CO₂.

[UU15: Control (Unadapted, unshocked for 15 minutes); UA15: Unadapted and acid shocked for 15 minutes; AA15: Acid adapted and acid shocked for 15 minutes; UU30: Control (Unadapted, unshocked for 30 minutes); UA30: Unadapted and acid shocked for 30 minutes; AA30: Acid adapted and acid shocked for 30 minutes]

* Significantly different from corresponding control (UU15 or UU30) ($p \leq 0.05$)

** Significantly different from corresponding control (UU15 or UU30) and corresponding unadapted treatment (UA15 or UA30) ($p \leq 0.05$)

3.1.2: Percent Survival of *Escherichia coli* O157:H7 CL56 After Acid Stress (as assessed by culturability)

The percent survival of *E. coli* O157:H7 CL56 subjected to Acid Treatment 2 is shown in Figure 3.1.2. Bacteria were adapted at 37°C and 5% CO₂ in either DMEM pH 5.0 (MES) or pH 7.0 (unbuffered) and acid shocking was done at pH 3.0 (unbuffered) at room temperature. The percent survival of acid adapted-acid shocked *E. coli* O157:H7 CL56 after 5, 15 or 30 minutes was significantly lower by 30-53% compared to the unstressed control ($p \leq 0.05$).

Figure 3.1.2: Percent Survival of *E. coli* O157:H7 CL56 after Acid Stress

Effect of acid adaptation plus acid shock or acid adaptation alone on the survival *E. coli* O157:H7 CL56 was assessed by plate count assay ($n \geq 2$). Data based on a minimum of 3 independent experiments and the values are means $\pm$ standard deviations. Acid Shock Treatment 2: 25 mM MES and adaptation at 37 °C and 5% CO₂ was used.

[UU: Control (Unadapted, unshocked); AA5, AA15, AA30: Acid adapted and acid shocked for 5, 15, and 30 minutes, respectively]

*Significantly different from control (UU) ($p \leq 0.05$)

3.1.3: Percent Survival of *Escherichia coli* O157:H7 GFP8624 After Acid Stress (as assessed by culturability)

Using Acid Treatment 2, the percent survival of *E. coli* O157:H7 GFP8624 after acid stress exposure was examined. Bacteria were adapted at 37°C and 5% CO₂ in either DMEM pH 5.0 (MES) or pH 7.0 (unbuffered) and acid shocking was done at pH 3.0 (unbuffered) at room temperature. The percent survival of acid adapted-acid shocked *E. coli* O157:H7 GFP8624 after 15 or 30 minutes was significantly lower by 42-44% compared to the unadapted-unshocked control ($p \leq 0.05$). These acid adapted-acid shocked treatments were also significantly lower (28-30%) than cells subjected to acid adaptation alone ($p \leq 0.05$). The results of acid stress on the survival of *E. coli* O157:H7 85170 is shown in Figure 3.1.3.

Figure 3.1.3: Percent Survival of *E. coli* O157:H7 GFP8624 after Acid Stress

Effect of acid adaptation plus acid shock or acid adaptation alone on the survival *E. coli* O157:H7 GFP8624 was assessed by plate count assay (n=3) and the values are means $\pm$ standard deviations. Acid Shock Treatment 2: 25 mM MES and adaptation at 37 °C and 5% CO₂ was used.

[UU: Control (Unadapted, unshocked) ; AU: Adapted and unshocked; AA15: Acid adapted and acid shocked for 15 minutes; AA30: Acid adapted and acid shocked for 30 minutes]

** Significantly different from control (UU) and Adapted, unshocked (AU) ($p \leq 0.05$)

3.2: Adhesion of Acid Stressed *Escherichia coli* to Human Epithelial Cells

Adherence of *E. coli* O157:H7 strains 85170, CL56, and GFP8624, and a non-pathogenic *E. coli* laboratory strain, HB101, to either HEp-2 or Caco-2 human epithelial cells after acid stress was examined. HEp-2 cells, human carcinomic laryngeal epithelial cells, are extensively used model systems for studying pathogenic *E. coli* adherence patterns (Foster *et al.*, 1999). However, since *E. coli* O157:H7 is an intestinal pathogen, adhesion assays were also conducted with a more physiological relevant cell line, Caco-2 cells, adenocarcinomic colonic epithelial cells (Torres and Kaper, 2003). The adhesion of viable bacteria to these cell lines was determined by plate count assays as previously described by De Jesus *et al.* (2005). To control for day-to-day variation, all of the results were reported normalized to the positive control, where the amount of viable bacteria bound to host cells was expressed as a percentage of initial viable inoculums incubated with the epithelial cells and the percentages were normalized to the control treatment(s), with the control treatments representing 100% (Boerlin *et al.*, 1999). The 3-hour Hep-2

adhesion assay results for strain 85170 subjected to Acid Treatment 1 and 3 can be seen in Figures 3.2.1 and 3.2.2 respectively. Figure 3.2.4 shows the results of a 3-hr adhesion assay of strain CL56 to Caco-2 cells, and Figure 3.2.5 compares the results of a 3 and 6-hour adhesion assay under the same conditions. The host adhesion capability of GFP8624 to HEp-2 cells was assessed by a plate count assay and by confocal microscopy and these results are presented in Figures 3.2.6 and 3.2.7 respectively. The adhesion ability of *E. coli* HB101 is presented in Figure 3.2.8.

Regardless of the *E. coli* O157:H7 strains, epithelial cell lines, and the different acid stress treatments used, the bacteria subjected to acid adaptation followed by a 30 minute acid shock adhered significantly better than the unadapted-unshocked control (by a factor of 1.6-3.2, $p \leq 0.05$). Table 3.2 below summarizes the plate count adhesion results obtained and these results are further outlined in the appropriate sections below.

Table 3.2: Summary of Acid Stressed *E. coli* O157:H7 Host Cell Adhesion

Treatment	85170 (Acid Treatment 1) (HEp2)	85170 (Acid Treatment 3) (HEp2)	CL56 (Acid Treatment 2) (Caco-2)	GFP8624 (Acid Treatment 2) (HEp-2)
UU15		100 ± 27		
UU30		100 ± 17		
UU	100 ± 23		100 ± 10	100 ± 18
AU				111 ± 16
UA15		23 ± 9		
UA30		21 ± 15		
AA5	153 ± 25			
AA15	246 ± 182	37 ± 7	236 ± 47	236 ± 48
AA30	322 ± 67	160 ± 37	294 ± 59	257 ± 43
Non-normalized Range of Percentage Viable Bacteria Bound to Host Cells	<2%	<2%	1-3%	4-11%

Results are expressed as means ± standard deviations. Normalized results for 85170 and CL56 are from a minimum of 3 and 2 independent experiments, respectively and GFP8624 results are from 1 independent experiment. A minimum of 3 replicates for each experimental treatment was completed. [UU15, UU30: Unadapted, unshocked for 15 and 30 minutes (controls); UU: Unadapted, unshocked (control); AU: Acid adapted, unshocked; UA15, UA30: Unadapted, acid shocked for 15 and 30 minutes; AA5, AA15, AA30: Acid adapted, acid shocked for 5, 15, and 30 minutes.]

Acid stressed bacteria incubated with 1% mannose did not affect the host adhesion capability of *E. coli* O157:H7 to HEp2 cells, however pre-treatment of the acid stressed bacteria with erythromycin resulted in a significant adhesion decrease ($p \leq 0.05$) and these results are found in Figure 3.2.9, and Figure 3.2.10 respectively.

3.2.1: Adhesion of Acid Stressed *Escherichia coli* O157:H7 85170 to Human Epithelial Cells

Using Acid Treatment 1, the host adhesion capability of *E. coli* O157:H7 85170 after acid stress exposure to HEp2 cells was examined. Bacteria were adapted at room temperature in either unbuffered DMEM pH 5.0 or pH 7.0 and acid shock treatments were carried out at pH 3.0 (unbuffered), also at room temperature. Acid adapted and acid shocked bacteria for 5 or 30 minutes showed significantly enhanced adhesion over the control treatment, 153% and 321%, respectively ($p \leq 0.05$). Results also indicate that the normalized adhesion of *E. coli* O157:H7 85170 acid adapted and acid shocked for 30 minutes was significantly higher than the treatments subjected to acid-adaptation and shocking for 5 minutes (by a factor of 1.7, $p \leq 0.05$).

Figure 3.2.1: Acid Stressed *E. coli* O157:H7 85170 Adhesion to HEp2 Cells (Acid Treatment 1)

Effect of acid adaptation plus acid shock of *E. coli* O157:H7 85170 on adhesion to HEp-2 cells was assessed by plate count assay (n= 3-6 for each experiment). The results of 4 independent experiments are shown (except AA5, N=2) and the values are means $\pm$ standard deviations. Acid Shocked Treatment 1: No buffering of all media and adaptation at room temperature. [UU: Control (Unadapted, unshocked); AA5, AA15, AA30: Acid adapted and acid shocked for 5, 15 and 30 minutes respectively.]

* Significantly different from control (Unadapted, unshocked) ($p \leq 0.05$)

** Significantly different from control (Unadapted, unshocked) and Acid adapted and acid shocked for 5 minutes (AA5) ($p \leq 0.05$)

The bacteria host-cell adhesion of acid stressed *E. coli* O157:H7 85170 subjected to Acid Treatment 3 was also determined and is shown in Figure 3.2.2. Bacteria were adapted at 37°C and 5% CO₂ in either DMEM pH 5.0 (MES) or pH 7.4 (MOPS) and acid shocking was done at pH 3.0 (unbuffered) at room temperature. Bacteria subjected to acid adaptation and a 30-minute acid shock showed significantly enhanced adhesion over the unstressed control (by a factor of 1.6, $p \leq 0.05$). Results also indicate that acid shocking alone decreased the host adhesion capability of this strain compared to the unstressed control. The adhesion of both unadapted-acid shocked bacteria for 15 and 30 minutes to host cells was significantly decreased to 23% and 21% (compared to 100% for controls) respectively ($p \leq 0.05$). A significant 8-fold increase in adhesion was seen when adaptation was preceded by treatments subjected to a 30-minute acid shock ($p \leq 0.05$). The host cell

adhesion capability of this strain was also influenced by length of exposure to acid. Results show that the normalized adhesion of *E. coli* O157:H7 85170 acid adapted-acid shocked for 30 minutes was significantly higher (4-fold) than the adhesion of acid adapted-acid shocked for 15 minutes treatments ($p \leq 0.05$).

Figure 3.2.2: Acid Stressed *E. coli* O157:H7 85170 Adhesion to HEp2 Cells (Acid Treatment 3)

Effect of acid adaptation plus acid shock and acid shock alone of *E. coli* O157:H7 85170 on adhesion to HEp-2 cells was assessed by plate count assay ($n = 4-6$ for each experiment). The results of 3 independent experiments for UU15, UA15 and AA15 and a minimum of 4 independent experiments for UU30, UA30 and AA30 are shown. The values are means $\pm$ standard deviations. Acid Shocked Treatment 3: 25 mM MES and MOPS used and adaptation at 37 °C and 5% CO₂. [UU15: Control (Unadapted, unshocked for fifteen minutes); UA15: Unadapted, shocked for fifteen minutes; AA15: Acid adapted and acid shocked for fifteen minutes; UU30 Control (Unadapted, unshocked for thirty minutes); UA30: Unadapted, shocked for thirty minutes; AA30: Acid adapted and acid shocked for thirty minutes]

* Significantly different from corresponding control (UU15 or UU30) ($p \leq 0.05$)

*** Significantly different from corresponding control (UU30), (UA30) and (AA15) ($p \leq 0.05$).

A control experiment was performed to determine the effect of acid stress on the growth of *E. coli* 85170 throughout the infection time in a three-hour bacterial-host cell adhesion assay and is shown in Figure 3.2.3. Following acid stress, the bacteria were normalized and added to a 24 well plate without epithelial cells, mimicking the procedure used in previous acid adhesion assays. After a 3 hour incubation at 37 °C and 5% CO₂,

the bacteria were collected and plated out on TP agar to assess any differences in viability (as assessed by culturability) or growth that may have arose due to acid stress. There were no significant differences in both the initial amount of bacteria loaded and the final viabilities after this three-hour incubation ($p \geq 0.05$).

Figure 3.2.3: The Effect of Acid Stress on *E. coli* O157:H7 85170 Culturability throughout a 3-hour infection time (Acid Treatment 3)

A

B

The effect of acid stress on *E. coli* O157:H7 85170 viability following a 3-hour incubation at 37 °C and 5% CO₂. **A:** Initial amount of bacteria loaded **B:** Culturability after a 3-hour incubation at 37 °C and 5% CO₂. The results of 2 independent experiments are shown the values are means ± standard deviations. Acid Shocked Treatment 3: 25 mM MES and MOPS used and adaptation at 37 °C and 5% CO₂. [UU30 Control (Unadapted, unshocked for thirty minutes); UA30: Unadapted, shocked for thirty minutes; AA30: Acid adapted and acid shocked for thirty minutes]. No significant differences were observed, $p \geq 0.05$.

3.2.2: Adhesion of Acid Stressed *Escherichia coli* O157:H7 CL56 to Human Epithelial Cells

The bacteria host-cell adhesion of acid stressed *E. coli* O157:H7 CL56 subjected to Acid Treatment 2 was also determined and is shown in Figure 3.2.4. Bacteria were adapted at 37°C and 5% CO₂ in either DMEM pH 5.0 (MES) or pH 7.4 (unbuffered) and acid shocking was done at pH 3.0 (unbuffered) at room temperature. Acid adapted and acid shocked bacteria for 15 and 30 minutes showed significantly enhanced adhesion to Caco-2 cells compared to the unadapted, unshocked control (236% and 294% respectively, $p \leq 0.05$). The host cell adhesion capability of this strain was also influenced by the length of time of the acid exposure. Results show that the normalized adhesion of *E. coli* O157:H7 CL56 acid adapted-acid shocked for 30 minutes was significantly higher (by a factor of 1.2) than the adhesion of acid adapted-acid shocked for 15 minutes treatments ($p \leq 0.05$).

Figure 3.2.4: Acid Stressed *E. coli* O157:H7 CL56 Adhesion to Caco-2 Cells (Acid Treatment 2)

Effect of acid adaptation plus acid shock of *E. coli* O157:H7 CL56 on adhesion to Caco-2 cells was assessed by plate count assay ($n \geq 5$). Data shown are the results of 2 independent experiments and the values are means $\pm$ standard deviations. Acid Shocked Treatment 2: 25 mM MES and adaptation at 37 °C and 5% CO₂.

[UU: Control (Unadapted, unshocked); AA15, AA30: Acid adapted, acid shocked for 15 and 30 minutes respectively.]

*Significantly different from UU (Unadapted, unshocked) ($p \leq 0.05$)

**Significantly different from UU (Unadapted, unshocked), and AA15 (Acid adapted, shocked for 15 minutes) ($p \leq 0.05$)

Using Acid Treatment 2, the host adhesion capability of *E. coli* O157:H7 CL56 after acid stress exposure to Caco-2 cells was examined at two different bacteria-host incubation times of 3 and 6 hours. All 3-hour and 6-hour adhesion results were expressed as a percentage of viable bacteria bound to host cells and normalized to the 3-hour control (unadapted-unshocked). For the 3-hour adhesion, acid adapted and acid shocked bacteria for 15 or 30 minutes showed significantly enhanced adhesion to host cells compared to the control (100%), 217% and 342% respectively, with acid adapted-acid shocked for 30 minutes adhering significantly greater than the acid adapted-acid shocked for 15 minutes treatment ($p \leq 0.05$). Both of these treatments also showed significantly higher adhesion compared to the adhesion of acid adapted-acid shocked for 5 minutes treatment ($p \leq 0.05$). The 6-hour adhesion results also show a similar trend, with both acid-adapted and acid shocked for 15 and 30 minutes treatments adhering significantly better than the 6 hour control, 307%, 486%, 115%, respectively ($p \leq 0.05$). The acid adapted-acid shocked for 30 minutes treatment also adhered significantly higher to host cells compared to the acid adapted-acid shocked for 15 minutes treatment ($p \leq 0.05$). These results also indicate that the infection time increased the adhesion of acid stressed bacteria to host cells. Both acid adapted-acid shocked for 15 and 30 minutes cells that were incubated with the host cells for 6 hours adhered significantly higher (by a factor of 1.4) to Caco-2 cells than the corresponding treatments that were incubated for 3 hours ($p \leq 0.05$). These results are shown in Figure 3.2.5 below.

Figure 3.2.5: The Effect of Incubation Time on the Adhesion of Acid Stressed *E. coli* O157:H7 CL56 to Caco-2 Cells (Acid Treatment 2)

Effect of acid adaptation plus acid shock of *E. coli* O157:H7 CL56 on adhesion to Caco-2 cells was assessed by plate count assay (n=6 for each treatment except for UU for 6 hour treatment, n=4) and the values are means $\pm$ standard deviations. Acid Shocked Treatment 2: 25 mM MES and adaptation at 37 °C and 5% CO₂.

[UU: Control (Unadapted, unshocked); AA5, AA15, AA30: Acid adapted, acid shocked for 5, 15 and 30 minutes respectively.]

* Significantly different from 3 hr control UU (Unadapted, unshocked) and AA5 Acid adapted, shocked for 5 minutes for 3 hours ($p \leq 0.05$)

** Significantly different from 3 hr control UU (Unadapted, unshocked), AA5 (Acid adapted, shocked for 5 minutes for 3 hours) and AA15 (Acid adapted, shocked for 15 minutes for 3 hours) ($p \leq 0.05$)

++ Significantly different from 6 hour control UU (Unadapted, unshocked) and 3 hour AA15 ($p \leq 0.05$)

+++ Significantly different from 6-hour control (Unadapted, unshocked), 6 hour AA15 (Acid adapted, shocked for 15 minutes) and 3 hour AA30 (Acid adapted, acid shocked for 30 minutes) ($p \leq 0.05$)

3.2.3: Adhesion of Acid Stressed *Escherichia coli* O157:H7 GFP8624 to Human Epithelial Cells

The bacteria host-cell adhesion of acid stressed *E. coli* O157:H7 GFP8624 subjected to Acid Treatment 2 was also determined by plate counts and is shown in Figure 3.2.6. Bacteria were adapted at 37°C and 5% CO₂ in either DMEM pH 5.0 (MES) or pH 7.0 (unbuffered) and acid shocking was done at pH 3.0 (unbuffered) at room temperature. According to Kanetani (2005), the inclusion of GFP in *E. coli* O157:H7 8624 did not affect the host-adhesion capabilities of GFP8624 compared to the parental 8624 strain. Acid adapted and acid shocked bacteria for 15 and 30 minutes showed

significantly enhanced adhesion to HEp-2 cells compared to the unadapted, unshocked control (236% and 256% respectively, $p \leq 0.05$). These treatments also showed significantly higher adhesion by a factor of 2.1-2.3 than acid adapted-unshocked cells ($p \leq 0.05$).

Figure 3.2.6: Acid Stressed *E. coli* O157:H7 GFP8624 Adhesion to HEp2 Cells (Acid Treatment 2)

Effect of acid adaptation plus acid shock of *E. coli* O157:H7 GFP8624 on adhesion to HEp-2 cells by plate count assay ($n=6$ for each treatment except for AU, $n=4$) and the values are means $\pm$ standard deviations. Acid Shocked Treatment 2: 25 mM MES and adaptation at 37 °C and 5% CO₂. [UU: Control (Unadapted, unshocked); AU: Acid adapted, unshocked, AA15, AA30: Acid adapted and acid shocked for 15 and 30 minutes respectively.]

** Significantly different from control (Unadapted, unshocked) and Acid adapted, unshocked (AU) ($p \leq 0.05$)

Confocal microscopy was used to qualitatively observe this enhanced adhesion after acid stress. The images of host-cell adhesion after 4-hour and 6-hour incubations are shown in Figure 3.2.7. It appears that acid stress enhances the host adhesion capability of *E. coli* O157:H7 GFP8624 and that a longer incubation time will also result in higher adhesion.

Figure 3.2.7: Qualitative Acid Stressed *E. coli* O157:H7 GFP8624 Adhesion to HEp2 Cells (Acid Treatment 2)

Effect of acid adaptation plus acid shock (Acid Treatment 2) of *E. coli* O157:H7 GFP8624 on adhesion to HEp-2 cells using Confocal microscopy. **A: E:** HEp-2 cells only; **B:** Unadapted unshocked control (4 hour incubation); **C:** Acid adapted-acid shocked for 30 minutes (4 hour incubation); **D:** Unadapted-unshocked control (6 hour incubation); **E:** Acid adapted-acid shocked for 30 minutes (6 hour incubation)

3.2.4: Acid Stressed *Escherichia coli* HB101 Adhesion to Human Epithelial Cells

The effect of acid stress on the host adhesion capability of a nonpathogenic *E. coli* strain (HB101) was examined. *E. coli* O157:H7 85170 was used as a reference strain. Using Acid Treatment 3, the normalized 3-hour adhesion to HEp-2 cells was expressed as a percentage of viable bacteria bound compared to the reference control (85170 unadapted-unshocked) with this control representing 100%. Results presented in Figure 3.2.8 show that both unstressed and acid stressed HB101 treatments adhered to host cells significantly lower than the unstressed reference strain 85170, 6.4% and 0.8% respectively, $p \leq 0.05$). However, the adhesion to HEp-2 cells by the acid adapted-acid shocked for 30 minutes HB101 cells adhered significantly lower than the unadapted unshocked HB101 control (by a factor of 7.8, $p \leq 0.05$).

Figure 3.2.8: Acid Stressed Adhesion of Pathogenic and Nonpathogenic *E. coli* to HEp-2 Cells (Acid Treatment 3)

Effect of acid adaptation plus acid shock of *E. coli* O157:H7 85170, HB101 on adhesion to HEp-2 cells was assessed by plate count assay ($n \geq 2$) and the values are means $\pm$ standard deviations. Acid Shocked Treatment 3: 25 mM MES and MOPS and adaptation at 37 °C and 5% CO₂. [UU30: Control (Unadapted, unshocked for 30 minutes); AA30: Acid adapted and acid shocked for 30 minutes]

* Significantly different from 85170 control UU30 ($p \leq 0.05$)

** Significantly different from 85170 control UU30 and 85170 AA30 ($p \leq 0.05$)

*** Significantly different from 85170 control UU30, 85170 AA30 and HB101 UU30 ($p \leq 0.05$)

Inset: Adhesion of Unshocked and Acid Shocked HB101

3.2.5: The Effect of Mannose on Acid Stressed *E. coli* O157:H7 85170 Adhesion to Human Epithelial Cells

To establish whether the enhanced adhesion to host epithelial cells seen with acid stressed *E. coli* O157:H7 is mediated by type 1 fimbriae, a mannose control adhesion assay was completed. α -D-Mannose is the primary bladder cell receptor for uropathogenic *E. coli* and mannose sensitive binding of FimH, an adhesin found at the tip of type 1 pili, requires a highly conserved FimH pocket (Hung *et al.*, 2002). Like uropathogenic *E. coli*, EHEC possess all the genes required for the type 1 pilus structure, and incubation of EHEC with α -D-Mannose will result in the binding of the sugar to the FimH pocket of the pili, which will therefore inhibit any bacterial adhesion due to type 1 fimbriae (Hung *et al.*, 2002). In this study, *E. coli* O157:H7 85170 were acid stressed using Acid Treatment 3 and incubated with HEp2 cells in the presence and absence of 1% mannose and the results are shown in Figure 3.2.9. Significant differences between similar experimental acid stress treatments in the presence and absence of 1% mannose were not seen ($p \geq 0.05$).

Figure 3.2.9: The Effect of Mannose on the Adhesion of Acid Stressed *E. coli* O157:H7 85170 to HEp-2 Cells (Acid Treatment 3)

Effect of 1% mannose on acid adaptation plus acid shock and acid shock alone of *E. coli* 85170 on adhesion to HEp-2 cells was assessed by plate count assay (n= 3). The results of 2 independent experiments are shown and the values are means $\pm$ standard deviations. Acid Shocked Treatment 3: 25 mM MES and MOPS used and adaptation at 37 °C and 5% CO₂. [UU30 Control (Unadapted, unshocked for thirty minutes); UA30: Unadapted, shocked for thirty minutes; AA30: Acid adapted and acid shocked for thirty minutes]

⁺Significantly different from UA30 (without mannose) (p $\leq$ 0.05)

^{*}Significantly different from UA30 (with mannose) (p $\leq$ 0.05)

3.2.6: The Effect of Erythromycin on Acid Stressed *E. coli* O157:H7 85170 Adhesion to Human Epithelial Cells

Using Acid Treatment 1, the host adhesion capability of *E. coli* O157:H7 85170 after acid stress exposure to HEp2 cells was examined in the presence of erythromycin. This antibiotic, below the minimal inhibitory concentration, inhibits protein synthesis (Huesca *et al.*, 1996). Erythromycin has been used to inhibit bacterial protein synthesis during acid shock treatment (De Jesus *et al.*, 2005). Bacteria were adapted at room temperature in either unbuffered DMEM pH 5.0 or pH 7.0 and acid shocked treatments were carried out at pH 3.0 (unbuffered) also at room temperature. Following stress, all treatments were incubated at room temperature for 30 minutes with or without 0.06

mg/mL erythromycin. Pre-treatment with erythromycin resulted in decreased adhesion for both acid adapted, acid shocked for 15 and 30 minutes cells, with a highly significant decrease for bacteria that were acid adapted acid shocked for 30 minutes (>2 fold decrease, $p \leq 0.05$). These results are shown in Figure 3.2.10.

Figure 3.2.10: The Effect of Erythromycin on the Adhesion of Acid Stressed *E. coli* O157:H7 85170 to HEp-2 Cells (Acid Treatment 1)

The effect of erythromycin on acid adaptation plus acid shock of *E. coli* O157:H7 85170 adhesion to HEp-2 cells was assessed by plate count assay ($n = 6$, except for UU and AA15 with erythromycin, $n = 4, n = 2$ respectively) and the values are means $\pm$ standard deviations. Acid Shocked Treatment 1: No buffering of all media and adaptation at room temperature.

[UU: Control (Unadapted, unshocked); AA15, AA30: Acid adapted and acid shocked for 15 and 30 minutes respectively.]

* Significantly different from control UU (Unadapted, unshocked), and AA15 (Acid adapted and acid shocked for 15 minutes) with erythromycin ($p \leq 0.05$)

** Significantly different from control UU without erythromycin (Unadapted, unshocked), AA15 without erythromycin (Acid adapted and acid shocked for 15 minutes), and AA30 with erythromycin (acid adapted and acid shocked for 30 minutes) ($p \leq 0.05$)

3.3: Change in Media pH During Acid Stress of *Escherichia coli* O157:H7 85170.

To investigate the differences seen in the host adhesion capability of acid stressed *E. coli* O157:H7 85170 to HEP-2 cells using different acid treatment protocols, the effects of buffering agents and adaptation temperatures on media pH was examined. *E. coli* O157:H7 85170 was subjected to each of the two Acid Treatment protocols and following the adaptation stage, shocking stage, and final neutralization stage, the media supernatants were sterile filtered and the pH was measured. The increase or decrease in pH from the initial pH for Acid Treatment 1 (all media unbuffered and adaptation at room temperature), and Acid Treatment 3 (DMEM pH 5.0 buffered with 25 mM MES, DMEM pH 7.4 buffered with 25 mM MOPS and adaptation carried out at 37°C and 5% CO₂) for all experimental treatments are shown in Figures 3.3.1, and 3.3.2, respectively.

For Acid Treatment 1, all treatments at every stage (after adaptation, shocking, and neutralization) show small differences of ± 0.2 pH units except for the supernatants after adaptation at room temperature for AA15 and AA30 treatments (acid adapted and acid shocked for 15 and 30 minutes). A decrease of -0.74 to -0.76 pH units in unbuffered media initially at pH 5.0 resulted after acid adaptation for these treatments. In the presence of 25 mM MES (Acid Treatment 3) and adaptation at 37°C and 5% CO₂, this extreme pH decrease was not observed.

The media pH changes after every step (after adaptation, shocking, and neutralization) for all experimental treatments subjected to the buffering conditions of Acid Treatment 3 (25 mM MES, 25 mM MOPS) varied only by ± 0.21 pH units.

Figure 3.3.1: Change in Media pH During Acid Stress of *E. coli* O157:H7 85170: Acid Stress Treatment 1

Change in media pH during Acid stress of *E. coli* O157:H7 85170 using Acid Stress Treatment 1: DMEM without any buffers and adaptation at room temperature. The supernatants after adaptation, acid shocking and ½ hour after neutralization were sterile filtered and measured with a pH meter. Initial adaptation pH for acid adapted and unadapted cells was pH 5.0 and 7.0, respectively. Initial acid shocking pH for acid shocked and unshocked cells was pH 3.0 and 7.0, respectively. The final neutralization pH for all treatments was 7.0. [UU15: Control (Unadapted, unshocked for fifteen minutes); UA15: Unadapted, shocked for fifteen minutes; AA15: Acid adapted and acid shocked for fifteen minutes; UU30 Control (Unadapted, unshocked for thirty minutes); UA30: Unadapted, shocked for thirty minutes; AA30: Acid adapted and acid shocked for thirty minutes]

Figure 3.3.2: Change in Media pH During Acid Stress of *E. coli* O157:H7 85170: Acid Stress Treatment 3

Change in media pH during Acid stress of *E. coli* O157:H7 85170 using Acid Stress Treatment 3: DMEM solutions at pH 7.4 were supplemented with 25 mM MOPS. For acid adaptation DMEM was supplemented with 25 mM MES and adaptation was carried out at 37°C and 5% CO₂. The supernatants after adaptation, acid shocking and ½ hour after neutralization were sterile filtered and measured with a pH meter. Initial adaptation pH for acid adapted and unadapted cells was pH 5.0 (MES) and 7.4 (MOPS), respectively. Initial acid shocking pH for acid shocked and unshocked cells was pH 3.0 and 7.4 (MOPS), respectively. The final neutralization pH for all treatments was 7.4 (MOPS). [UU15: Control (Unadapted, unshocked for fifteen minutes); UA15: Unadapted, shocked for fifteen minutes; AA15: Acid adapted and acid shocked for fifteen minutes; UU30 Control (Unadapted, unshocked for thirty minutes); UA30: Unadapted, shocked for thirty minutes; AA30: Acid adapted and acid shocked for thirty minutes]

3.4: Real Time RT-PCR Analysis of Acid Stressed *Escherichia coli* O157:H7 85170 Virulence Factors

The transcriptional levels of known virulence factors, intimin and EspA, were examined after acid stress by real time RT-PCR. These two virulence factors were chosen since intimin is the primary mediator of host-cell attachment and EspA as the translocon, has also been known to mediate initial transient cell attachment. Reductions in host cell attachment/adhesion have been reported for EspA mutants and decreased Hep2 cell adherence of *E. coli* O157:H7 (strain 8624) incubated with anti-intimin has also been documented (Beltrametti *et al.*, 1999; Gansheroff *et al.*, 1999). In the current study, *E. coli* O157:H7 was acid stressed using Acid Treatment 4, and total RNA was extracted. The integrity and concentration for each RNA sample was determined by agarose gel electrophoresis and spectrophotometric measurements respectively (data not shown).

Total isolated RNA was DNase treated and assessed once again for integrity and concentration (data not shown). Using a 1st Strand cDNA Synthesis kit, the cDNA for each sample was synthesized. Prior to RT-PCR assays, several optimization procedures were carried out. Gradient PCR was completed to ensure that the correct gene/amplicon was amplified and to also determine the best annealing (T_A) and melting (T_M) temperatures. Standard curves using genomic DNA were constructed for each target and reference gene to determine PCR efficiency. Table 3.3 summarizes these optimization experiments.

Table 3.3: Real Time PCR Optimization Results

<i>Gene</i>	T_A (°C)	T_M (°C)	<i>Std. Curve Efficiency</i>
<i>gapA</i> (gapA)	59	69	1.982
<i>eae</i> (Intimin)	59	69	1.980
<i>espA</i> (EspA)	56	66	1.978

One important criterion in relative quantification calculations is amplification efficiency of the PCR reaction. Initial calculations assumed the amplification efficiency of the PCR reaction to be ideal or 1, thus implying that the PCR product concentration doubles during every cycle within the exponential phase (Wong and Medrano, 2005). However, this assumption may not always hold true since many PCRs do not have ideal amplification efficiencies and an overestimation of starting concentrations can occur if an appropriate correction factor is not used. Amplification efficiencies are calculated from PCR standard curves and in LightCycler quantification, the amplification efficiencies of the target and reference/housekeeping genes should be approximately equal and close to the ideal amplification efficiency of 2. The amplification efficiencies determined from the standard curves in this study for *gapA*, *espA*, and *eae*, were 1.982, 1.978, and 1.980 respectively. These amplification efficiencies were approximately equal and close to 2.0.

In relative mRNA quantification, housekeeping genes are used as reference genes to correct for sample-to-sample variations which can result from differences in: (1) initial

sample amount, (2) nucleic acid recover, (3) RNA integrity and quality, (3) cDNA efficiencies, and (4) loading/pipetting errors (Wong and Medrano, 2005). In order to achieve relevant results, it is necessary that the chosen reference/standards be tailored to the specific experiment because various experimental treatments and physiological conditions and different stages in cell cycle can affect even the expression of ubiquitous transcripts and housekeeping genes (Reue, 1998).

To validate that the chosen housekeeping gene, *gapA*, was suitable for these acid stress studies, a modified method outlined in the Roche Applied Science Technical Note No LC15/2002 was used. The *gapA* Cp values from five different cDNA pools were obtained and compared across all experimental treatments and these results are shown in Figure 3.4.1. Results show that acid stress did not significantly change the *gapA* expression level for these treatments ($p \geq 0.05$). In recent *E. coli* O157:H7 microarray and relative gene quantification RT-PCR studies, *gapA* was used as a housekeeping gene (Noller *et al.*, 2003; Fitzmaurice *et al.*, 2004). In addition, Maurer *et al.* (2005) have shown that *gapA* expressions of logarithmic *E. coli* K-12 subjected to either pH 5 or pH 7 showed no significant differences.

Figure 3.4.1: Acid Stressed *E. coli* O157:H7 *gapA* Expression Level

Validation of *gapA* as a housekeeping gene for acid stressed *E. coli* 85170. These results are from 5 independent experiments and the values shown are means $\pm$ standard deviations. [UU15: Control (Unadapted, unshocked for fifteen minutes); UA15: Unadapted, shocked for fifteen minutes; AA15: Acid adapted and acid shocked for fifteen minutes; UU30 Control (Unadapted, unshocked for thirty minutes); UA30: Unadapted, shocked for thirty minutes; AA30: Acid adapted and acid shocked for thirty minutes].

No significant differences, $p \geq 0.05$.

To ensure the integrity of every step in a real-time RT-PCR analysis several controls such as the “No RT” and “No template” controls are used. The use of minus reverse transcription control in the real-time PCR run can allow for detection of any DNA contamination in the samples which can affect the overall results. To ensure that all cDNA samples were free from contamination, RT-PCR runs for all undiluted cDNA negative controls (“No RT controls”) were completed using *gapA* primers. The majority of samples did not contain any contamination, only 2 samples did (data not shown). However, for the 2 samples that did test positive for contamination, the contamination was deemed insignificant since the samples were undiluted. If these samples were subjected to the same dilution as the “template positive” samples, the contamination would not be evident and thus would not have a detrimental effect on the validity of the results.

Real-time PCR was carried out for all experimental samples using Quantitect SYBR Green PCR kit (Qiagen) as previously described (Korithoski *et al.*, 2005). The crossing points (Cps) obtained for the target and reference genes, along with the standard curve PCR efficiencies were used in the LightCycler Relative Quantification Software (v. 4.0)/ Pfaffl mathematical model for gene normalization and relative quantification.

Relative expression ratios of acid stressed *E. coli* 85170 virulence genes were first subjected to parametric statistical analysis and are reported in Figure 3.4.2. Using these statistics, both intimin and EspA normalized gene expression ratios for unadapted and acid shocked for 30 minutes treatments were significantly higher than the control, 2.14 and 1.61 respectively ($p \leq 0.05$). Also, the EspA gene ratio was significantly decreased by a factor of 2.6 in comparison to the control for cells that were acid adapted and subjected to a 15-minute acid shock ($p \leq 0.05$).

A recent Excel based software tool for RT-PCR relative expression was designed by Pfaffl *et al.* (2002). This software tool, named Relative Expression Software Tool or REST© and its derivatives incorporate the Pfaffl mathematical model for gene normalization. REST© - MCS Software (Relative Expression Software Tool – multiple condition solver) allows for up to 10 target genes to be analyzed with 10 replications per group and also provides analysis for multiple experimental conditions. All REST© analyses incorporate a statistical model, which allows for Pair Wise Fixed Reallocation Randomization Tests©. The rationale for this test stems from the assumptions made by parametric tests such as analysis of variance and t-tests, which are dependent on normality of distributions and are most likely not suitable for relative gene quantification since ratios and variances can be high and normal distributions are unlikely. According to Pfaffl *et al.* (2002), permutations and randomization tests are more suitable for these analyses since distributional assumptions about the data are avoided and as a result these tests are believed to provide better indications of a treatment effect than what is in fact observed. Many other studies on bacterial gene expression have incorporated this statistical tool to analyze RT-PCR results (Bron *et al.*, 2004; Kolayli *et al.*, 2004; Nielson and Bove, 2005; Van Schaiket *et al.*, 2005; Xue *et al.*, 2005).

Relative Expression ratios of acid stressed *E. coli* 85170 virulence genes normalized to *gapA* and to the relative unadapted unshocked controls were determined using REST© - MCS Software (Relative Expression Software Tool – multiple condition solver). The results are shown in Table 3.4. According to the pair wise fixed reallocation randomization tests used in this software, both the intimin and EspA gene expression ratios were significantly increased in the unadapted, acid shocked for 30 minutes treatment (UA30) by a factor of 2.143 and 1.606, respectively. The EspA gene expression ratio was also upregulated by a factor of 1.675 in the unadapted, acid shocked for 15 minutes (UA15) treatments.

Both the parametric and nonparametric statistical tests showed that the relative ratios of EspA and intimin for cells that were subjected to acid shock only for 30 minutes were significantly higher relative to the control ($p \leq 0.05$). However, only the nonparametric tests found a significant increase in EspA levels in comparison to the control for cells that were unadapted and acid shocked for 15 minutes ($p \leq 0.05$). Also, this randomization test did not find a significant decrease compared to the control for EspA ratios in the acid adapted and acid shocked for 15 minutes treatment, whereas the parametric analysis did ($p \leq 0.05$).

Figure 3.4.2: Relative Expression Ratios of Acid Stressed *E. coli* O157:H7 Virulence Factors

Relative expression ratios of acid stressed *E. coli* 85170 virulence genes normalized to *gapA* and to the relative unadapted unshocked controls. These results are from a minimum of 3 independent experiments (for intimin N=3, EspA N=5) and the results were obtained using the Pfaffl mathematical model for gene normalization and relative quantification. Statistical analysis was done using parametric t-tests. [UU30 control (Unadapted, unshocked for 30 minutes) for: UA30: Unadapted, acid shocked for 30 minutes and AA30: Acid adapted, acid shocked for 30 minutes; UU15 control (Unadapted, unshocked for 15 minutes) for: UA15: Unadapted, acid shocked for 15 minutes and AA15: Acid adapted, acid shocked for 15 minutes.

* Statistically significant result (compared to control), $p \leq 0.05$

Table 3.4: Relative Expression Ratios of Acid Stressed *E. coli* O157:H7 Virulence Factors (Non-parametric Analysis)

Experimental Treatment	Intimin	EspA
UA15 – Unadapted, acid shocked for 15 minutes	0.514 (0.484)	1.675 (0.001)*
AA15 – Acid adapted, acid shocked for 15 minutes	0.574 (0.492)	0.368 (0.509)
UA30 – Unadapted, acid shocked for 30 minutes	2.143 (0.001)*	1.606 (0.001)*
AA30 – Acid adapted, acid shocked for 30 minutes	1.417 (0.492)	0.753 (0.509)

Relative expression ratios of acid stressed *E. coli* 85170 virulence genes normalized to *gapA* and to the relative unadapted unshocked controls. (p values are indicated in brackets). These results are from a minimum of 3 independent experiments (for intimin N=3, EspA N=5) and the results were obtained using REST© - MCS Software (Relative Expression Software Tool – multiple condition solver).

* Statistically significant result (compared to control) according to software pair wise randomization tests, $p \leq 0.05$.

3.5: Verotoxin Production of Acid Stressed *Escherichia coli* O157:H7 8624

It has been suggested by Karmali *et al.*, (1985) that the flow of VT from the periplasmic space into the culture supernatant is most likely due to a “leaky” outer membrane. It has also been recently reported that acid stress affects membrane fluidity (Yuk and Marshall, 2004). This study looked at the effect of acid stress on both the verotoxin secreted and retained in the periplasmic space of *E. coli* O157:H7 8624 using the vero cell cytotoxicity assay.

Verotoxin production has been reported to be strain variable and strains have been categorized as either trace-, low- to moderate-, and high-level toxin producers, based on detectable toxin titers in the culture supernatant after overnight incubation (Karmali *et al.*, 1985). Given the possibility that strain 8624 was a high VT producer, dilutions of both the filtrates and extracts were completed to ensure that any significant difference in undiluted verotoxin production/secretion due to acid stress could be determined.

In this study, the bacteria, subjected to Acid Treatment 3, were normalized based on prior viability experiments and grown overnight in rich media to induce verotoxin production. Various dilutions of the control standard VT1, supernatant filtrates, and periplasmic extracts were incubated with semi-confluent monolayers of vero cells for 2-3 days at 37°C and 5% CO₂. The remaining live cells were fixed with 2% formalin and stained with crystal violet. The absorbance of crystal violet was read with an ELISA plate reader and the graphical representation of these results are found in Figures 3.5.1, 3.5.2 and 3.5.3 corresponding to the positive control (standard VT1), verotoxin secreted in the supernatant, and verotoxin retained in the periplasmic space respectively.

The shape of the VT1 standard curve in Figure 3.5.1 is similar to cytotoxicity curves obtained by Gentry and Dalrymple (1980). The CD₅₀ (dose in ng/mL to cause death in 50% of cells) was calculated to be 5.3×10^{-4} ng/mL. Hoey *et al.*, (2003) reported a similar vero cell CD₅₀ for VT1, 2.7×10^{-4} ng/mL and the differences in these CD₅₀ values are likely due to experimental conditions since this value is dependent on cell

concentration, and the incubation time of the toxin with cells (Gentry and Dalrymple, 1980). None of the acid stress treatments showed a significant change in verotoxin production either in the periplasmic extracts or in the supernatant filtrates compared to the unadapted, unshocked controls ($p \geq 0.05$). To confirm that these experimental results were not the product of significantly different initial bacteria inoculums, the initial and final CFU/mL for each treatment prior and after overnight growth were measured, and these results are shown in Figure 3.5.4.

Figure 3.5.1: Vero Cell Cytotoxicity Standard Curve: Absorbance vs. Log [VT1] (ng/mL)

Standard VT1 vero cell cytotoxicity curve. Results are expressed as the mean absorbance $\pm$ standard deviations (corresponding to the remaining live vero cells) vs. the log concentration of VT1 (ng/mL) and are representative of 3 independent experiments with $n=6$ replicates for each dilution except for the most concentrated standard (1.1×10^{-1} ng/mL).

Figure 3.5.2: Secreted Verotoxin Produced by Acid Stressed of *E. coli* O157:H7 8624

A

B

The effect of acid stress (Acid Treatment 3) on the verotoxin secreted in the supernatant by *E. coli* O157:H7 8624 was measured by the vero cell cytotoxicity assay. Results are expressed as the mean absorbance $\pm$ standard deviations (corresponding to the remaining live vero cells) vs. 1/dilution of verotoxin in the supernatant and are representative of 3 independent experiments. **A:** Experimental treatments: UU15-control: unadapted, unshocked for 15 minutes; UA15: Unadapted, acid shocked for 15 minutes; and AA15: Acid adapted, acid shocked for 15 minutes. **B:** Experimental treatments: UU30-control: unadapted, unshocked for 30 minutes; UA30: Unadapted, acid shocked for 30 minutes; and AA30: Acid adapted, acid shocked for 30 minutes.

Figure 3.5.3: Periplasmic Verotoxin Production of Acid Stressed of *E. coli* O157:H7 8624

A

B

The effect of acid stress (Acid Treatment 3) on the verotoxin produced in the periplasmic space by *E. coli* O157:H7 8624 was measured by the vero cell cytotoxicity assay. Results are expressed as the mean absorbance $\pm$ standard deviations (corresponding to the remaining live vero cells) vs. 1/dilution of verotoxin in the periplasmic extract and are representative of 3 independent experiments. **A:** Experimental treatments: UU15-control: unadapted, unshocked for 15 minutes; UA15: Unadapted, acid shocked for 15 minutes; and AA15: Acid adapted, acid shocked for 15 minutes. **B:** Experimental treatments: UU30-control: unadapted, unshocked for 30 minutes; UA30: Unadapted, acid shocked for 30 minutes; and AA30: Acid adapted, acid shocked for 30 minutes.

Figure 3.5.4: Initial and Final Bacteria Concentrations of Acid Stressed of *E. coli* O157:H7 8624 during Verotoxin Production

The initial and final bacteria concentrations of acid stressed (Acid Treatment 3) *E. coli* O157:H7 8624 were measured either by plate count or OD600 to confirm that the initial inoculums and growth during verotoxin production were the same across all treatments. After acid stress, a plate count assay (n=2) was completed to determine the initial CFU/ml for each treatment. Following overnight growth, the OD600 of each treatment was taken and converted to a final CFU/mL using a standard curve previously completed. OD 600 results are from 2 independent experiments and the results are the means $\pm$ standard deviations.

For both final and initial bacteria concentrations, $p \geq 0.05$.

4.0 Discussion

4.1: Effect of Acid Stress on the Percent Survival of *E. coli* O157:H7 (as assessed by culturability)

The ability to withstand stress exposure is variable and is dependent on many factors including the type and severity of the stress, prior adaptation or habituation, the growth phase of the organism, and the inherent ability of different strains to survive the encountered stress. In addition to these factors, various experimental conditions and methodologies such as the growth media, temperature, pH, and length of the challenge and or adaptation, make it difficult to compare the acid stress responses across similar strains and species.

In this study, regardless of the *E. coli* O157:H7 strain and acid treatments used, acid adaptation of mid log phase *E. coli* O157:H7 for 1 hour at pH 5.0 followed by a 5, 15 or 30 minute acid shock at pH 3.0 in minimal media DMEM, which contained L-glutamate and high glucose, resulted in significantly lower percent survivals compared to unstressed controls, by approximately 30%, 44-53%, and 42-62%, respectively ($p \leq 0.05$). Bhagwat (2003) reported a similar survival phenotype of 47.2% for *E. coli* O157:H7 grown to exponential phase in EG media pH 5.5 supplemented with glutamate, and subsequently acid shocked for 1 hour at pH 2.0.

The percent survival of unadapted, acid shocked strain 85170 was approximately 71-81% (71% for a 30 minute acid shock and 81% for a 15 minute acid shock) relative to the survival of the unstressed control. Although significantly lower than the unstressed controls, this survival was significantly higher than cells that were previously adapted by a factor of 1.4 - 1.5 ($p \leq 0.05$). Brudzinski and Harrison (1998) found that acid adaptation in some cases, provided protection to subsequent acid stress compared to unadapted cells, and that a wide range of acid tolerance phenotypes exists among different strains and *E. coli* isolates. In their studies, they reported that unadapted *E. coli* O157:H7 incubated statically at pH 3.5 at 32°C, survived better than those bacteria previously adapted at pH 5.0 in minimal media with glucose. Additionally, a recent study by Seputiene *et al.*, (2006) has shown that unadapted log phase *E. coli* K-12 subjected to

an acid challenge for 2 hours at pH 2.5 demonstrated significant acid resistance when grown to mid log phase in minimal media pH 7.0. Approximately 50-60% of the cells survived and it was observed that this AR response required the glutamate-dependent AR system. In the present study, this AR system was also induced by preinduction of the bacteria to mid log phase growth in DMEM containing high glucose and glutamate. Although the bacteria that were only acid shocked survived better than their acid adapted counterparts in this study, more research is needed to examine the effects of acid habituation under these conditions since only brief acid stress was examined. Perhaps prior acid adaptation may confer better survival when cells are exposed to pH 3.0 for longer than 30 minutes and this may be significant given that the average time in the stomach has been reported to be approximately 2 hours (Gorden and Small, 1993).

For the most part, acid adaptation or habituation of log phase *E. coli* confers better protection (up to 10, 000 fold better) to severe acid stress than unadapted cells and this has been reported by Seputiene *et al.*, (2006) and Paul and Hirshfield (2003), though these results were obtained with *E. coli* strains grown in rich media. However, Seputiene *et al.*, (2006) also investigated the effect of acid adaptation on the survival of log phase *E. coli* grown in minimal media. They demonstrated that the survival of acid adapted *E. coli* in minimal media at pH 4.5 for 1 hour and subsequently shocked for 2 hours at pH 2.5 was considerably improved after adaptation relative to the unadapted cells and a higher protection was seen when the media was supplemented with glutamate. Therefore, not only is the glutamate decarboxylase system induced in log phase *E. coli* cells during growth in minimal media at pH 7.0, but also under similar conditions during acid adaptation. Their results also provide evidence that the alternative RNA polymerase sigma factor, RpoS, is involved in acid protection of both adapted and unadapted cells in minimal media. RpoS is a regulatory factor involved in stress responses and is responsible for the transcriptional activation of various stress related genes, including catalase, during osmotic shock, heat shock, and acid stress (Jordan *et al.*, 1999; Bhagwat *et al.*, 2005).

Important biochemical reactions, transport systems and protein secretion are coordinated with cell membrane functions, which are dependent on membrane fluidity. The ratios of unsaturated and saturated fatty acids are known to affect membrane fluidity and the changes induced by acid adaptation are thought to protect cells from lethal pH (Brown *et al.*, 1997; Yuk and Marshall, 2004). Recently, Yuk and Marshall (2004) have found that acid adaptation of *E. coli* O157:H7 resulted in decreased membrane fluidity. This decrease in membrane fluidity may be affiliated with changes in proton flux and may confer better protection to acid adapted cells compared to non-adapted cells by hindering proton flow into the cell. These researchers also found that acid adaptation of an RpoS mutant strain resulted in increased membrane fluidity compared to the wild type parental strain, suggesting that RpoS may affect the regulation of genes involved in membrane fluidity. One such gene thought to play a role in membrane fluidity during acid stress is cyclopropane fatty acid (CFA) synthase. CFA synthase, regulated by RpoS, is thought to convert monounsaturated fatty acids to their cyclopropane derivatives during mild acid stress and confer enhanced survival to cells subjected to acid shock. However, there is some controversy surrounding the role cyclopropane fatty acid (CFA) synthase in acid habituated cells. In a study by Chang and Cronan (1999), the transcription of CFA synthase was reported to be enhanced during acid adaptation. Similarly, Brown *et al.* (1997) found that acid adapted *E. coli* cells had higher levels of CFAs relative to unadapted cells. On the other hand, Yuk and Marshall (2004) have recently reported that acid adaptation resulted in decreased levels of CFAs compared to the levels observed in cells grown at pH 7.3.

In this study, even though the reductions in viability (or percent survival) for acid stressed bacteria were deemed significant, cells that survived still represented a majority, a fact which is consistent with the low oral infectious dose seen with this serotype. Survival after acid stress involves complex phenotypical changes and is dependent on many environmental conditions and signals.

4.2: Effect of Acid Stress on *E. coli* O157:H7 Adhesion to Host Epithelial Cells

Like many bacteria, gastrointestinal bacteria including *E. coli* O157:H7, are faced with constant changing environments and are subjected to a variety of different stresses, including acid stress. In order for *E. coli* O157:H7 to colonize the large intestine and establish infection, the bacteria must first be able to breach the lethal pH of the stomach and subsequently survive the various environmental stresses associated with the small and large intestines. Only those organisms that survive will be able to cause disease. As such, many enteric pathogens have evolved several complex acid defense mechanisms, which are induced under different environmental conditions dependant on many factors such as growth phase, culture conditions, and the severity of stress encountered. These important stress responses of *E. coli* O157:H7 enhance virulence and correlate to the low infectious dose of approximately 100 organisms (De Jesus *et al.*, 2005).

Other gastrointestinal pathogens have been reported to show enhanced host-cell adhesion following exposure to acid stress. In a study by DeJesus *et al.* (2005), the host adhesion of EPEC was significantly enhanced by a factor of 1.5 to 2.7 after acid adaptation at pH 5 for 1 hour, followed by brief acid shock at pH 3. The observed enhanced adhesion was dependent on the different culture conditions employed and their studies also provided evidence that heat-shock protein HSP 70 plays a role in the enhanced adhesion of acid stressed EPEC. In addition, another study by Waligora *et al.* (1999) reported that *Clostridium difficile* host-cell attachment was increased by a factor of 8.3 following acid stress at pH 4 for 45 minutes in comparison to the adhesion that resulted under standard conditions.

Prior ingestion stress has been shown to play a role in host cell adhesion for several gastrointestinal pathogens including *Citrobacter rodentium*, *Vibrio cholerae*, and enterohemorrhagic *E. coli* (Bauer and Welch 1996; Merrell *et al.*, 2002; LaRocque *et al.*, 2005; Wiles *et al.*, 2005). Following passage through the host gastrointestinal tract, it has been suggested that exposure to various stresses encountered during infection, including acid stress, may render the pathogens more virulent by inducing a 'hyperinfectious'

phenotype. For example, a study by Bauer and Welch (1996) found that the host-cell adhesion of EHEC was higher for clinical isolates compared to isolates obtained from food. In another study by Wiles *et al.* (2005), *C. rodentium* was reported to have a lower oral ID following passage through the murine gastrointestinal tract, which influenced the tissue distribution of the colonizing bacteria. In addition, research by LaRocque *et al.*, (2005) has shown that the gastrointestinal pathogen, *V. cholerae* recovered from patient stools during the early stages of infection had a significant increase in *tcpA*, a major subunit of toxin-coregulated pilus (TCP) required for intestinal colonization.

In this study, regardless of the acid treatment protocols, *E. coli* O157:H7 strains, and the epithelial cell lines used, acid adaptation for 1 hour at pH 5.0 followed by a 30 minute acid shock at pH 3.0, resulted in significantly enhanced adhesion to host cells compared to the unstressed controls ($p \leq 0.05$). Even though the viabilities for these treatments were all significantly lower relative to the control cells ($p \leq 0.05$), *E. coli* O157:H7 strains subjected to this acid treatment were better able to adhere to host cells by a factor of 1.6 to 3.2. According to a study by Winsor *et al.*, (1992), EHEC exhibit strain-related differences in adherence to colonic epithelial studies and this may be one major factor that can account for the differences in the enhanced adhesion observed among the *E. coli* O157:H7 strains. In addition, Sherman *et al.*, (1987) also reported inherent strain differences with respect to adhesion to both HEp-2 and Intestinal 407 cells for clinical O157:H7 isolates. Of course the different acid treatment protocols and cell lines used may also play a role in the observed differences associated with the host-cell adhesion capabilities.

According to the non-normalized adhesion results, *E. coli* O157:H7 strain 85170 had the lowest percentage of viable bacteria bound to host cells after a 3-hour incubation (<2%), strain CL56 adhered moderately (1-3%), while strain GFP8624 showed the highest adherence levels (4-11%). These adhesion results are comparable to results obtained by Sherman *et al.*, (1987). These authors report that the adhesion to HEp-2 cells of several clinical O157:H7 isolates from the stools of children diagnosed with HUS, ranged from 0.2 to 7.4 % of the initial viable inoculums.

In this study, all of the strains used in the adhesion assays were LEE positive and capable of expressing intimin. There were differences in verotoxin gene expression; *E. coli* O157:H7 85170 is a verotoxin-negative strain, CL56 produces both VT1 and VT2, and GFP8624 is a VT2 positive strain. Interestingly, it has been suggested that a possible synergism exists between the expression of the adhesin intimin and Shiga toxin 2 (Boerlin *et al.*, 1999). Perhaps this synergism is one factor that may contribute to the differences among strain adhesion phenotypes and ultimately severe EHEC-induced diseases.

The results of this study also suggest that previously acid adapted cells subjected to lethal acid stress enhances host-cell adhesion and may be dependent on the length of acid shock exposure. Significantly enhanced adhesion to the Caco-2 cell line (by a factor of 1.2) was observed for acid adapted *E. coli* O157:H7 CL56 that were exposed to a 30-minute acid shock compared to those that were acid shocked for 15 minutes ($p \leq 0.05$). These acid adapted cells that were acid shocked for 15 minutes also showed significantly enhanced adhesion relative to the control cells, greater than 2 fold, ($p \leq 0.05$). In addition, *E. coli* O157:H7 85170 cells previously adapted and subjected to a brief 5 minute acid shock using Acid Treatment 1, resulted in a significant 1.5 fold increase in adhesion to HEp-2 cells compared to the unadapted, unshocked control, and cells that were acid shocked for an additional 25 minutes (acid adapted-acid shocked for 30 minutes) adhered better to the epithelial cells by a factor of 2.1 ($p \leq 0.05$).

The effect of host-cell incubation time was also observed to enhance host-cell adhesion of acid stressed *E. coli* O157:H7. A 6-hour acid adhesion assay of strain CL56 to Caco-2 cells resulted in similar enhanced adhesion trends for both the acid adapted-acid shocked for 15 and 30 minutes compared to the 3-hour adhesion treatments. However, the previously adapted cells subjected to either a 15 or 30-minute acid shock and incubated with host cells for 6 hours adhered significantly better by a factor of 1.4 than the corresponding 3-hour treatments ($p \leq 0.05$). Confocal microscopy of the host cell

adhesion of *E. coli* O157:H7 GFP8624 also demonstrated increased adherence after acid stress exposure and this adhesion increased with incubation time.

The effect of acid shock alone on the host adhesion capability of *E. coli* O157:H7 85170 subjected to Acid Treatment 1 was also examined. Under these conditions, unadapted cells subjected to a brief acid shock of either 15 or 30 minutes resulted in significantly decreased adhesion to HEp-2 cells by 4 fold despite ($p \leq 0.05$). Further experimentation is needed to examine the effect of acid shock alone on other *E. coli* O157:H7 strains and on the acid stress methodologies used. Interestingly, the different acid stress treatments used had a profound effect on the acid stressed adhesion of *E. coli* O157:H7 85170 to HEp-2 cells. Both acid adapted cells exposed to acid shock for either 15 or 30 minutes using Acid Treatment 3 resulted in a significant 2 to 6 fold decrease in adhesion compared to similar experimental treatments subjected to Acid Treatment 1 ($p \leq 0.05$). There are 2 major methodological differences between these two acid stress protocols. The first pertains to buffering agents; Acid Treatment 3 incorporated media buffering agents, DMEM pH 5.0 was buffered with 25 mM MES and DMEM pH 7.4 was buffered with 25 mM MOPS, whereas the DMEM used in Acid Treatment 1, both pH 5.0 and pH 7.0 were unbuffered. The second protocol difference surrounds the acid adaptation conditions; acid adaptation for Acid Treatment 3 was carried out at 37°C and 5% CO₂, whereas in Acid Treatment 1, this process was done at room temperature. It was hypothesized that in the absence of the buffers, the media pH would decrease and this increase in acid exposure perhaps accounted for the increased host cell adhesion observed. An experiment was devised to investigate the effect of buffering agents and adaptation conditions on media pH. Results show that in the absence of the MES buffer (Acid Treatment 1), a decrease of -0.74 to -0.76 pH units in unbuffered media initially at pH 5.0 resulted after acid adaptation. Minor fluctuations of ± 0.21 pH units were observed at every other stage in Acid Treatment 1 and in all stages for Acid Treatment 3.

According to Buchanan and Edelson (1996), supplementing the media with glucose allows for glucose fermentation, which results in media acidification. In the present study, the DMEM used contained high glucose, which satisfies this criterion.

However, during the brief 1 hour adaptation at room temperature for Acid Treatment 1, only cells exposed to DMEM pH 5.0 substantially decreased the media pH (-0.7 pH units), whereas the unadapted cells in DMEM pH 7.0 only resulted in minor decreases of -0.2 pH units. A recent study by Paul and Hirshfield (2003) has proposed that acid adaptation of *E. coli* between pH 4.0 and 6.0 does not result in a single flat protective stress response, and in fact, cells previously adapted at pH 4.3 rather than at pH 5.5 survived better when subsequently acid shocked at pH 3. According to these authors, pre-exposure of exponential phase *E. coli* to pH 5.5 and 4.3 resulted in the production of various acid shock proteins and depending on the adaptation pH, different acid shock proteins were produced which can confer differences in protection efficiency. Interestingly, *E. coli* O157:H7 strain 85170 when subjected to unbuffered DMEM initially at pH 5.0 and adapted for 1 hour, resulted in a -0.7 decrease in media pH to a final pH of 4.3, the most 'superior' adaptation pH for log phase *E. coli* according to Paul and Hirshfield (2003). These authors reported this acid protection for log phase *E. coli* occurred with acid pretreatment in rich media, and in this study, acid adaptation was carried out in minimal media. However, Lin *et al.*, (1995) previously reported that a toxigenic *E. coli* strain grown to mid log phase in minimal media supplemented with glucose was able to confer higher protection to cells shocked at pH 3.3 for 2, 4, and 6 hours if the cells were previously adapted at pH 4.3. If pH 4.3 is the 'superior adaptation pH', possible explanations as to why no apparent significant differences in viabilities were found between pre-adapted treatments using either Acid Stress Treatment 1 or 3 needs to be further explored. Perhaps the adaptation time and or shocking times in this study needed to be extended to observe this enhanced protection. Nevertheless, these preliminary results may suggest that the *E. coli* O157:H7 acid stress response may possibly be comparable to the biphasic ATR of *Salmonella typhimurium*. Perhaps several acid shock proteins are induced at two stages of adaptation, which may play an important role in host cell adhesion. Future research should consider this possibility.

Even though the majority of EHEC strains show mannose-resistant adherence to epithelial cells, it has been reported that mannose-sensitive pili in EHEC have been bound to both isolated rabbit colonocytes and isolated human intestinal epithelial cells

(Sherman *et al.*, 1987; Windsor *et al.*, 1992). The type 1 pili adhesin, FimH, in uropathogenic *E. coli* has been reported by Hung *et al.*, (2002) to have a highly conserved mannose binding FimH pocket. These researchers also found that three *E. coli* O157 strains, including *E. coli* O157:H7 EDL933, had a mutation in this mannose binding pocket at position 135 and according to the crystal structures of the mannose-FimH interactions, this mutation was predicted to exclude mannose from the binding pocket. In addition, Hung *et al.*, (2002) found that UPEC strains mutated at this site, abolished mannose sensitive binding to bladder cells. In this study, the acid stressed adherence of *E. coli* O157:H7 to HEp-2 cells was not affected by the addition of mannose, suggesting that surface structures and adhesins other than type 1 pili are responsible for the increased adhesion to host cells following acid adaptation and subsequent lethal acid shock exposure.

Given that the enhanced host cell adhesion of acid stressed *E. coli* O157:H7 could result from either upregulated adhesin expression and or modifications to existing adhesins, we sought to clarify the role of protein synthesis in the enhanced adhesion by erythromycin pretreatment. Erythromycin, one of the macrolides, used at concentrations below the minimal inhibitory concentration (MIC), can inhibit bacterial protein synthesis (Huesca *et al.*, 1996). This antibiotic has been used to prevent bacterial protein synthesis in acid stressed EPEC (DeJesus *et al.*, 2005). Acid adapted and acid shocked *E. coli* O157:H7 85170 pretreated with erythromycin below the MIC resulted in decreased adhesion to HEp-2 cells. A highly significant decrease in the host cell adhesion capability, greater than 2 fold, was seen for acid adapted cells subjected to a 30 minute acid shock ($p \leq 0.05$). The unadapted, unshocked control cells pretreated with erythromycin had no effect on adhesion compared the control cells not treated with erythromycin. These results suggest that the enhanced adhesion of acid stressed *E. coli* O157:H7 is likely to be the result of enhanced adhesin expression and or novel adhesin expression.

The effect of acid stress on host cell adhesion of a non-pathogenic *E. coli* HB101 was included in this study as a control. Both acid stressed and unstressed HB101

adhesion to HEp-2 cells was significantly decreased compared to an *E. coli* O157:H7 unstressed control by a factor of 15 and 125, respectively ($p \leq 0.05$). However, acid stress significantly decreased the adhesion of this nonpathogenic strain to host cells by a factor of 8 ($p \leq 0.05$). Conversely, a recent study by DeJesus *et al.*, (2005) reported that acid stressed HB101 resulted in significantly enhanced adhesion to HEp-2 cells. Differences in acid stress treatment protocols may be a factor for these discrepancies. The HB101 acid treatment protocol used by DeJesus *et al.*, (2005) is similar to Acid Treatment 1 used to acid stress *E. coli* O157:H7 85170 in this study. This treatment included unbuffered DMEM pH 5.0, and pH 7.0, and the acid adaptation step, was completed at room temperature. In the present study, HB101 were acid stressed using Acid Treatment 3, which incorporated buffered DMEM pH 5.0 (MES) and pH 7.4 (MOPS) and the adaptation step was carried out at 37°C and 5% CO₂. Results of this study have shown that *E. coli* O157:H7 85170 subjected to Acid Treatment 1, have significantly enhanced host-cell adhesion capabilities relative to bacteria subjected to Acid Treatment 3. As previously outlined, this increased adherence may be a result of lower acid adaptation pH due to glucose fermentation in the media without buffers and this may be a possible reason why DeJesus *et al.*, (2005) observed highly enhanced adhesion with HB101 that were acid adapted and acid shocked using this acid treatment protocol. Regardless, acid stressed and unstressed pathogenic *E. coli* strains, both *E. coli* O157:H7 (in this study) and EPEC adhere to host epithelial cells significantly better than the nonpathogenic HB101 strain, indicating that mechanisms of enhanced adhesion correlate to their pathogenesis (DeJesus *et al.*, 2005).

To date, the intestinal colonization process of *E. coli* O157:H7 is poorly understood. Intimin is the only well documented adhesin associated with intimate adherence to host cells *in vivo*. The ability of EHEC to adhere to host cells and subsequently colonize the large intestine is a key virulence factor and a requirement for the production of diarrhea. Several proteins have been implicated as novel EHEC adhesion factors, both fimbrial and non-fimbrial including long polar fimbriae 2, outer membrane protein A (OmpA), Iha, and Efa-1 (EHEC factor for adherence) (Torres *et al.*, 2005). In addition, EspA filaments are also important adhesion factors (Garmendia *et al.*, 2005). The enhanced host cell

adhesion observed with acid stressed *E. coli* O157:H7 may possibly result from the upregulation of one of these adhesion factors. In the next section, two of these virulence factors, intimin and EspA, were examined under unshocked and acid shocked treatments.

4.3: Real Time RT-PCR Analysis of Acid Stressed *Escherichia coli* O157:H7 85170 Virulence Factors

Since acid stressed *E. coli* O157:H7 85170 (Acid Treatment 3) resulted in significantly different adhesion to host cells depending on treatment, real time RT-PCR relative quantification was completed to investigate the gene expression levels of 2 known virulence factors, intimin and EspA.

Cellular behaviour pertaining to survival, growth and differentiation are often mirrored in changing patterns of gene expression and the ability to quantitate transcription levels of specific genes is key to any research into gene function (Bustin, 2000). Reverse-transcription polymerase chain reaction (RT-PCR) remains the technique of choice to quantitate rare mRNA targets and detect mRNA in a small number of cells (Bustin, 2002). The application in a “real-time” setting has made this technique the most popular technique for mRNA detection and quantification and it is the preferred method of choice for validating microarray analyses (Bustin and Nolan, 2004). However, real-time RT-PCR is very complex and is fraught with many problems associated with its true sensitivity, reproducibility and specificity. In order to obtain biologically relevant results, careful assay design and optimization procedures are required.

Prior to obtaining any relevant RT-PCR results, several optimization procedures were completed. RNA integrity was assessed by agarose gel electrophoresis and spectrophotometric analysis at every stage of the isolation and was found to be intact and pure. The results of gradient PCR ensured that the primers used did in fact amplify the correct gene/amplicon and also provided the best annealing (T_A) and melting (T_m) temperatures used in the RT-PCR assay. The housekeeping gene *gapA* was chosen and tested across every experimental condition and according to the results, *gapA* was found to be a suitable housekeeping gene since there were no significant differences observed

across treatments ($p \geq 0.05$). This is consistent with a recent report, which indicated no change in *E. coli* K-12 *gapA* expression level after acid stress (Maurer *et al.*, 2005). The amplification efficiencies determined from the standard curves for *gapA*, *espA*, and *eae*, were 1.982, 1.978, and 1.980 respectively. These amplification efficiencies were approximately equal and close to 2.0, a requirement for LightCycler relative gene quantification. Finally, the majority of cDNA samples were free from DNA contamination, and for the 2 samples were contaminated, the contamination was deemed insignificant in the undiluted cDNA pool.

According to the real time RT-PCR results using both parametric and nonparametric statistical analyses, bacteria that were unadapted and subjected to a 30-minute acid shock had significantly higher normalized EspA and intimin expression levels ($p \leq 0.05$). For this treatment, the mRNA levels for intimin (*eae*) and EspA (*espA*) were upregulated by a factor of 2.143 and 1.606 respectively. The nonparametric statistical analysis, which is thought to be more suitable for these analyses, also revealed statistically valid upregulation for EspA in the unadapted and shocked for 15 minutes treatment ($p \leq 0.05$). Interestingly, for both of these treatments, the bacteria host adhesion capabilities were significantly decreased by a factor of 2 to 6 ($p \leq 0.05$).

Like other mRNA quantification techniques, it is important to bear in mind that real-time RT-PCR quantitates steady mRNA levels and no more. Not all signaling changes occur at the RNA level and accurate mRNA quantification may not truly reflect the levels of protein produced due to any posttranscriptional and posttranslational regulation (Bustin, 2002).

Several recent studies have proposed that LEE-encoded virulence factors (including intimin and EspA) may be regulated at the posttranscriptional level (Roe *et al.*, 2003; Stevens *et al.*, 2004; Deng *et al.*, 2005). In one study, Deng *et al.*, (2005) observed differences in translocator and effector secretion in growth media in the presence and absence of calcium for several A/E pathogens, including EHEC, EPEC and CR (*Citrobacter rodentium*). However, these researchers suggest that these proteins are

regulated at the posttranscriptional level since no significant differences in the expression of *tir*, *espA* and *espB* by conventional RT-PCR was observed for these bacterial pathogens. In another study, Stevens *et al.*, (2004) found that *E. coli* O157:H7 *toxB* and *efa-1* mutants exhibited decreased adherence to host epithelial cells and had lower expression and secretion levels of LEE-encoded type III proteins. However, they report that the promoter activities of LEE1, LEE4 and LEE5 were not significantly altered in these mutants, therefore suggesting that the regulation of these LEE-encoded proteins occurs posttranscriptionally.

Roe *et al.*, (2003) have recently reported that *E. coli* O157:H7 EspA secretion is phase variable and controlled posttranscriptionally. According to these authors EspA filaments are expressed by a sub-population of the bacteria, and certain strains, high secretors, have a higher proportion of bacteria producing EspA filaments compared to low secretor strains. In their study, it was found that an inverse relationship existed between secreted EspA and EspD proteins and the detectable mRNA transcripts. High secretor strains, which produced high levels of EspA and EspD proteins were found to have lower mRNA levels of the *espADB* transcript relative to low secretor strains which secreted low EspA, and EspD proteins. According to the authors, this inverse relationship could be due to differences in the half-lives of the mRNA transcripts. It was also suggested that perhaps secretion and translation are coupled at the type III secretion system, making this transcript difficult to extract. These authors contend that LEE4 is most likely transcribed at the same level between low and high secretor strains. However translation and secretion is restricted in the low secretors. It was also speculated that a phase-variable factor allows for translation of the *espADB* transcript and this factor may be encoded outside of the LEE pathogenicity island. In addition, they have also proposed that *E. coli* O157:H7 expression of EspA, Tir and intimin is heterogeneous and possibly co-ordinated (Roe *et al.*, 2004).

Similar to Roe *et al.*, (2003), an inverse relationship between adherence phenotypes and LEE mRNA virulence factors was observed. Acid shocked bacteria, previously shown to have decreased host-cell adhesion capabilities, had significantly

higher normalized mRNA ratios for both LEE-encoded virulence factors, intimin and EspA. Maybe the different acid treatments elicited different phase variation responses in *E. coli* O157:H7 85170 which resulted in different secretion phenotypes for this strain. Perhaps the acid adapted bacteria subjected to a 30-minute shock were “high secretors” of LEE-encoded virulence factors and this may account for the enhanced adhesion to host epithelial cells. Similarly, it is possible that the “low secretor” phenotype was induced in cells that were unadapted and subjected to a brief acid shock, which ultimately resulted in decreased host cell adhesion. Roe *et al.*, (2003b), have recently proposed that secretion and translation is coupled to the type III secretion system, making the mRNAs difficult to extract and may be one reason why the mRNA levels of these genes were not significantly upregulated for bacteria that were acid adapted-acid shocked for 30 minutes. In addition, it is possible that acid shock alone inhibits the secretion of these virulence factors. It seems that the regulation and secretion of these virulence factors is extremely complex.

It has been recently reported by Elhanafi *et al.*, (2004) that acid adaptation of exponential *E. coli* O157:H7 at pH 5.5 increased the protein expression intimin and in conjunction with the results in this study, it seems that these virulence factors may be controlled at the posttranscriptional level. However to confirm this statement, protein expression under the same conditions needs to be examined. Further research is needed to fully explore the roles that these virulence factors have, if any, in the host adhesion capability of acid stressed *E. coli* O157:H7.

4.4: The Effect of Acid Stress on *Escherichia coli* O157:H7 8624 Verotoxin Production

While adhesion to host cells is considered to play an important role in *E. coli* O157:H7 infection, the production of verotoxins or Shiga toxins is the primary virulence factor associated with EHEC-induced renal disease and vascular complications of hemorrhagic colitis. Secretion of these toxins occurs through the cell envelope using spherical fragments of the bacterial outer membrane, called vesicles (Yuk and Marshall,

2004). Membrane fluidity may be an important factor in verotoxin secretion. Alteration of the membrane lipid composition, and hence membrane fluidity, generally are induced by various environmental stresses, including acid stress. It has been recently reported that acid adaptation decreased membrane fluidity and may be one factor why decreased verotoxin secretion has been reported in acid adapted cells (Yuk and Marshall, 2004).

Several studies have been completed examining the effect of acid adaptation only on *E. coli* O157:H7 verotoxin production and results have been equivocal. In these studies, the effect of acid stress has been reported to either decrease verotoxin production or not have any significant effect at all.

One study by Yuk and Marshall (2004) found that acid adaptation at pH 5.0 for 24 hours of stationary phase *E. coli* O157:H7 reduced the total amount of verotoxin produced relative to cells grown at pH 7.3 and 8.3. Duffy *et al.*, (2000) were also in agreement with this finding. These authors reported that after 48 hours, cells adapted at pH 5.6 produced less toxin than non-adapted cells at pH 7.4. However, Elhanafi *et al.*, (2004) reported that stationary cells adapted at pH 5.5 at 4°C for 4 weeks did not have any effect on post stress *stx*₂ expression or Stx2 production. Similarly, Leenanon *et al.* (2002), reported that acid adaptation enhances *stx*₂ toxin mRNA levels but does not enhance subsequent Stx toxin production.

The results of this study examined the effect of acid shock alone and adaptation followed by acid shock of exponential *E. coli* O157:H7 8624 on verotoxin production and secretion. No significant differences were found across all the various treatments, suggesting that acid stress does not augment the severity of EHEC induced renal disease. Further research is needed to examine the effect of acid adaptation alone under these experimental conditions and also if other *E. coli* O157:H7 strains produce the same observed results noted here.

4.5: Significance and Summary of Results

This study has shown that acid adapted and subsequently acid shocked *E. coli* O157:H7 show significantly enhanced adhesion to host epithelial cells, in an infection time-dependent manner. This enhanced adhesion after acid stress was not a result of type 1 fimbriae alone and pretreatment of the bacteria with erythromycin suggests that the enhanced adhesion is likely due to other known or novel surface structures and adhesins. On the other hand, a nonpathogenic *E. coli* strain demonstrated decreased adhesion after acid stress. However adhesion levels of both unstressed and stressed nonpathogenic HB101 were significantly lower than an *E. coli* O157:H7 strain. Acid shock alone decreased *E. coli* O157:H7 85170 adhesion to HEp-2 cells. The variations of the host adhesion capability of *E. coli* O157:H7 85170 to HEp-2 cells were dependent on the experimental conditions of acid protocols used. Preliminary results suggest that in the absence of buffering agents during the acid adaptation stage, the bacteria acidified the media pH by glucose fermentation, which may have enhanced the virulence of this pathogen through increased adhesion. Real time RT-PCR analysis of virulence factors intimin and EspA mRNA levels show no significant upregulation for the acid stressed treatments previously shown to enhance host-cell adhesion. On the contrary, the bacteria treatments subjected to acid shock only, were shown to have elevated mRNA levels for both intimin and EspA, even though the host-cell adhesion ability of the strain was significantly decreased with these treatments. It is obvious that complex regulation mechanisms for LEE-encoded virulence factors exist, and the role of intimin and EspA in enhanced adhesion after stress remains to be elucidated. Finally, acid stress of *E. coli* O157:H7 subjected to acid shock alone, or acid shock preceded by acid adaptation did not effect verotoxin production.

The results of this study provide further insight into the pathogenic mechanisms of *E. coli* O157:H7. Future research dedicated to developing new therapies to treat and prevent EHEC-related diseases and various food decontamination and cattle husbandry procedures should consider the results found in this study. The production of verotoxin is the major virulence factor in EHEC infection since it can ultimately lead to several life threatening diseases such as hemolytic uremic syndrome and thrombic thrombocytopenic

purpura. In this study, acid stress did not affect verotoxin production. This negative finding is significant and suggests that exposure to acid stress will not lead to increased cases of HUS due to increased verotoxin production. Adhesion to host cells is another important virulence factor of EHEC infection. Although not associated with the severe life threatening disease, adherence to host epithelial cells is still a requirement for the development of diarrhea (Tzipori *et al.*, 1987; Campellone and Leong, 2003). Even though acid stress did not appear to enhance verotoxin production, the research findings in this study not only show that the survival of acid stressed organisms remains high, but those that have survived have enhanced adhesion capabilities to host epithelial cells. It is well documented that many opportunities for acid stress exposure exists for this enteric pathogen, including the anorectum of cattle, animal feed, food-processing conditions, acidic foods, and the gastrointestinal tract. Given the low oral ID of this pathogen, the implications and significance of acid stress is serious. Bacteria that survive acid stress exposure during ingestion will still be able to produce potent cytotoxins and subsequently induce severe disease. Furthermore, since acid-stressed bacteria have enhanced adhesion capabilities, the severity of EHEC-induced diarrhea may be increased and may occur more quickly in infected individuals.

Future studies should build on the findings documented in this research. It was shown that in the absence of buffers, *E. coli* O157:H7 acidified the media during acid adaptation, which correlated to higher enhanced host cell adhesion compared to cells acid adapted in buffered media. These results suggested that lower adaptation pH or biphasic ATR may exist, which may play an important role in the induction of various virulence factors and ultimately host cell adhesion. The increase or decrease in media pH in both unbuffered and buffered media should be completed with several different *E. coli* O157:H7 strains, in the presence and absence of glucose, and compared to the host cell adhesion capabilities. In addition, adhesion experiments and western blot analysis should be conducted at different acid adaptation pHs, buffered DMEM pH 4.3 and DMEM 5.0.

Since the results of this study highlighted the complex regulation mechanisms for LEE-encoded virulence factors, the role of intimin and EspA in enhanced adhesion after

acid stress needs to be clarified. Acid adhesion assays with EspA and intimin mutants should be completed. Additionally, western blot analysis should be conducted to assess expression levels of intimin and EspA on acid-stressed organisms, and the secretion phenotypes of acid-stressed *E. coli* O157:H7 should also be examined to further explore the possible posttranscriptional regulation of these virulence factors after acid stress.

The role of other potential adhesins, such as OmpA, Iha, long polar fimbriae 2, and Efa-1 should also be examined after acid stress. Acid adhesion assays with parental and mutant strains for each adhesin should be conducted.

Since acid stress may be inducing novel adhesin expression, microarray analysis should be completed and any potential adhesins upregulated could be confirmed with real time RT-PCR and acid adhesion assays.

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