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Role Of Selected Fimbrial Adheins In Pathogenesis Of Acid-Induced Eneterohemorrhagic Escherichia Coli 0157:H7

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ROLE OF SELECTED FIMBRIAL ADHEINS IN PATHOGENESIS OF ACID-INDUCED ENETEROHEMORRHAGIC *ESCHERICHIA COLI* O157:H7

By

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Abstract

Enterohemorrhagic ***Escherichia coli*** (EHEC) O157:H7 is a food-borne pathogen that causes hemolytic uremic syndrome and hemorrhagic colitis. The mechanisms underlying the adhesion of EHEC O157:H7 to intestinal epithelial cells are not well understood. Like other food-borne pathogens, EHEC O157:H7 must survive the acid stress of the gastric juice in the stomach and short chain fatty acid in the intestine in order to colonize the large intestine. We have found that acid stress and short chain fatty acid stress significantly enhance host-adhesion of EHEC O157:H7 and also upregulates expression of EHEC fimbrial genes, *lpfA1*, *lpfA2* and *yagZ*, as demonstrated by our DNA microarray. We now report that disruption of the *yagZ* (also known as the *E. coli* common pilus A) gene results in loss of the acid-induced and short chain fatty acid-induced adhesion increase seen for the wild type strain. When the *yagZ* mutant is complemented with *yagZ*, the stress-induced adhesion phenotype is restored, confirming the role of *yagZ* in the acid as well as short chain fatty acid induced adhesion to HEp-2 cells. On the other hand, neither disruption in the long polar fimbriae genes *lpfA1* or *lpfA2* in the wild type showed any effect in adherence to HEp-2 cells; rather displaying a hyperadherant phenotype to HEp-2 cells after acid-induced or short chain fatty acid-induced stress. These results suggest that the absences of LpfA1 or LpfA2 are not critical for acid-induced adherence. However, YagZ may play an essential role in the adhesion of EHEC to epithelial cells upon exposure to acid or short chain fatty acid stress. The results also indicate that acid or short chain fatty acid stress, which is a part of the host's natural defense mechanism against pathogens, may regulate virulence factors resulting in enhanced bacteria-host attachment during colonization in the human or bovine host.

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List of Abbreviations

AA	Acid adapted acid stress
AA30	Acid adapted acid stress
A/E	Attaching and effacing lesions
AcrAB pump	Drug efflux pumps
ATCC	American Type Culture Collection
AR1	Acid resistance 1
AR2	Acid resistance 2
AR3	Acid resistance 3
AR4	Acid resistance 4
ATR	Acid tolerance response
CaCo-2	Colonic adenocarcinoma cells
CFU	Colony forming units
DMEM	Dulbecco's MEM
EDTA	Ethylenediaminetetraacetic acid
EHEC	Enterohemorrhagic Escherichia coli
FBS	Fetal bovine serum
Gb-3	Glycolipid globotriaosylceramide-3 receptor
GIT	Gastrointestinal tract
HC	Hemorrhagic colitis
HEp-2	Laryngeal carcinoma cells
HUS	Hemolytic-uremic syndrome
Iha	IrgA homologue adhesion
LB	Luria-Bertani
LEE	Locus of enterocytes effacement
LPS	Lipopolysaccharide
MEM	Minimal essential media
MLC	Myosin light chain
N-WASP	Neuronal Wiskott-Aldrich syndrome protein
PBS	Phosphate buffered saline
RpoS	Stress response regulon
SCFA	Short chain fatty acids (stress)
STEC	Shiga toxin producing E. coli
Stx	Shiga toxin
TccP	Tir-cytoskeleton coupling protein
Tir	Translocated intimin receptor
TTSS	Type III secretion system
UU	unshocked control for its counterpart SCFA stress
UU30	unadapted unstressed counterpart for AA30 stress
Vero cells	African Green Monkey Kidney cells
WT	Wild type

1.1 Introduction

The gastrointestinal tract (GIT) is a natural habitat for approximately 10^{12} commensal microbes (26). However, gastrointestinal diseases occur when pathogens are transmitted through the fecal-oral route (3). EHEC is an example of a food-borne pathogen that causes hemolytic uremic syndrome (HUS). The disease is characterized by bloody diarrhea that can be accompanied by microangiopathic heamolytic anaemia, thrombocytopenia and renal failure (68). The following sections briefly describe different aspects of the virulence and pathogenesis of EHEC.

1.2 Epidemiology of EHEC-mediated gastroenteritis

EHEC-mediated diarrhea constitutes a serious health concern world-wide. Populations affected by EHEC-induced infections include those from Europe as well as the United States. Several aspects of the epidemiology of EHEC-related disorder will be discussed in the following sections.

1.2.1 The etiological agent

EHEC is a Shiga Toxin (STx) producing *E. coli* family, which can cause HUS and hemorrhagic colitis in human (14). Of the EHEC family, EHEC O157:H7 is the predominant cause of HUS. EHEC O157:H7 was first identified in two independent

outbreaks in the United States as early as the 1980s (14). However, the abundant detection of this serotype could largely be due to the diagnostic procedures developed for efficiently detecting this particular serotype as compared to others (45).

1.2.2 Infectious dose

The infectious dose of EHEC is quite low, about 10^2 (36). Certain sources have even reported the infectious dose to be as low as ten microorganisms (http://www.hi-tm.com/Documents/Ecoli98.html). This very low dose provides an additional challenge for the development of an antimicrobial.

1.2.3 Routes of Transmission

The natural environmental reservoir of EHEC is the anorectum of cattle. Modes of transmission of EHEC can be through the consumption of contaminated food like beef, raw milk, non-pasteurized fruit juice, unchlorinated water and through person-to-person contact during activities like swimming or day care activities (14). Additionally, transmission by consumption of food contaminated with bovine manure as in crop cultivation indicates direct zoonotic and environmental transmission (14).

1.2.4 Prevalence and Incidence

Prevalence and incidence worldwide of Shiga Toxin-producing *E. coli* (STEC) infection was found to be 17-40% in beef mince and other types of raw meat products in US, Canada and England (4). However, all the isolates of the study lacked the Locus of Enterocyte Effacement Pathogeneicity Island (LEE PAI), and therefore these isolates might not cause severe disease in humans (4). STEC can produce two types of Shiga Toxin (STx), Stx1 and STx2. Leotta *et. al.* (2008) stated that STx2-positive strains are highly prevalent in Western Europe while strains positive for both STx1 and STx2 are more prevalent in US and Australia. Their study indicate that phage types PT2, PT4, PT8, PT14, PT21, PT32 and PT54 are harbored in more than 75% of the STEC O157:H7 in Europe and Canada, PT2 and PT8 being the predominant phage types in England and many other European countries (33).

1.2.5 Mortality and Morbidity

An estimated 8-10% of individuals infected by EHEC can experience the severe complications of HUS (9). Children usually suffer from abdominal pain or tenderness, nausea or vomiting, fever and anemia while affected adults remain asymptomatic (55). Mortality of EHEC-mediated HUS is approximately 2% during the acute phase of infection (7). According to a more recent report, the mortality caused by HUS is 3-5% and nearly two thirds of the infected children require dialysis (61).

1.2.6 Colonization Site

EHEC colonizes the distal ileum and colon (26). However, disruption of certain fimbrial adhesins like long polar fimbriae can extend the adherence of selected strains to the small intestine (20). Deletion of these genes may unmask other adhesin genes that are part of the bacteria's survival and virulence strategy during several ingestion stresses discussed below.

1.3 Responses to Ingestion Stress

EHEC must adapt to various stresses in order to successfully establish colonization in the gastrointestinal tract (GIT) (28). It must survive several ingestion stresses including the acute acidic environment of the stomach as well as exposure to short chain fatty acids (SCFA) in the large intestine. This pathogen has numerous stress-inducible mechanisms to survive the challenges of these ingestion stresses, as will be discussed in the following sections.

1.3.1 Acid Tolerance Response

Acid tolerance response (ATR) is vital for EHEC's journey through the stomach. ATR is an adaptive response to acid stress. It has been shown that acid stress-associated response offers resistance to other stresses like heat, salt and hydrogen peroxide (5). However, resistance to other stresses does not offer protection against acid (5). House *et. al.* (2009) have shown that EHEC pre-adapted to mild acidic conditions are able to tolerate acute acidity of as low as pH3.0 and pre-adaptation contributes to enhancement in the adhesion

phenotype compared to both the unshocked and the unadapted/shocked counterparts. *E. coli* O157:H7 is adapted to better survive gastric acidity upon pre-exposure to mildly acidic foods which might be a reason why they have such a low infectious dose (28). The acid resistance can be attributed to arginine and glutamate decarboxylase-dependent systems and a glucose catabolite-repressed system which will be explained briefly in the following sections. Moreover, adaptation in mild acidic conditions provides comparable resistance to freeze-thaw stress, possibly because acid adapted bacterial membranes are less fluid and therefore less susceptible when it comes to freeze-thaw disruption of the plasma membrane (32). As early as 1999, Buchanan *et. al*. (2004) has shown that acid resistant strains also gain resistance to inactivation by irradiation in food processing. It has been shown that EHEC are more tolerant to low pH than other enteric pathogens like *Salmonella* and *Shigella* (54). Exposure to acid stress has been shown to allow EHEC O157:H7 better adherence although the Shiga Toxin (STx) production remained unaffected (28). In order to tolerate and overcome acid stress, several acid resistance response systems identified in EHEC are discussed in the following sections.

1.3.2 Acid Response 1

Acid resistance 1 (AR1), also known as the oxidative repressed acid resistance, is one of the most poorly understood response systems. An acid response is the bacteria's ability to survive acid challenges (13). *E. coli* grown to stationary phase in buffered LB media at mildly acidic pH (pH 5.5) are significantly resistant to acute acidic conditions of pH 3.0 compared to those grown under more neutral pH and this is the basis of AR1 (2). *E. coli*

grown at pH 8.0 develop inhibitory response to AR1 while at lower pH of 5.5, the inhibitory effect of AR1 is not evident. However, when media are supplemented with glutamate, even at pH 8.0, the inhibition of AR1 is eliminated, implying that media composition and environmental conditions cumulatively play a role in the regulation of AR1. It is believed that an inducible glutamate decarboxylase consume protons during decarboxylation of glutamate. The final product glutamate/γaminobutyric acid (GABA), is then exported out of the cell as an exchange for new substrates; the result being preventing decrease of cytosolic pH to lethal levels (13).

1.3.3 Acid Response 2

Acid resistance 2 (AR2), also know as the glutamate-dependent acid resistance is one of the most well characterized systems. It is believed to provide optimal protection to EHEC in acute acidic environments of pH 2.0 or lower. AR2 functions by converting glutamate to GABA, consuming a proton in the process in order to maintain the cytosolic pH at 4.2 (2, 13, 21).

1.3.4 Acid Response 3

Acid resistance 3 (AR3), known as arginine-dependent system is dependent on the presence of arginine in acidic minimal media for induction (13, 21). Bacteria previously grown in rich media under anaerobic conditions only can induce this system. In a manner similar to that of AR2, AR3 converts arginine to agmatine, consuming a proton in the

process in order to maintain the intercellular cytosolic pH at 4.7 (13, 21).

1.3.5 Acid Response 4

Acid resistance 4 (AR4), also known as the lysine-dependent acid resistance is a lysine dependent decarboxylation system (17, 34). Like AR2 and AR3, AR4 consumes a proton during the decarboxylation of lysine. AR4 provides protection only at mildly acidic environments (17, 34).

1.3.6 Short Chain Fatty Acid Response

Composition of bacterial species in the microbiota greatly influences the amount and types of SCFA that are produced in healthy people (41). Most SCFA in the GIT are derived from breakdown of complex carbohydrates by bacteria. However, digestion of proteins and peptides also makes a significant contribution to SCFA production in the intestine (41).

The main constituents of SCFA are acetate, butyrate and propionate. The concentration of short chain fatty acid are low (20 to 40 mM) in the small intestine and increases towards the large intestine (130 to 300 mM) where pathogens like EHEC colonize (30a).

EHEC O157:H7 from fecal samples showed a significant reduction of growth upon exposure to acetic, propionic and butyric acids compared to exposure to hydrochloric

acid and lactic acid. Concentrations of 40 mM SCFAs at pH 5.5 almost completely inhibits growth (64). Propionic acid appeared to be more growth-suppressing than acetic and butyric acids. The production of Stx2 corresponded to the amount of growth in those samples (64). Fu *et. al.* (2003) showed that acetic and propionic acid increases low pH resistance in EHEC O157:H7 isolates from cattle. Research had shown that there is enhanced adherence to human epithelial cells and STx production of EHEC upon exposure to 90 mM SCFA (63). This is consistent with findings by Carey *et al*.(2008) who showed that there was varying but considerable upregulation of STx in EHEC O157:H7 (12). Additionally Arnold *et. al.* (2001) had shown that RpoS and metabolic enzymes are upregulated in response to SCFA when exposed to acetate. In fact, in the presence of acetate as the sole source of carbon, genes needed for replication and transcription are down-regulated while the ones for metabolic processes are up-regulated (47). Another component of SCFA, butyrate, has been shown to affect the binding, internalization, translocation and activation of Shiga Toxin within eukaryotic cells (37). The role of Shiga Toxin and other virulence factors in the pathogenesis of EHEC will be briefly discussed below.

1.4 Virulence factors and associated properties

Several virulence factors play a role in the successful infection of EHEC in the host. These include the virulence plasmid, several known and putative potential adhesins and Shiga Toxin, all of which will be discussed in the following sections.

1.4.1 Pathogenicity Island

The EHEC genome contains a 43 kb pathogenecity island, the locus for enterocyte effacement (LEE) of O157:H7 which encodes for genes that play important roles in A/E lesion formation during pathogenisis (52, 76). The LEE regions are made up of five operons, LEE1-5. LEE1, LEE2 and LEE3 enocde the type III secretion system (T3SS), a molecular syringe that is used to inject effector proteins into host cells upon intial attachment. LEE4 encodes *E. coli* secreted proteins Esp(s) like EspA, EspB and EspD, all of which contribute to the injection process mentioned above (52, 76). The last operon, LEE5, encodes translocated intimin receptor (Tir) which facilitates intimate attachment to host epithelium in concert with other LEE-encoded proteins (52, 76).

1.4.2 Virulence plasmid

A 92kb plasmid, pO157 exists in clinical isolates of EHEC O157:H7 (35). The plasmid pO157 in EHEC O157:H7 encodes EHEC-haemolysin that can be used by the bacteria to acquire iron in the blood and has been shown to be cytotoxic *in vitro*. It also encodes the serine protease, EspP, which could cause mucosal hemorrhage characteristic of hemorrhagic colitis (11, 50, 59, 62, 76). Intimin adherence protein-deficit, *eae*-negative serotypes have larger plasmids compared to *eae*-positive serotypes. The plasmid pO157 has 100 open reading frames, of which only 19 have been characterized. Amongst the characterized ORFs are ones that encode a type II secretion system, a peroxidase (KatP), a zinc metalloprotease (StcE) and a toxin (ToxB) (10, 11, 25, 67, 78).

1.4.3 Hemolysin

In addition to Stx which is a primary cause of HUS, some Stx-negative strains produce cause HUS (1). Stx-negative *E. coli* O26 strains have also been found to be the causative agents of HUS in a study on a group of patients. In the study, the Stx genes were absent from the clinical isolate (1). However, all the isolates of that study produced a pore-forming cytolysin, Hly. EHEC-Hly is cytotoxic to human microvascular endothelial cells. Immune response to EHEC-Hly was evident from the serum samples of HUS patients. Moreover, *hly* is expressed by the EHECO157:H7 isolates from patient. Thus EHEC-Hly could be a vital non-STx factor playing a major role in the pathogenesis of EHEC (1).

1.4.4 Shiga Toxin

Shiga Toxin producing EHEC, also known as STEC, produce two types of Shiga Toxin, Stx (STx1 and STx2) that are encoded by *stxA* and *stxB* by two types on integrated lambda lysogenic phages in the bacterial genome under the control of SOS response (22). More than 200 serotypes of *E. coli* have been identified with the ability to produce Shiga-Toxin. Of them, serotype O157:H7 is the STEC most highly associated with HUS (4).

STx is a 70 KDa protein composed of a single A subunit surrounded by five identical B subunits (18, 27, 45, 51, 59, 60, 69, 70). The A subunit can be excised to generate the catalytically active A1 (27kDa) and A2 (4KDa) units. The A1 subunit interacts with the 60S ribosomal subunit and prevents protein translation leading to death of the host cell

(Figure 1.1). The five 7.7 KDa B subunits bind to the glycolipid globotriaosylceramide-3 receptor on the host cell, Gb3 (18, 27, 45, 51, 59, 60, 69, 70). When the complex enters the host cell, the A subunit inactivates ribosomes and halts protein synthesis leading to death of the cell. The cell death of the vascular endothelium leads to a breakdown of the lining and hemorrhage which contributes to the bloody diarrhea in EHEC-mediated gastroenteritis (18, 27, 45, 51, 59, 60, 69, 70).

1.4.5 Adhesins

EHEC needs to establish initial and intimate attachment to host cells as part of its multiple-step infection process in order to successfully colonize the large intestine. The attachment to host epithelial cells is established by non-fimbrial as well as fimbrial adhesins. Analysis of the genomic sequence from EHEC O157:H7 shows it bears at least 24 regions encoding putative adhesions of which 10 belong to potential fimbrial adhesin gene clusters, 13 encode putative non-fimbrial adhesins while one encodes LEE-encoded intimin (74). The presence of numerous, uncharacterized putative fimbrial clusters in EHEC, suggest that several adhesions, in addition to intimin participate in binding of the pathogen to the host (74).

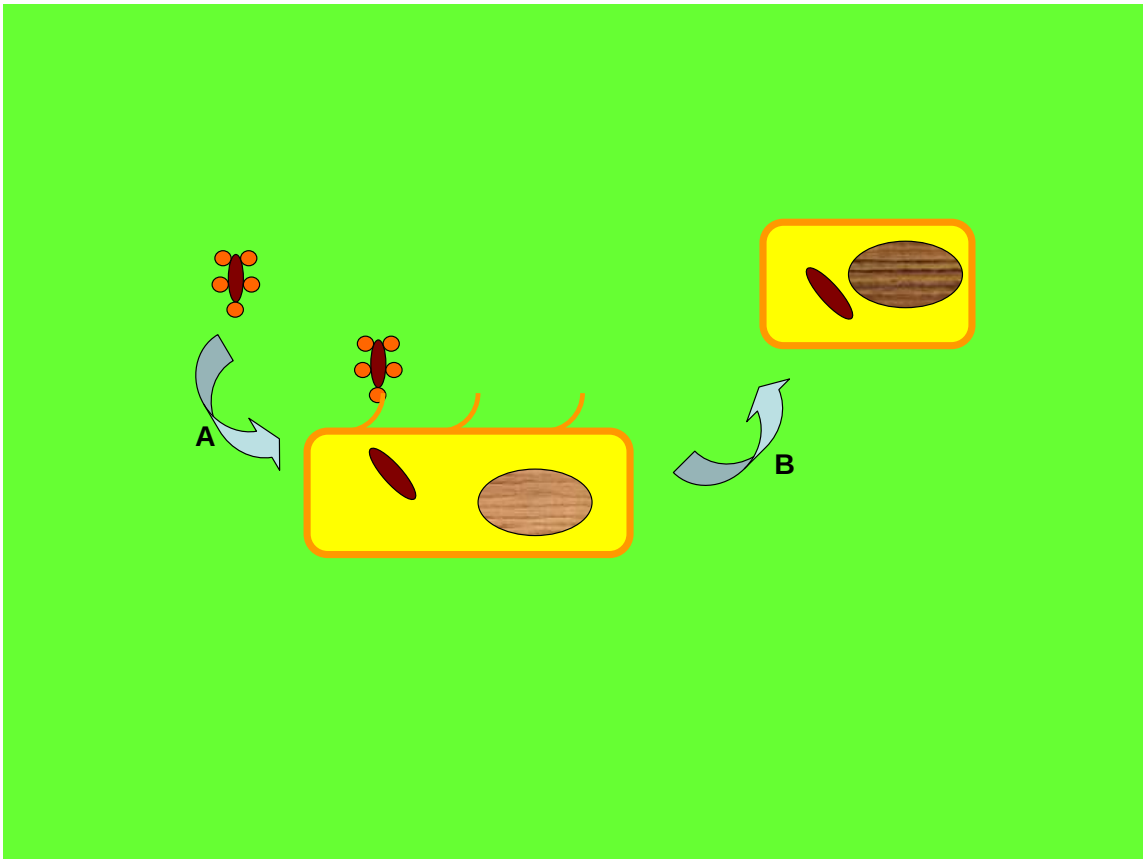


Figure 1.1: Mechanism of action of Shiga Toxin. Secreted shiga toxin by EHEC attaches to the host cell globotriaoslceramide (Gb3) or globotetraosylceramide (Gb4) receptors ( ) and using the B subunit ( ) (A). The catalytic A subunit

( ) enters the host cell and prevents protein translation, resulting in shrinkage of host cell and condensation of chromatin in the nucleus, all of which lead to apoptosis of the infected host cell (B). *Adapted from (14).*

1.4.5.1 Fimbrial Adhesins

As many as 16 fimbrial clusters have been identified in EHEC O157:H7 (39). Of these 16 clusters, 13 are predicted as clusters of chaperone and usher genes and 4 are distinct in EHEC O157:H7 serotypes (39). Though EHEC encodes several of the known fimbrial proteins including type 1 fimbriae, curli, long polar fimbriae-1 (LpF1) and long polar fimbriae-2 (LpF2), only LPF1/2 and curli clusters have been found to be functionally expressed in EHEC (39).

1.4.5.2 Long Polar Fimbriae

Torres *et. al. (2002),* identified two EHEC loci that have strong homology to the long polar fimbriae in *Salmonella* Typhimurium. The six ORFs of the first *lpf* operon are similar to the corresponding *Salmonella* gene cluster. The nomenclature of EHEC *lpf-1*, *lpf*ABCC'DE is also similar to that in *S. enterica* (Figure 1.2 and Table 1.1). The first gene in the operon, LpfA, has high homology to FimA; additionally LpfB and LpfC/LpfC' are homologous to FimC and FimD, the usher-chaperone subunits of the corresponding Fim in *Salmonella*. The *lpf* form surface structures that are 7–8 nm in diameter and 2–10 μm in length extending from bacterial cell surfaces. Both the *lpf* loci are flanked by sequences homologous to those in *E. coli* K-12, which is suggestive of the *lpf* loci having been acquired by horizontal transfer during the evolution of *S. enteric* serovar Typhimurium and *E. coli* O157:H7 (73).

Although the long polar fimbriae were visualized by electron microscopy in cloned *E.*

coli strain ORN172, the polar distribution was different from that previously observed for the *S. enterica* serovar *Typhimurium* (73, 74). The clone EHEC long polar fimbriae, was apparently distributed peritrichously on the bacteria. Another unusual characteristic of the *lpf* operon is the presence of two ORFs for disrupted *lpfC* (*lpfC* and *lpfC*') predicted to be encoding putative outer membrane components of 40.2 and 17.8 kDa, respectively the fimbrial structure compared to one protein of 94.4 kDa in its *Salmonella* counterpart.

Research has linked the role of long polar fimbriae to STEC pathogenesis (71). Strong correlations between STx production and presence of lpfAs have been reported, suggesting that it is a characteristic feature of STx producing strains (71). Introduction of the EHEC *lpf* operon into a non-fimbriated *E. coli* K-12 strain resulted in increased adhesion to tissue culture cell, thereby indicating a role in adherence to host epithelium (73). Although, the adhesin, intimin is important for adherence, other factors that initiate adherence in EHEC strains are not known (73). *In vitro* results suggest that long polar fimbriae may participate during adherence at some stage of the process (20, 73). When the bacteria-eukaryotic cell interactions are evident, the expression of long polar fimbriae seems to favor the formation of microcolonies, but it is yet to be known if the expression of long polar fimbriae participates in bacteria-to-bacteria interactions or if their presence enhances adherence to tissue epithelial cells (73).

When compared with the first *lpf* operon, the *lpf2* region encodes an extra ORF, renamed as *lpfD'2*, which encoded a second putative minor fimbrial subunit (73, 74). The role of the *lpfD'2* ORF in O157:H7 lpf2 biogenesis is unknown. In EHEC O113:H21, *lpf* and

cryptic operon of *S. typhi* is absent; on the other hand, in EHEC O157:H7 and *S. typhimurium*, the *lpfD'2* gene is replaced by *lpfE* (73, 74).

Long polar fimbriae have been showed to be required for adherence to Caco-2 cells and probably play a role in adherence during the very early stages of infection when intimin is not required for the initial adherence or for the formation of the A/E lesion (20, 73). However, other studies have found that *lpfA1; lpfA2* double mutant was shed in significantly lower numbers than the wild type, and tissues from infected gnotobiotic pigs had a significant reduction in A/E lesions (20, 73, 74). However, there was not any demonstrated difference in adherence of the EHEC O157:H7 lpfA2 single and double mutants (*lpfA1;lpfA2*) at later time points of infection (20, 73, 74).

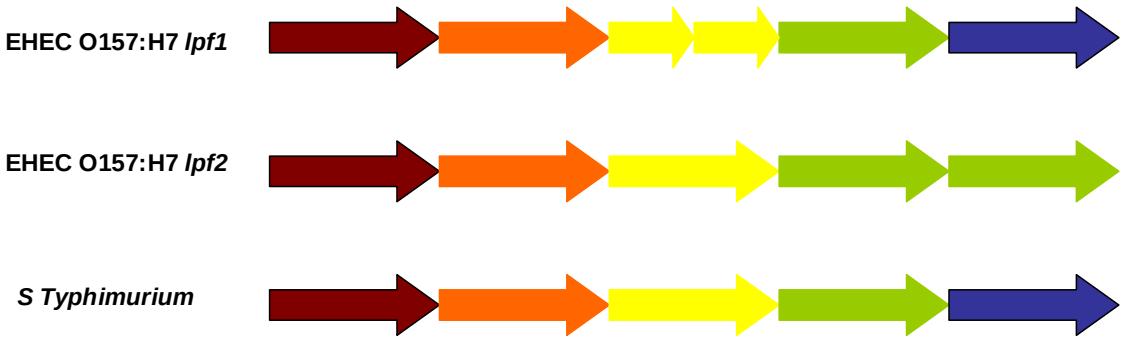


Figure 1.2: Comparison of the genetic organization of the *lpf* regions from EHEC O157:H7 and *Salmonella serovar Typhimurium*. Genes are shown as arrows with patterns representing their putative function: red (major fimbrial subunit, *lpfA*), orange (chaperone, *lpfB*), yellow (outer membrane usher chaperone, *lpfC*), green (minor fimbrial subunit, *lpfD*) and blue (fimbrial subunit, *lpfE*). *Adapted from (20).*

Table 1.1: Percentage similarity of EHEC LPF and *Salmonella typhimurium* LPF to EPEC LPF proteins

Protein	% identity of EPEC LPF proteins to proteins of other AE strains		
	O157:H7-1	**O157:H7-2**	***S Typhimurium***
LpfA	89	32	75
LpfB	79	41	65
LpfC	74	38	69
LpFD	46	27*	33
LpfE	52	NA	43

*LpfD is duplicated
NA-lacks lpfE

Adapted from (67).

1.4.5.3 *E. coli* Common Pilus

E. coli common pilus (Ecp) is produced by commensal as well as pathogenic strains of *E. coli* (56). Therefore it is likely that the pathogenic strains use Ecp to mimic commensal strains, thus gaining an advantage in host colonization and evasion of the host immune system (8, 53). It is located in a putative fimbrial operon, MAT operon (Figure 1.3) (39).

yagZ (also known as *ecpA* or *matB)* encodes the major subunit of 195 amino acid which contains a 22 residue signal sequence at the N-terminal in *E. coli* associated with newborn meningitis and septicemia (53). This 22-residue signal sequence is not related to its type IV fimbrillin counterpart. The 195 amino acid sequence lacked cysteine residues. The C-terminal lacked the β-zipper motif which is needed for recognition by fimbrial chaperones. The expression of the gene was found to be temperature-dependent and was under the regulation of transcriptional as well as post-transcriptional events (53). In this study, there was no or little expression of *yagZ* at 37ºC in *E. coli* associated with newborn meningitis and septicemia. Both the genes upstream and downstream, *matA* and *matC*, respectively, affect the formation of Mat fimbria (Figure 1.3 and 1.4). It has also been found that *matB (yagZ)* and *matC* are part of one transcript while *matA* is transcribed independently (53).

yagZ has been reported to promote adherance of EHEC to cultured epithelial cells in synergism with other adhesins, like bundle-forming pilus, in enteropathogenic *E coli*

(58). EcpA was detected upon infection with human HeLa and HT-29 tissue cultures (58). EcpA in EHEC has been shown to be expressed under anaerobic conditions at 26ºC and 37ºC as well as upon infection with HEp-2 cells (56). Research with enterotoxigenic *E coli* has revealed that Ecp is not related to any known colonization factor. The major subunit gene, *yagZ,* is found in 80% of enterotoxigenic strains and 58% of these strains produce Ecp irrespective of the expression of other known colonization factors (8). In accordance, previous studies have shown that the 21 KDa EcpA protein promotes direct binding of the EHEC to the cell membrane by the recognition of specific host cell receptors or by building physical bridges between adhering bacteria. However, in other studies transcriptional analysis and transposon or signature-tagged mutagenesis have provided contradictory results, ruling out any role of EcpA in adhesion to cultured Caco-2 cells and the bovine GIT (56).

Rendon *et. al*. (2007), have shown that the presence of host cells is not a prerequisite for the production of EcpA. They have also shown that EcpA is not produced during cultivation in Luria Bertani but rather when the bacteria is grown in DMEM, preferably at 5% CO_2 which is contradictory to what was seen in the study with *E. coli* associated with newborn meningitis and septicemia by Pouttu *et. al*. (2001) (53, 56) . A reduction in adhesion of EHEC to tissue culture cells has been reported in previous findings by Rendon et. al. (2007). EcpA is also produced at temperatures below 37ºC; it is, therefore, likely to play a role in the persistence of the bacteria outside the host (56).

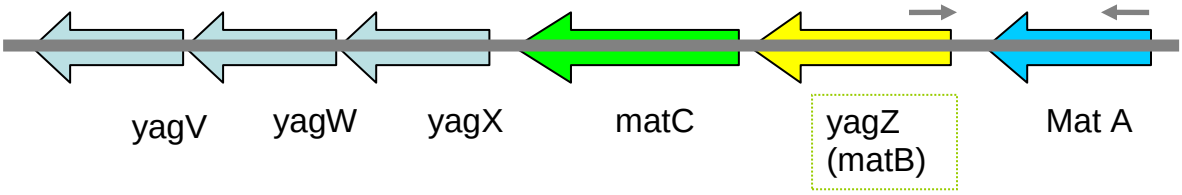


Figure 1.3: Gene organization of the Mat fimbrial cluster. The Mat fimbrial cluster consists of the regulator *matA*, the major pilin subunit *matB* (also known as *yagZ* or *ecpA*), the chaperones *matC* and *yagV*, the usher *yagX*. The function of *yagW* remains unpredicted at present. The arrows in grey were used to amplify the predicted promoter region of *matB* in the current research project; diagram not to scale. *Adapted from (56).*

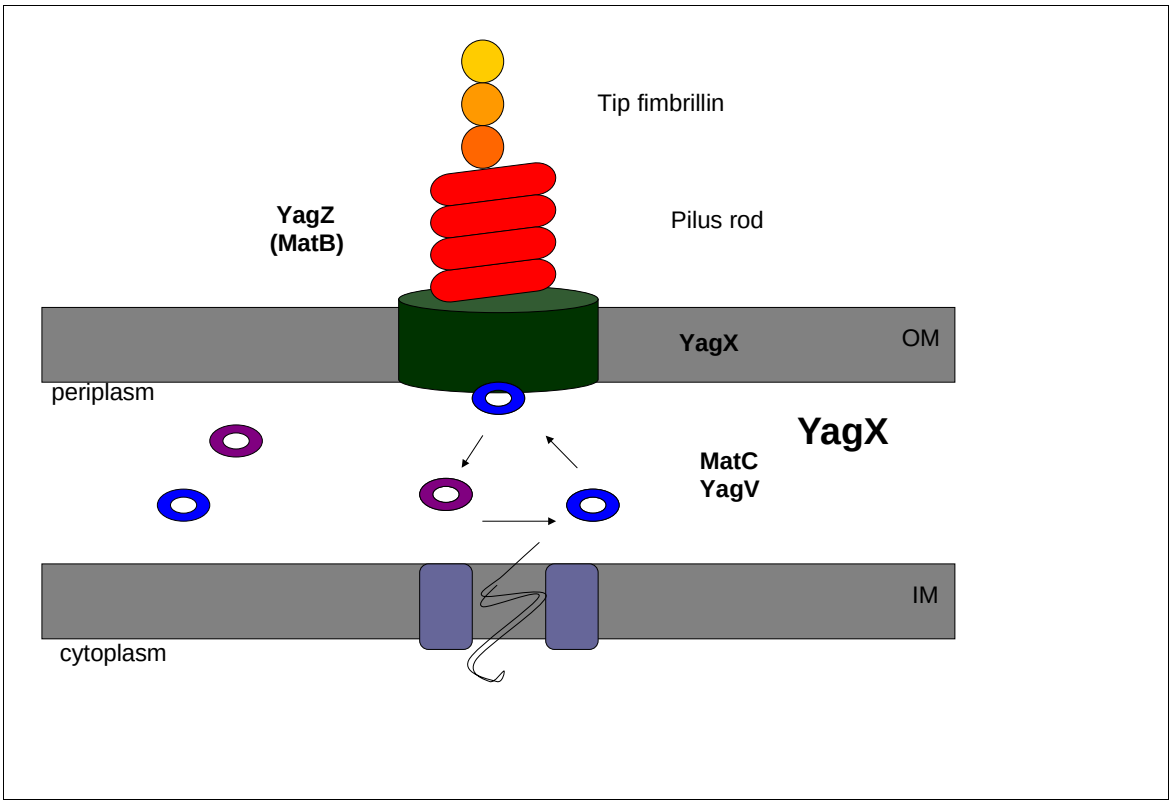


Figure 1.4: Model of the Mat fimbrial cluster. The Mat fimbrial cluster is made up of the major fimbrial subunit MatB (also known as YagZ or EcpA), the chaperones MatC and YagV, the usher YagX. The minor pilin subunit remains unpredicted at present. OM: outer membrane; IM: inner membrane. *Adapted from (56).*

1.4.5.4 Regulation of Fimbrial Adhesin Gene Expression

Researchers have shown that the microenvironment of EHEC is crucial to the regulation and expression of fimbrial adhesins as well as other virulence factors (39). Low *et al*. (2006) have investigated the potential of several environmental conditions that favour expression of several adhesin operons. Among the conditions tested are suboptimal temperature, pH and media composition (39).

Expression analysis on *lpf2* suggests lpf2 is expressed best when induced in media at pH 6.7 compared to pH 5.5 (72). β-galactosidase assays of promoter expression also supports the finding that *lpf2* expression is favoured in media supplemented with NaCl (72). The level of *lpf2* expression was reduced to half in media with high salt molarity compared to their counterpart controls (72).

Expression of *ecpA* has also been found to be influenced by temperature and growth media. EHEC is able to produce EcpA at temperatures below 37°C, suggesting the fimbriae play a role in EHEC adhesion also when outside the host environment. Additionally, DMEM is a better media than LB for EcpA expression (56). The expression of adhesins along with toxin production at various steps of infection lead to successful colonization and delivery of toxin to host cells. A brief mechanism of EHEC pathogenesis will be discussed below.

1.5 Pathogenic Mechanism of EHEC

EHEC is a non-invasive pathogen and does not enter the host cell cytoplasm. For successful establishment of infection, EHEC initially adheres non-intimately and then intimately to the intestinal epithelium surface (Figure. 1.5). EHEC initial attachment is facilitated by EHEC adhesins like EspA filaments. The intimate attachment is facilitated by non-fimbrial adhesin intimin receptor that interacts with its own injected receptor, Tir (30). Intimin also interacts with integrin and nucleolin which are upregulated by Stx on the host cell (18, 44, 57).

EHEC adherence to host epithelial cells leads to formation of A/E lesions characterized by loss of intestinal microvilli (Figure 1.5) (68). It also causes rearrangement of the host cytoskeleton to form pedestal-like structures underneath the pathogen where they adhere and permits delivery of Stx (63, 68). A study in neonatal calves has shown that the A/E lesions consist of areas with degenerated epithelium accompanied by intimately-attaching EHEC and sloughing epithelial cells (16). Virulence factors that aid in A/E lesion formation include LEE encoded proteins like EspA, EspB, EspD, EspG and Map accompanied by non-LEE encoded factors like Iha, LPF, curli and F9 fimbriae (19, 38, 65). EspA filaments and fimbrial adhesin allow initial attachment. EHEC then translocates various other Esp effectors and Tir into the host cell and intimate attachment occurs through binding of the bacterial non-fimbrial adhesion, intimin to its receptor, Tir. Once Tir is translocated through the T3SS, Tir moves to the cell membrane where it forms a hairpin loop structure. The central region of Tir allows binding with intimin while the cytosolic domain of Tir binds with cytoskeletal proteins leading to formation of actin pedestal structures (23).

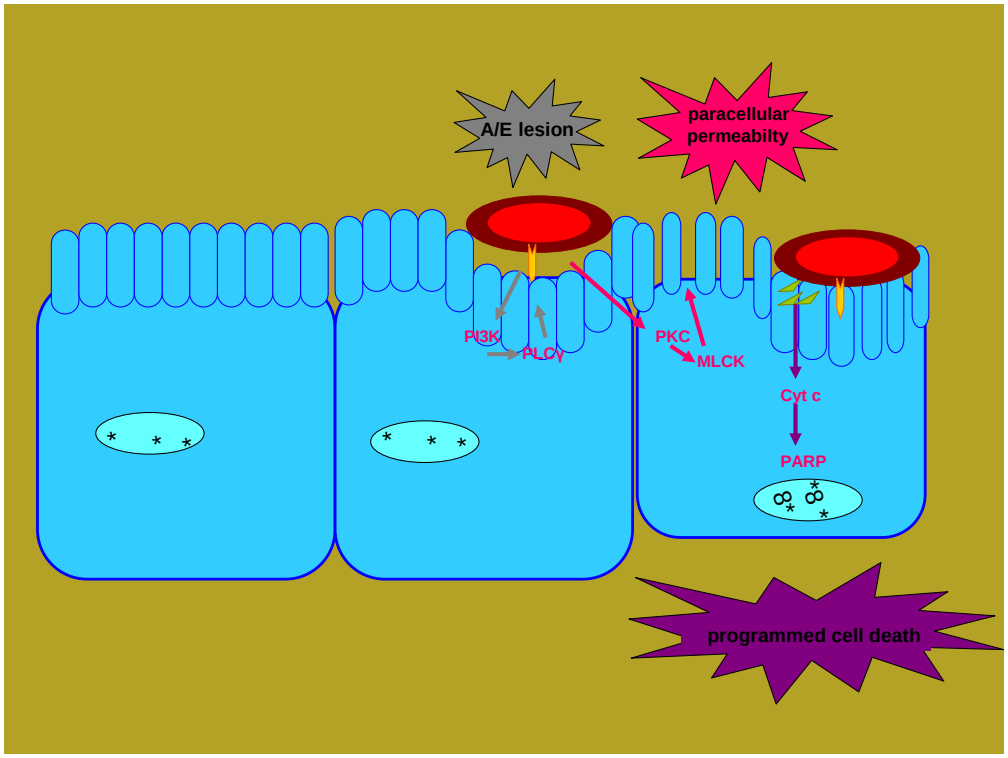


Figure 1.5: Mechanism of pathogenesis of EHEC. EHEC ( ) binds to host epithelial cells using the T3SS ( ) to inject effector proteins into the host cytosol. The effector proteins contribute to cup-like pedestal formations and A/E lesions thorough activation of phosphatidyl- inositol-3-kinase (PI3K), phospholipase C (PLC) signaling pathway. EHEC also activates protein kinase C (PKC) and myosin light chain kinase (MLCK) leading to deregulation of host cellular permeability. Following intimate attachment to host cell, EHEC secretes STx ( ) that leads to activation of cytochrome c (Cyt c) and degradation of DNA repair enzyme (PARP). The host cell shrinks and the nucleus ( ) appears to be more condensed with chromatin (8) induced by STx, all of which contributes to the programmed cell death of the infected host cell. *Adapted from (14).*

In addition, intimin binds to the host cell receptors such as integrin and nucleolin. In addition to Tir-intimin binding pair, TccP binds indirectly with Tir and activates the N-WASP and stimulates Nck-independent actin polymerization leading to pedestal formation of the A/E lesions, mentioned above (77). EHEC also uses T3SS effectors like EspH induced filipodia in Cdc42 dependent events which is followed by actin pedestal elongation (75).

As part of the pathogenic mechanism, EHEC disrupts the epithelial barrier and tight junction which causes an imbalance in the ion secretion, loss of fluid into the lumen that leads to watery diarrhea (6). In order to achieve that EHEC activates ezrin by phosphorylation, while occludin is dephosphorylated (6). Moreover, phosphorylation of myosin light chain (MLC) by protein kinase C (PKC) disrupts the epithelial barrier (42).

After colonization on the cells with altered cytoskeleton structure, the bacteria produce STx which crosses the epithelial barrier of the intestine to reach the endothelium of small blood vessels that supply the gut, kidney and other viscera (29, 65). The STx can then bind to their receptor Gb3 and act on these underlying cells of the endothelium and damage the microvasculature cells of the blood vessels supplying these organs, and activate prothrombic and proinflammatory events leading to the clinical manifestations of HUS (29, 65).

A/E lesion formation may not be an absolute requirement of causing disease. In fact, Taylor (2008) stated that A/E is not essential but Stx production is a requisite for

occurrence of HUS. It has been reported that HUS-causing EHEC produce STx2 more frequently compared to Stx1. Moreover, research with a pig model has revealed that STx2d, a member of the subdivision of STx2 do not require attachment and effacement to result in HUS (68). A/E lesions, therefore, might be an added virulence property that facilitates STx delivery to the enterocytes (68). Although Stx production appears to be sole cause of the detrimental effects characteristics of HUS. Stx-negative organisms have been detected to be etiological agents of HUS, a probability being through subversion of species with STx-producing strains.

1.6 Subversion with other species

EHEC O26 is the leading cause of HUS in non-O157 mediated HUS in Europe and the US (7). *E. coli* serogroup O26 includes EHEC which are STx-postive and well as atypical enteropathogenic *E. coli* (aEPEC) which are Stx-negative. Clinical isolates from patients in Germany have been found to carry EHEC O26 during the early phase of infection while it was largely replaced by aEPEC O26 during the latter phase of infection (7). Analysis of the *stx* showed that EHEC O26 lost the *stx* to aEPEC O26 and aEPEC accepted the *stx* by being lysonized by stx-harbouring prophages in the EHEC isolates *in vitro* (7). This study signifies the fact that many strains could perhaps exhibit ephemeral interconversions through loss and gain of *stx*-harbouring phages. STx-producing phages can cause EHEC O26 and O157 strains to transduce commensal *E. coli* and EPEC in GIT of mice and sheep (7). Thus there may be a possibility that stx-negative strains isolated in HUS patients gain *stx* in some phase of the infection. Subversion with other

species could add to further complication in treating HUS patients. Currents status of available treatment options for HUS patients are discussed below.

1.7 Recent Progress in Treatment

Antibiotics treatment is not advisable for EHEC-induced gasteroenetritis (14, 46). The antibiotic administration may even increase the risk of HUS in infected children (14, 46). As seen with a study using sulfa-containing and beta-lactam antibiotics, antibiotics can release preformed periplasmic toxin into the gut lumen, leading to increased toxin delivery into the systemic circulation and facilitating transport of the toxin to susceptible vascular endothelial cells in the renal glomerulous (14, 46). Therefore it is essential to develop alternate modes of medication. Analogues to Gb3 and Stx-neutralizing monoclonal antibodies that can bind STx, and STx1/2 toxoids are the subject of research aimed at treatment strategies for EHEC infection. However these await appropriate clinical trials (48, 49). Neri *et. al.* (2007) have shown the Gb3 copolymer can neutralize the cytotoxic effect of Stx *in vitro* and when intravenously introduced in mice protected them from death (46). Moreover galacto-trehalose copolymers have been shown to neutralize Stx1 *in vitro* and could be a better choice for oral administration than the Gb3 copolymers due to their stability in the intestine (46). More research is needed to see if this effect could be translated into humans. Other agents like heparin and heparin sulfate have been shown to reduce EHEC colonization by blocking interactions of EHEC intimin with epithelial integrins both *in vitro* as well as *in vivo.* Thus these two molecules are potential therapeutic candidates (24). Ma *et. al.* (2008) have developed four anti-Stx2-

McAbs which have successfully neutralized the toxin in *in vitro* as well as *in vivo* studies (40). The engineering of the anti-Stx2 antibody in their study could be tested for effectiveness in humans for the prevention of EHEC O157:H7 infection. As far as the prevention to EHEC infection is concerned, Marcato *et. al.* (2005) have demonstrated that conjugation of Stx2 B-subunit with adjuvants and carrier proteins could serve as potential vaccine candidate. In fact, such a preparation has been approved to be used on humans (43). For instance as much as 9% of antibiotic resistant clinical isolates of EHEC O26 and O111 were found to have multiple antibiotic resistance to ampicillin-tetracycline-streptomycin-cephalothin-sulfisoxazole-ticarcillin-kanamycin-minocycline-pperacillin-chloramphenicol (31). About 79.8% of STEC O157:H7 from various sources like dairy cows, cull dairy cow feces, cider, salami, human feces, ground beef, bulk tank milk, bovine feces, and lettuce have been shown to be resistant to one or more antibiotics which includes amikacin, carbenicillin, ceftriaxone, cefuroxime, ciprofloxacin, fosfomycin, moxalactam, norfloxacin, streptomycin, tobramycin, trimethoprim, and tetracycline (66). The emergence of multiple resistance and production of STx upon antibiotic administration in EHEC is making it even more difficult to treat the disorders caused by this pathogen. Vaccination with crucial colonization factor has also failed to prevent colonization in the intestine in a study with calves (19).

1.8 Work leading to the project

EHEC is challenged with acid in the environment as well as in the host system. As it enters the host, EHEC must be able to survive the acidity of the gastric acid and the stress

of short chain fatty acids as it makes its journey to the colonization site in the large intestine.

Although a vast amount of research is ongoing to clarify the mechanisms of EHEC pathogenesis, the role of acid and short chain fatty acid stress in the GIT on EHEC virulence is far from clear. Recent research has indicated that acid-adapted acid stress (one hr adaptation at pH 5.0 followed by 30 min acid shock at pH 3.0) leads to enhanced adhesion to host epithelial cells (Figure. 1.6). Identifying the role of candidate adhesins in bacteria-host cell adhesion will improve our understanding of EHEC pathogenicity. Recent data on the effect of acid on candidate adhesins expression has been published by Foster's group (28). The microarray profiling from the published data reported upregulation of several genes in acid-adapted acid-induced EHEC and raised questions of the role of these genes in EHEC pathogenicity (28).

Among the genes that were upregulated in the microarray data of acid stressed EHEC are adhesins shown in the table below (Table 1.2). Expression of genes encoding fimbrial proteins *lpfA1* and *lpfA2* were found to increase by 1.85 and 1.99 fold following acid-adapted acid stress. The expression of *yagZ* and *yadK* expression after acid-adapted acid stress increased by 4.98 and 4.94 fold respectively. Recent research with *yadK* mutant have confirmed the role of YadK, in adhesion of acid-adapted acid induced EHEC to Hep-2 cells in vitro (15). It is therefore worth investigating if the increased expression of *yagZ, lpfA1* and *lpfA2* correlates with increased adhesion under acid-induced stress conditions in bacteria-host adhesion experiments.

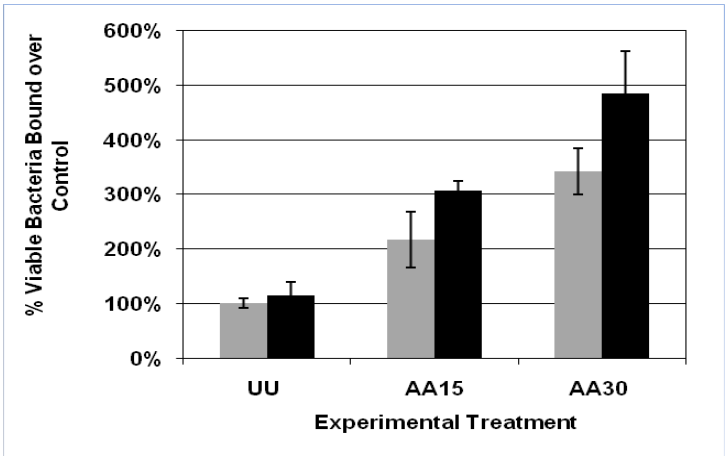


Figure 1.6: Adhesion of acid-induced EHEC to CaCo2 cells. Adhesion of acid adapted acid shocked CL56 to CaCo2 cells in a 3 hour (grey) or 6 hour (black) adhesion assay. UU: Unshocked; AA 15: Acid Adapted Acid Shocked 15 min; AA30: Acid Adapted Acid Shocked 30 min. Values are mean +/_ St Dev.

*Significantly different from 3 hour control UU **Significantly different from 6 hour control UU *** Significantly different from 6 hour control and 6 hour AA15.

Adapted from (28).

Table 1.2: Selected EHEC O157:H7 adhesin genes with their expression fold-change following acid adapted acid stress as determined by micro array analysis (28).

Gene Name	Gene ID	Hypothetical Function	Acid-adapted Acid Induced Expression fold change
yagZ	B0293	orf, hypothetical protein	4.98
lpfA1	Z4971	putative fimbrial major protein precursor	1.85
lpfA2	Z5225	type I fimbrial protein	1.99
yadK	B0136	putative fimbrial adhesion	4.94

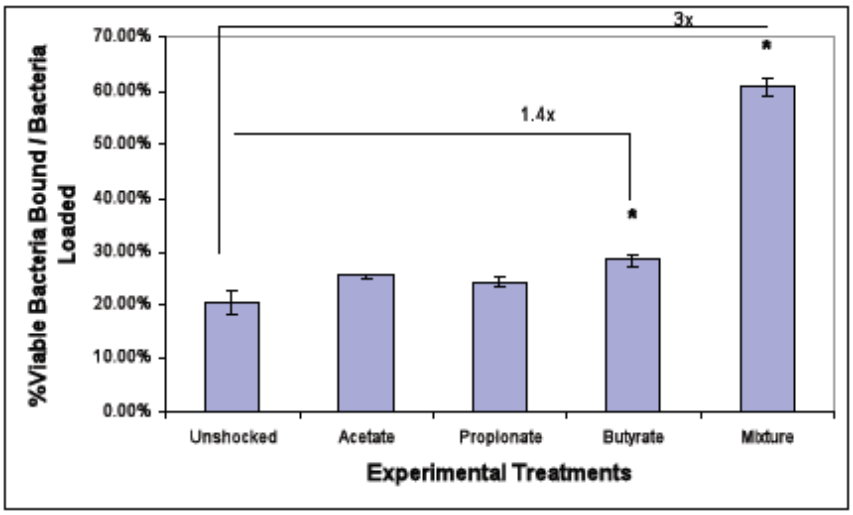


Figure 1.7: Adhesion of EHEC 8624 to HEp-2 cells upon exposure short chain fatty acid. Effect of SCFA stress on *E. coli* O157:H7 8624 in adhesion to HEp-2 cells was assessed by plate count assay (n= 3). Results representative of 2 independent experiments are shown and the values are means ± standard deviations. Short chain fatty acid stress treatment: 16 hr, 37°C, acetate: 110 mM; propionate: 70 mM; butyrate: 20 mM; Mixture of combined acetate-propionate-butyrate: ~ 90 mM

*Significantly different from corresponding control ($P < 0.05$)

Adapted from (63).

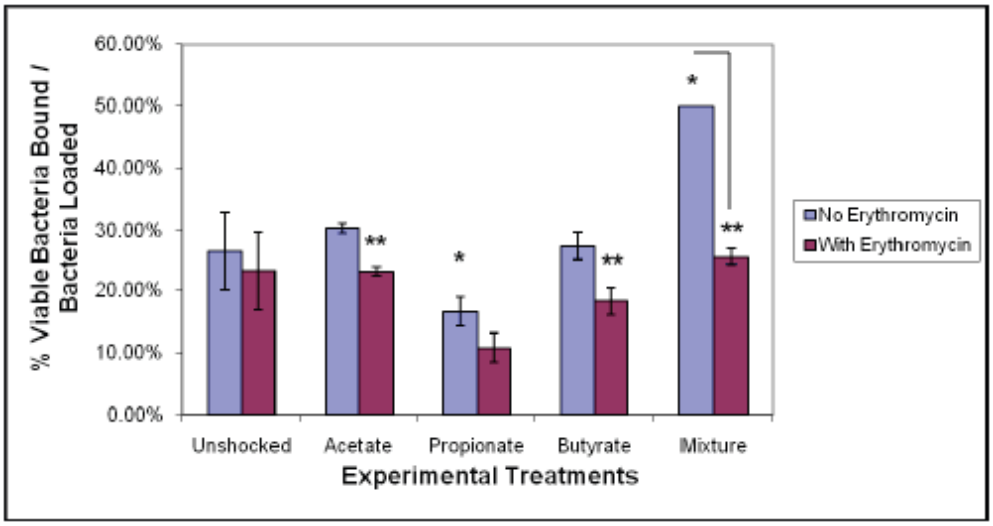


Figure 1.8: Adhesion of SCFA-induced EHEC 8624 to HEp-2 cells with and without Erythromycin Treatment. Effect of erythromycin pretreatment on ~90mM SCFA induced EHEC 8624 in adhesion to HEp-2 cells was assessed by plate count assay (n= 6). Values are means ± standard deviations.

* Significantly different from unshocked control (p= 0.05).

** Significantly different from corresponding SCFA control without erythromycin (p< 0.05)

Adapted from (63).

Previous research by Foster's group has also shown that exposure to a 90 mM SFCA mix (composed of 110 mM acetate, 70 mM propionate and 20 mM butyrate) resulted in a 3 fold increase in adhesion to HEp2 cells (63). Moreover, a more modest increase in adhesion to Hep-2 cells of 1.4 fold was observed after exposure to 20mM butyrate (Figure 1.7). Pretreating the bacteria with erythromycin which has been shown to disrupt bacterial protein synthesis, eliminated the adhesion enhancement seen after SFCA treatments (Figure 1.8) (63).

Expression of novel fimbrial adhesins under either acid-adapted acid stress or short chain fatty acid stress may enhance adhesion of the pathogen to human epithelial cells**.** Therefore, the aim of this thesis was to establish the role of selected fimbrial adhesions *lpfA1, lpfA2* and *yagZ* in acid-adapted acid and short chain fatty acid stress-induced adhesion.

This lead to the hypotheses that:

1. **The fimbrial adhesins *lpfA1, lpfA2* and *yagZ* contribute to the enhanced adherence to host cell seen after exposure to these stress treatments.**

2. **The expression of the fimbrial adhesins *lpfA1, lpfA2* and *yagZ (matB)* are enhanced under each of the acid-adapted acid stress and short chain fatty acids stress.**

1.9 Thesis Objectives

1. **Determine how the adhesins *lpfA1, lpfA2, ecpA* affect the host adhesion phenotype of the pathogen before and after stress treatments**

   The adhesion properties of each of Δ*lpfA1,* Δ*lpfA2 and* Δ*yagZ* mutants relative to the isogenic parent wild type strain will be assessed by adhesion experiments following each of the acid-adapted acid and short chain fatty acid stress.

2. **Determine the expression of selected adhesins of EHEC O157:H7 following stress treatments using promoter**

   β-galactosidase promoter fusion constructs will be made. Subsequently the promoter activity will be determined by β-galactosiadase assay following each of acid-adapted acid stress and short chain fatty acid stress.

2.1 Strains used in this study

Strains used in this study include 8624 wild type parent strain of EHEC ; 8624 lpfA1::cat isogenic *lpfA1* mutant of EHEC 8624 (73); 8624 lpfA2::te isogenic *lpfA2* mutant of EHEC 8624 (73) 8624 ΔecpA::Kan isogenic yagZ mutant of EHEC 8624 (56); EHEC-PA containing galatosidase fusion promoter to *acrA* (personal communication, J V Kus); EHEC-PZ containing galatosidase fusion promoter to *yagZ*; 8624 pMC transformed with pMC1403 (this study) (Table 2.1). A list of plasmids used in this study is provided in Table 2.2.

2.2 Propagation of bacterial cultures

A loopful of respective culture was streaked LB (Luria bertani) plates (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.1 mL 1N NaOH, 1.5% agar, supplemented with desired amount of required antibiotic (100 µg/mL ampicillin, 50 µg/mL kanamycin and 100 µg/mL streptomycin). This was then allowed to incubate overnight. Subculturing was done, if necessary, to get isolated colonies. These were then left in 4˚C and used for no more than one month, after which the process of subculturing was repeated if the bacteria of concern was to be used in further experiments.

2.3 Epithelial cell culture

The human epithelial cells, HEp-2 (**ATCC**CCL-23) were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Winsent Inc.) supplemented with 10% fetal calf

serum (Fetal calf serum, Winsent Inc.) and 10 µg/mL gentamycin and incubated at 37°C and 5% CO_2. Following microscopic confirmation of the confluent status of cells, the medium was discarded and 5 mL phosphate buffered saline (PBS, Winsnent Inc.) was added which was also discarded after a few seconds for rinsing. Then 2 mL of trypsin was added and allowed required incubation interval so that the cells could detach from the T25 cm^2 flask (TPP). Thereafter 1 mL of the HEp-2 cells was transferred into 7 mL Dulbecco's Modified Eagle's Medium (DMEM, Winsent Inc.) supplemented with 10% fetal calf serum (FCS, Winsent. Inc), to propagate a new passage, supplemented with gentamycin and incubated at 37°C and 5% CO_2 to replenish the media (Table 2.3).

The cells were washed with 10 mL PBS (Winsent Inc.), trypsinized as before and resuspended in 10ml DMEM (Winsent Inc.). 10µl of this sample were taken for counting with the help of a microscope (Olympus) by using a haemocytometer (Bright-Line). Cell culture grown in a 25 cm^2 vented culture flask from section 2.4 were counted using a haemocytometer (supplier) and added to 24-wells of flat bottom plates so that a cell monolayer of $2x10^5$ cells/well could be achieved by the next day. Dilutions of HEp-2 cells were done in DMEM. DMEM (Winsnet Inc.) was then added to each well to make a total volume of 500µl. The plates were incubated overnight at 37°C and 5% CO_2.

2.4 Acid Stress Treatment

A single isolated colony of bacteria was inoculated into 10 mL LB broth (with or without antibiotic) at 37°C with agitation, and grown overnight as previously described (28). The culture was diluted 10-fold in DMEM (pH 7.4) and subcultured without shaking at 37°C with 5% CO_2 to log phase.

Table 2.1: List of bacterial strains used in this study

Strains of EHEC O157:H7	Features	Features	Reference
8624	Wild Type	N/A	Rendon et. al., 2007
8624 Δ*ecpA*::Kan	*yagZ* mutant	Kanamycin^R	Rendon et. al., 2007
8624 C ΔecpA::Kan	*yagZ* complemented mutant	Kanamycin^R	Rendon et. al., 2007
8624 *lpfA1*::cat	*lpfA1* mutant	Streptomycin^R	Torres et. al., 2002
8624 *lpfA2*::te	*lpfA2* mutant	Streptomycin^R	Torres et. al., 2002
8624 pSE	pSE380 transformed 8624	Kanamycin^R	This study
8624 pSE-Δ*ecpA*	pSE380 transformed 8624 ΔecpA	Kanamycin^R	This study
8624 pMC	8624 transformed with pMC1403	Ampicillin^R	This study
8624-PA	8624 transformed with PacrA-pMC1403	Ampicillin^R	J V Kus
8624-PZ	8624 transformed with PMatB-pMC1403	Ampicillin^R	This study

Table 2.2 List of plasmids used in the study

Name of plasmid	**Function**	**Reference**
pMC1403	Promoter-less *lacZ*	J V Kus
PMatB-pMC1403	Encodes *lacZ* under the control of promoter of *yagZ*	This study
PacrA-pMC1403	Encodes *lacZ* under the control of promoter of *acrA*	J V Kus

Two mL of the subculture were resuspended with 10 mL DMEM + 25 mM MES (pH 5.0) and acid adapted statically at 37 °C with 5% CO_2 for 1 h. After adaptation, the bacteria cells were resuspended in 10 mL DMEM (pH 3) and acid shocked for 30 min at 37 °C with 5% CO_2. Unadapted, unshocked controls were resuspended in 10 mL DMEM with 25 mM MOPS (pH 7.4). The cell pellets were resuspended with 10 mL DMEM (pH 7.4) prior to use for infection of HEp-2 cells. Alternatively, the bacterial cells were resuspended in PBS following use in β-galactosidase assays.

2.5 SCFA Stress Treatment

A single isolated colony of bacteria was inoculated into 10 mL LB broth (untreated) or 10 mL LB supplemented with 90 mM SCFA (110 mM acetate, 70 mM propinate, 20 mM butyrate) with or without antibiotic, at 37°C with agitation, and grown overnight as previously described (63). The respective cultures were subcultured statically in DMEM (unshocked) or DMEM supplemented with 90 mM SCFA (shocked) at 37 °C with 5% CO_2, until OD600 of 0.4-0.6 was reached. The cell pellets were resuspended with 10 mL DMEM (pH 7.4) prior to subsequent use for infection of HEp-2 cells. Alternatively, the bacterial cells were resuspended in PBS for following use in β-galactosidase assays.

2.6 Adhesion Assay

Bacteria were grown in liquid broth (LB) for 5-6 h and centrifuged at 3500 x g for 10 minutes and the pellets resuspended in 90 mM SCFA and LB while the control had no

SCFA. The LB culture was incubated at 37°C for 16 -18 h. 1/10th subculture was resuspended in SCFA (110 mM acetate, 70 mM propionate, 20 mM butyrate) and DMEM and allowed two hrs of induction at 37°C. For the acid stress protocol, 1 mL of overnight unshocked culture was grown in DMEM to a 1/10th dilution until the culture reached an OD600 of 0.4-0.6 and then the 2 mL of the cultures used each for unadapted unstressed and acid adapted acid stress treatment as described earlier in section 2.5. The OD600 was measured once again (to make sure that OD600 did not exceed 0.4-0.6 and that the culture is in log phase). At the same time, the monolayer of cell lines were washed twice with PBS and plain DMEM at pH 7.4 was added to the wells. The required volume of bacterial suspension to get multiplicity of infection 50~100:1 was used to infect the monolayer of cell lines. The volume of DMEM pH 7.4 and bacterial suspension was adjusted to a total volume of 500 µl/well in the well plate by adding DMEM pH 7.4 as necessary. Three hours of infection was allowed at 37°C and 5% CO_2. At the same time, serial dilutions of bacterial sample in DMEM at pH 7.4 were made to determine viability of the bacterial suspensions after stress treatments (Figure 2.1). After the 3 hrs of infection the wells were washed with PBS 3 times and trypsinized with 250 µl of trypsin/EDTA (Winsent Inc.) for 5 minutes at 37°C. Aliqiout 50 µl of FBS (Winsent Inc.) was added then to each well and allowed incubation for a further 2-3 minutes at 37°C. Serial dilutions were prepared from each samples and 15µl of appropriate dilutions were plated on LB agar or tryptone agar media as required to determine the number of adherent bacteria. These along with the plates for viability mentioned earlier were incubated overnight at 37°C. The numbers of colony forming units (CFU) in the dilutions were used to determine the percentage of bacteria that adhered to the cell line cultures.

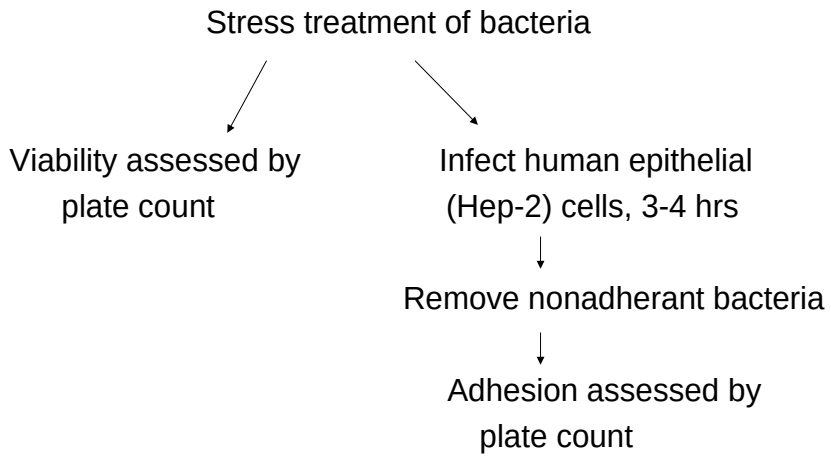


Figure 2.1: Simplified flowchart to represent the methodology for adhesion assays. Bacteria are grown overnight and stressed (acid adapted acid stress or short chain fatty acid stress). The stress treated bacteria are used to infect monolayers of HEp-2 cells. At the same time viability of the bacteria after stress treatments and before infection are assessed by plate count. Post infection of 3 hrs, number of adherent bacteria are assessed by plate count method.

2.7 Preparation of PMatB β-galactosidase construct

The predicted promoter region of *yagZ* was identified from regulon database (http://regulondb.ccg.unam.mx/). It matched the promoter mentioned in previous publication (39). The promoter region was amplified and ligated into pMC1403 and subsequently transformed into chemically competent DH5-α E. coli. After confirmation of a mutation free-sequence, the β-galactosidase promoter fusion construct was electroporated into electrocompetent EHEC 8624. β-galactosidase assay was performed following acid-adapted acid and short chain fatty acid stress to determine the promoter activity of *yagZ* (*matB*).

2.7.1 Purification of plasmid pMC1403

An isolated colony of *E. coli* K-12 (J V Kus) was inoculated in 10 mL of LB overnight at 37°C in a shaking incubator. 1.5 mL of the overnight culture was centrifuged at 130000 x g for 2 min and used with a miniprep kit (Bio Basic Ltd). 100 µl of Soultion I was added and incubated at room temperature for a min. 200 µl of Solution II was added and allowed another min of incubation at room temperature followed by addition of Solution II and incubation for one min at room temperature. The sample was centrifuged for 5 min at 12,000 rpm. The supernatant was transferred to EZ-10 column and centrifuged at 10,000 rpm for 2 min and this step was repeated thrice. The clean column was transferred to a sterile 1.5 ml centrifuge tube and 50 µl elution buffer added and the sample incubated at room temperature for 2 min. Centrifugation at 10,000 rpm was repeated for 2 min. The pMC1403 DNA plasmid prep thus obtained was stored at -20°C for future use (Table 2.1, Figure 2.2).

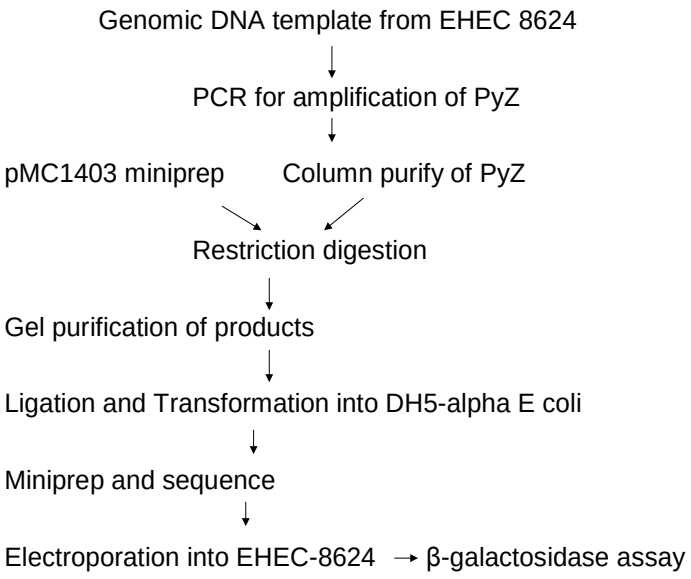


Figure 2.2: Simplified methodology for the expression of the promoter of *yagZ/matB*. PMatB, the predicted promoter region of matB, was amplified from EHEC 8624 and the PCR product obtained cleaned using a PCR clean-up kit (Biorad). Miniprep of pMC1403 and the clean PCR product was double digested (with BamHI and EcoRI, Fermentas). The digested products were run on an 1% agarose gel and extracted from gel (kit from Biorad). The clean digested plasmid and amplified produce were ligated using T4 DNA ligase (Fermentas) and transformed into chemically competent *E. coli.* Miniprep of the liagted pMC1403 was sent for sequencing and provided the sequence read was mutation-free, the plasmid was electroporated into electrocompetent EHEC 8624.

2.7.2 PCR Analysis

The promoter region of *yagZ (matB)* was identified using regulon database (http://regulondb.ccg.unam.mx/) and compared with previous findings by Low et. al (39). Polymerase chain reaction (PCR) was routinely used to amplify the promoter region of *matB* using Taq DNA Polymerase (Genescript Corp.),as per protocols described by Genescript Corp. All PCR reactions were performed in a 96-well Biorad PCR machine with an annealing temperature of 50.7ºC. PCR products (10 uL) were resolved on 1% agarose gels and visualized after ethidium bromide-staning under UV illumination. The primers used for this study are listed on Table 2.3.

2.7.3 PCR clean up

100 uL of PCR product of the predicted promoter region of *matB* (PmatB) was transferred into a microcentrifuge tube for clean-up using PCR clean-up kit (Bio Basic Inc). Five times of DF buffer was added to the volume of the DNA sample. A DF column was added to a collection tube and the sample transferred to the DF column. Centrifugation was done at 13,000 rpm for half a min and the flow-through discarded. 500 µl of wash buffer was added to the DF column and centrifugation repeated as before. The flow-through was discarded and centrifugation was repeated as before. The DF column was transfreed to a sterile 1.5 mL centrifuge tube and 40 µl of elution buffer added to the column matrix for am incubation period of 2 min at room temperature. Centrifugation was repeated as mentioned earlier. The eluted purified DNA was stored at -20°C for future use.

Table 2.3: Primers used for PCR in this study.

Primer	Primer sequence	Tm (° C)	Product
pMatB-F-EcoRI(2)	**5'AAGAATTCAAAATGATTACAGCAGGG**	**50.7**	**PMatB, predicted promoter of *matB*** (this study)
pMatB-R-BamHI	**5'AAGGATCCAGAGCTATTGCCAGAAC**		
pMC1403-F	**5'TGCCACCTGACGTCTAAGAA**	**55**	**Multiple cloning site of pMC1403** (J V Kus)
pMC1403-R	**5'GTTTTCCCAGTCACGACGTT**		

2.7.4 Restriction Digestion

Double digestion of DNA sample: 5 uL of DNA sample (of both pMC1403 and PmatB) was mixed with 4 uL of 2xTango buffer (Fermentas) and 9.5 uL of nuclease-free water. Then, 0.5 uL of EcoRI (Fermentas) and 1 uL of BamHI (Fermentas) was added to the sample; the sample was then incubated for an hour at 37°C in a water bath. An 8 uL of the digested sample (to check the size of the digested band) was loaded on 1 % agarose gel and the rest stored at -20°C for future use.

Single digestion of DNA sample: 5 uL of DNA sample (pMC1403) was mixed with 11 uL of nuclease-free water and 2 uL of 2x Tango buffer (Fermentas) and 2 uL of target restriction enzyme. The sample mixture was incubated for one hour at 37°C in a water bath. An 8 uL of the digested sample (to check the size of the digested band) was run on 1 % agarose gel.

2.7.5 DNA Extraction

Agarose gel containing the relevant DNA band was excised and the excess of agarose was excised to minimize the gel slice weight. 300mg of the slice was transferred into a microcentrifuge tube for DNA extraction with the gel extraction kit (Bio Basic Inc.). The eluted pure DNA products were stored at -20°C for future use.

2.7.6 Preparation of chemically competent DH5-α *E.coli*

A single colony of DH5-α *E.coli* was inoculated in 50 mL of LB at 37°C in a shaking incubator. 4 mL of the overnight culture was subcultured in a 400 mL of LB under the

same conditions until the OD600 reached 0.400. Aliquots of 50 mL were made and kept on ice for 5-10 min and then centrifuged for 7 mins at 3000 rpm at 4°C. The supernatant was discarded and the pellets gently resuspended in 10 mL of ice-cold $CaCl_2$ solution (60mM $CaCl_2$, 15% glycerol, 10mM PIPES; filter sterilized and stored at 4°C). The samples were centrifuged at 2500 rpm for 5 min. The supernatant was discarded and the pellets resuspended in 10 mL of $CaCl_2$ and the samples were incubated for 30 min on ice. The samples were then centrifuged at 2500 rpm at 4°C for 5 min as above. The pellets were resuspended in 2 mL of ice-cold $CaCl_2$ as above. Aliquots of 200 uL was stored in pre-chilled eppendorf tubes and stored at -80°C for future use.

2.7.7 Preparation of electrocompetent EHEC-8624

An isolated colony of EHEC 8624 was inoculated in LB overnight at 37°C in a shaking incubator. 1 mL of the overnight culture was subcultured in 100 mL of LB under same conditions until the OD600 reached 0.400. The subculture was incubated on ice for 15 min and 50 mL aliquots centrifuged at 3000 rpm for 10 min. The pellets were resuspended in 25 mL ice-cold sterile water and centrifuged as before. The pellets were resuspended in 10 mL of cold 10% glycerol and centrifuged again. Resuspension of pellets in 10% glycerol was repeated followed by centrifugation. The pellets were finally resuspended in 1 mL of 10% glycerol. Aliquots of 200 uL were stored at -80°C for future use.

2.7.8 Ligation

5 uL of vector DNA (clean double-digested pMC1403) was mixed with 10 uL of insert

DNA (clean double-digested PmatB). 4 uL of 5X buffer and 1 uL of ligase was added to the mixture (Fermentas; rapid DNA ligation kit). The mixture was incubated at room temperature for less than an hour. 5 uL of this mixture was added to 50 uL of chemically competent DH5-α *E. coli* (this study) for transformation.

2.7.9 Transformation and Electroprataion

Transformation of DH5-α *E. coli* : 5 uL of ligated plasmid DNA was mixed with 50 uL of chemically competent DH5-α *E. coli* and allowed to stand on ice for 30 mins. The sample was heat shocked at 42°C for 45s and allowed to stand at room temperature for 2 mins. 1 mL of SOC (0.5% yeast extract, 2% tryptone, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM glucose) was added to the sample and incubated at 37°C for 1.5 hrs in a shaking incubator. 200 uL of the sample was plated on to ampicillin agar plates and incubated at 37°C overnight. The predicted promoter region of *yagZ* that was transformed into *E coli* DH5-α, was sequenced by ACGT Corp and compared with the established *yagZ* gene sequence using ClustalW sequence alignment program. The alignment confirmed that the predicted PMatB was mutation-free (Figure 2.3).

Electroporation of EHEC 8624: 50 uL of electrocompetent EHEC-8624 was mixed with 10 uL of plasmid (miniprep from the transformed DH5-α *E. col)* in a pre-chilled eppendorf tube. The mixture was transferred in an electroporating cuvette. The electroporator was used to electroporate for a pulse and the sample mixture added to 1 mL of SOC. 1 hour incubation at 37°C in a shaking incubator followed. 200 uL of sample was grown on ampicillin agar plates to cultivate the recombinant EHEC 8624 containing the galactosidase fusion construct bearing the promoter of *matB,* EHEC 8624-PZ.

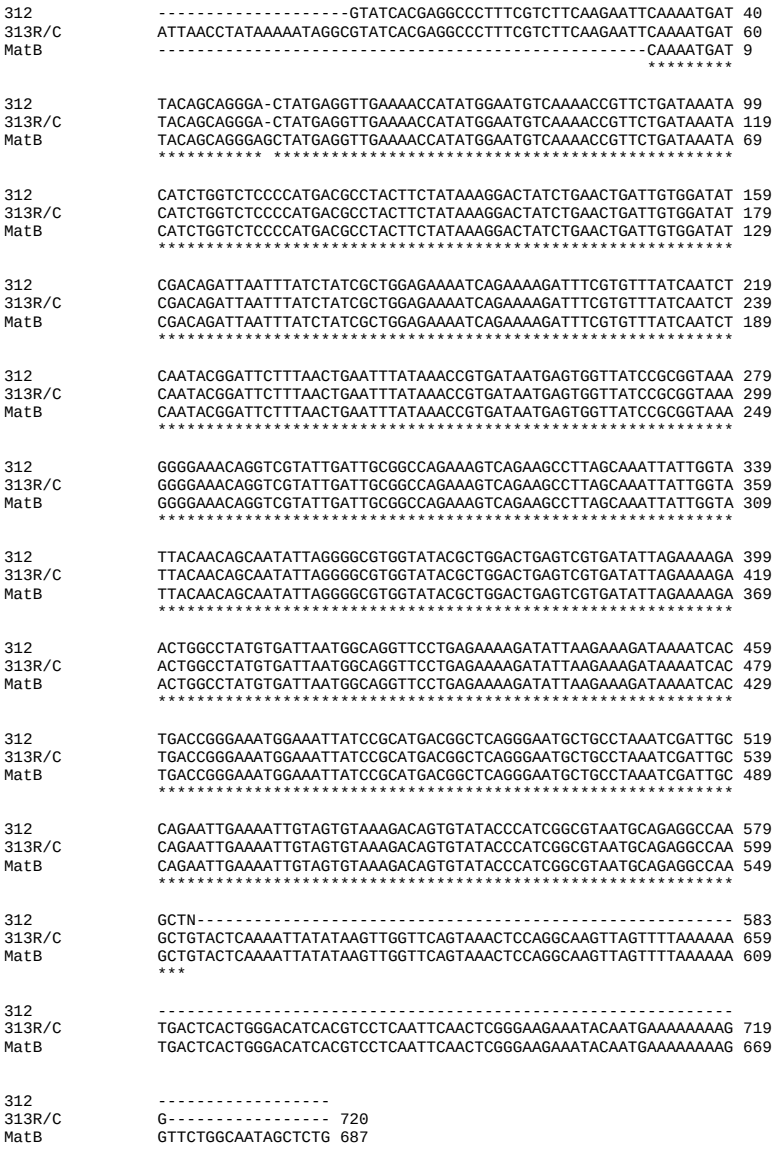


Figure 2.3: Sequence Alignment of promoter of *matB*. The predicted promoter region of *matB*, PMatB was amplified using the forward primer 5' AAG/ AAT TCA AAA TGA TTA CAG CAG GG and reverse primer 5' AAG/ GAT CCA GAG CTA TTG CCA GAA C from EHEC 8624 wild type. PMatB was ligated into pMC1403 plasimd and transformed into chemically competent *E. coli* DH5α, propagated in ampiciliin-selective media. Miniprep obtained from the overnight thereafter was sent to ACGT for sequencing. The sequence obtained was aligned using ClustaIW multiple alignment software. 312: sequence using with the forward primer; 312R/C: reverse and complemented sequence from the read of the reverse primer, Gene Runner software used; MatB: template sequence of PMatB

2.8 Analysis of β-galactosidase activity

Overnight culture was grown from isolated colonies EHEC 8624 P (bearing pMc1403 only), PZ (bearing the fusion construct of β-gal PyZ) or PA (bearing the fusion construct of β-gal P*acrA*) and the culture were stressed according to the stress protocols. Following stress, the subcultures were suspended in PBS. Aliquots of the subcultures were added to 20 uL of chloroform, 20 uL 1% SDS and Z-buffer (0.06 M Na_2HPO_4, 0.04 M NaH_2PO_4, 0.01 M KCl, 0.001 M $MgSO_4$ and 0.05 M beta-mercaptoethanol; stored at 4°C; personal communication Dr Kus). The aliquots and Z-buffer (J V Kus) volume were adjusted so that the total sample mixture is 1040 uL. The mixture is vortexed and allowed to settle for 2 mins at room temperature. 100 uL of the supernatant is transferred to a well of the 96 well-plate. The samples were incubated for 15 mins at 28°C and 150 uL of ONPG (0.933 mg/mL suspended in Z-buffer; stored at 4°C) added to the well. The OD570 and OD414 were recorded with a plate reader. The β-galactosidase activity was determined by the equation below (personal communication, J V Kus):

Miller Units = $1000 \times [(OD_{414} - 1.7 \times OD_{570})] / (T \times V \times OD_{600})$ where,

OD_{414} and OD_{570} are read from the reaction mixture.

OD_{600} reflects cell density in the PBS cell suspension.

T = time of the reaction in minutes.

V = volume of culture used in the assay in mLs.

Statistical analysis of the data was done with a student's TTest.

2.9 Statistical analysis

Statistical analysis was done with student's TTest. Average and standard deviation of three technical replicates from one biological sample were considered for viability assays. Student's TTest was performed to compare between untreated control and treatment counterparts. Statistical analysis was done in a similar manner for the adhesion assays. 2-way ANOVA was done to compare the effect of deletion of genes of the mutant stress with response as treatments and category as independent variables.

For the β-galactosidase assay, average and standard deviation was performed on four replicates from biological samples. Student's TTest was performed to compare promoter activity statistical significance difference between untreated control and treatment counterparts.

3.1 Role of selected adhesins in host adhesion with and without stress

Adhesion to host cells is a requisite in the multiple step of pathogenesis of EHEC and colonization in the large intestine. The following section deals with potential role of selected adhesins of EHEC in adherence to human epithelial cells *in vitro.*

3.1.1 Viability of EHEC 8624 and isogenic mutants after acid adapted acid stress

There was a significant decrease in the viability of 8624 wild-type following acid-adapted acid stress. A similar pattern was seen with the Δ *yagZ,* Δ*lpfA1 and* Δ*lpfA2* mutants after acid stress treatments (Figure 3.1). Although there was a reduction in the viability of stressed bacteria following acid adapted acid stress, there was a considerable amount of stress-exposed viable bacteria.

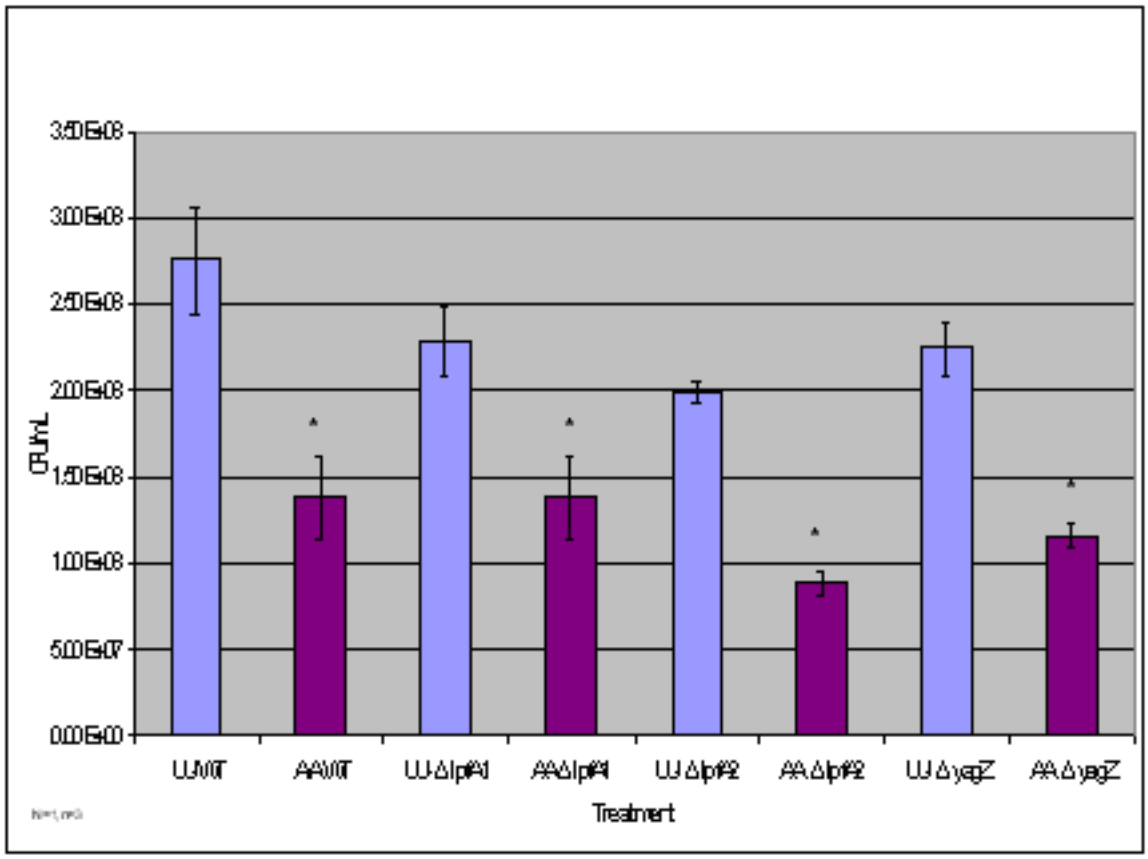
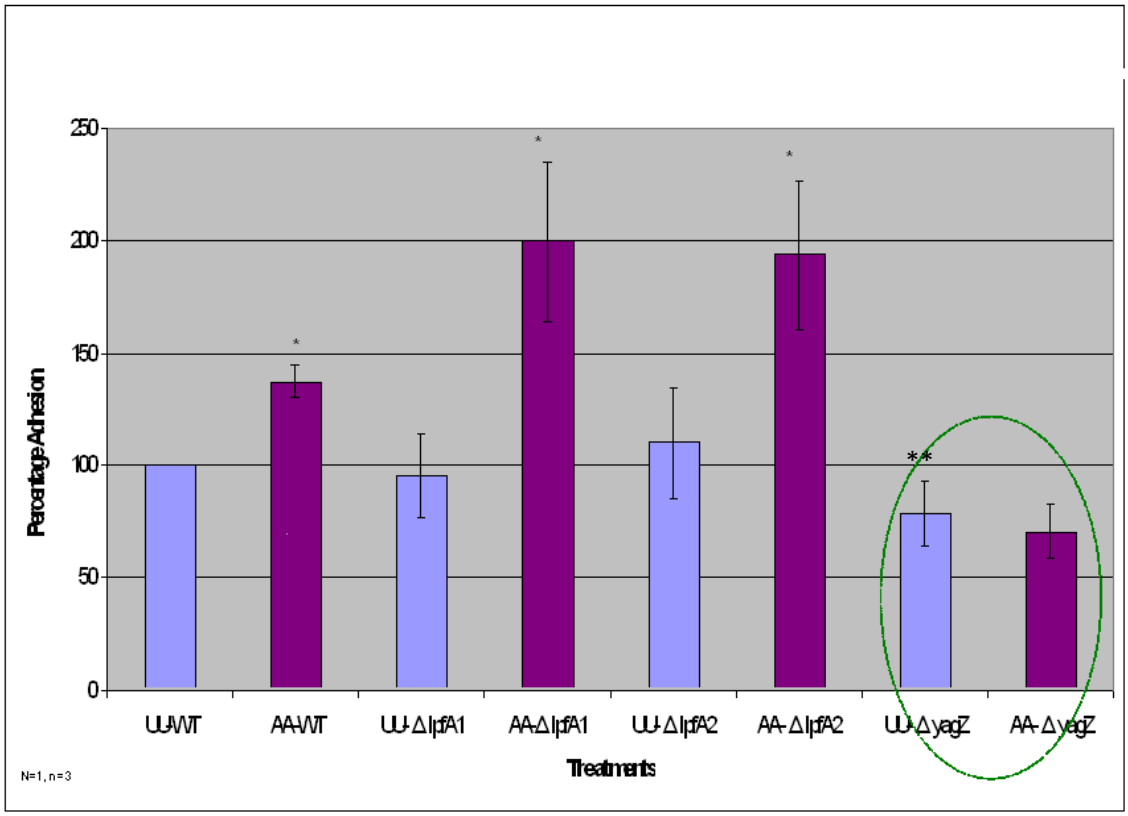


Figure 3.1: Viability of EHEC 8624 and mutants of *yagZ*, *lpfA1* and *lpfA2* following acid adapted acid stress. Effect of acid adapted acid stress on viability was assessed by a plate count assay (N (number of biological samples) =1, n (number of counts per biological sample) =3). Results representative of 2 independent experiments. Values are means ± standard deviations. Bacteria were adapted for 1 hr at pH 5.0 and shocked for 30 min at pH 3.0. Controls were adapted as well stressed at pH 7.4. Results representative of 2 independent experiments. Values are means ± standard deviations. UU: unadapted/unstressed; AA: acid adapted/acid stressed. WT: wild type; ΔyagZ: *yagZ* mutant; Δ lpfA1- *lpfA1* mutant; ΔlpfA2: *lpfA2* mutant. *Significantly different from corresponding control (P≤0.10)

3.1.2 Adhesion of EHEC 8624 and mutants to human epithelial cells after acid adapted acid stress

The adhesion of 8624 wild-type, Δ*lpfA1 and* Δ*lpfA2* mutants to Hep2-cells increased after acid adapted acid stress. In fact, the mentioned mutants were hyperadherant suggesting that deletion of *lpfA1* or *lpfA2* may result in upregulation of other genes that can enhance adherence or the absence of *lpfA1* and *lpfA2* may unmask other adhesins. On the other hand, adhesion of theΔ *yagZ* mutant was significantly decreased compared to wild-type cells following acid stress treatment (Figure 3.2).



Fre 3.2: Adhesion of acid-adapted acid stress *EHEC* 8624 and mutants to HEp-2 Cells. Effect of acid adapted-acid stress on *E. coli* O157:H7 in adhesion to HEp-2 cells was assessed by plate count assay (N=1, n= 3). Results representative of 2 independent experiments are shown and the values are means ± standard deviations. Bacteria were adapted for 1 hr. at pH5.0 and stresses for 30 min at pH 3.0 at 37°C, 5% CO_2. Controls were adapted as well as stressed at pH 7.4 37°C, 5% CO_2. Hep-2 cells were infected with bacteria for 3-4 hrs to determine adhesion at 37°C, 5% CO_2. Adhesion to Hep-2 has been normalized to the unadapted, unstressed wild-type. Results representative of 2 independent experiments are shown and the values are means ± standard deviations. UU: unadapted/unstressed; AA: acid adapted/acid stressed. WT: wild type; ΔyagZ: *yagZ* mutant; ΔlpfA1 - *lpfA1* mutant; ΔlpfA2: *lpfA2* mutant. **AA significantly different from corresponding control UU (P≤0.10); ** significantly different from UU wild type (P<0.10)

3.1.3 Viability of EHEC 8624 and mutants after short chain fatty acid stress

There was a significant decrease in the viability of 8624 wild-type following SCFA stress. A similar pattern was seen with the Δ *yagZ*, Δ*lpfA1 and* Δ*lpfA2* mutants following 90mM SCFA stress treatments (Figure 3.3). Although there was a reduction in the viability of stressed bacteria following short chain fatty acid stress, there was a considerable amount of stress-exposed viable bacteria. There was also a decrease in viability of the Δ*yagZ*.

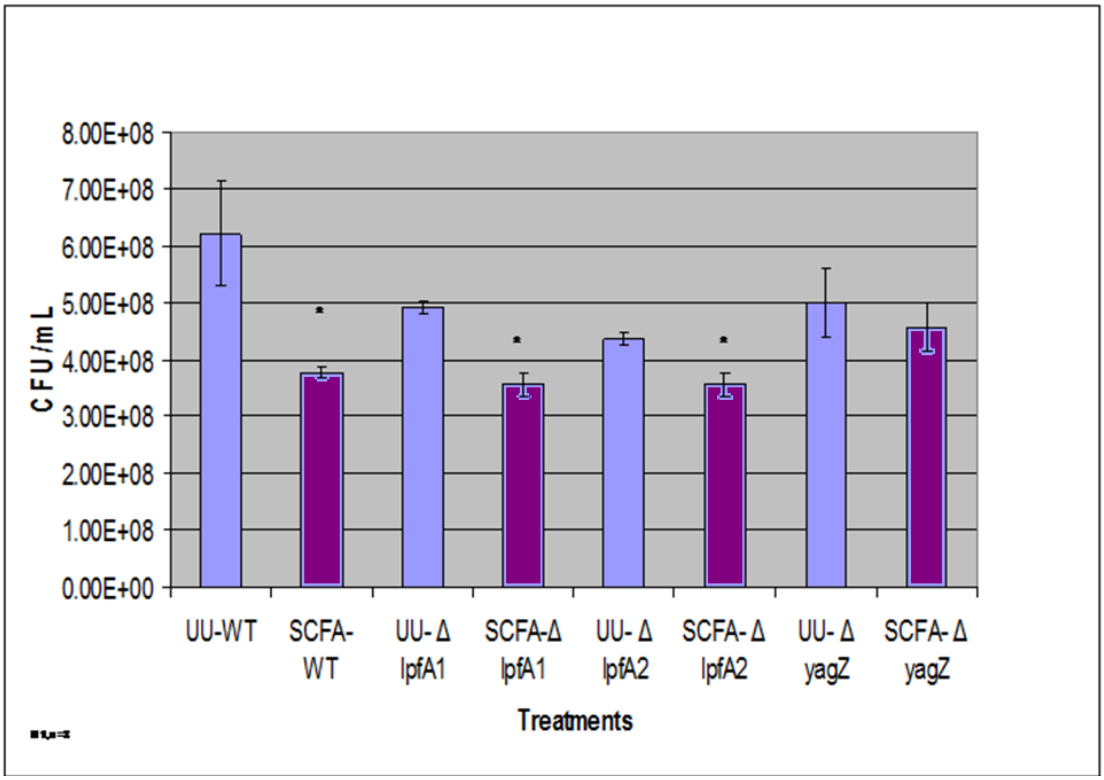


Figure 3.3: Viability of EHEC 8624 strain 8624 and mutants of *yagZ, lpfA1* and *lpfA2* following SCFA stress. Effect of acid adapted acid stress on viability was assessed by a plate count assay (N (number of biological sample) =1, n (number of counts per biological sample) =3). Result representative of 2 independent experiments. Values are means ± standard deviations. Bacteria were grown overnight in 90mM SCFA and stressed in DMEM supplemented with 90 mM SCFA at 37°C, 5% CO_2. Controls were adapted as well stressed at pH 7.4 37°C, 5% CO_2. UU: unstressed; SCFA: SCFA stressed. WT: wild type; ΔyagZ: *yagZ* mutant; ΔlpfA1 - *lpfA1* mutant; ΔlpfA2: *lpfA2* mutant. *Significantly different from corresponding control (P≤0.10)

3.1.4 Adhesion of EHEC 8624 and mutants to human epithelial cells after SCFA stress

The adhesion of 8624 wild-type, Δ*lpfA1 and* Δ*lpfA2* mutants to Hep2-cells increased after SCFA stress. In fact, the mentioned mutants were hyperadherant suggesting that deletion of *lpfA1* and *lpfA2* may upregulate other genes that can enhance adherence. The unshocked mutants of lpfA1 and lpfA2 were more hyperadherant relative to the wild type suggesting they are not critical for adhesion to host cells whether induced or non-induced by SCFA (Figure 3.4). On the other hand, adhesion of the *yagZ* mutant was significantly decreased compared to wild-type following SCFA stress treatment. This implies that YagZ might play a role in the SCFA-induced adhesion of 8624 to human epithelial cells *in vitro*.

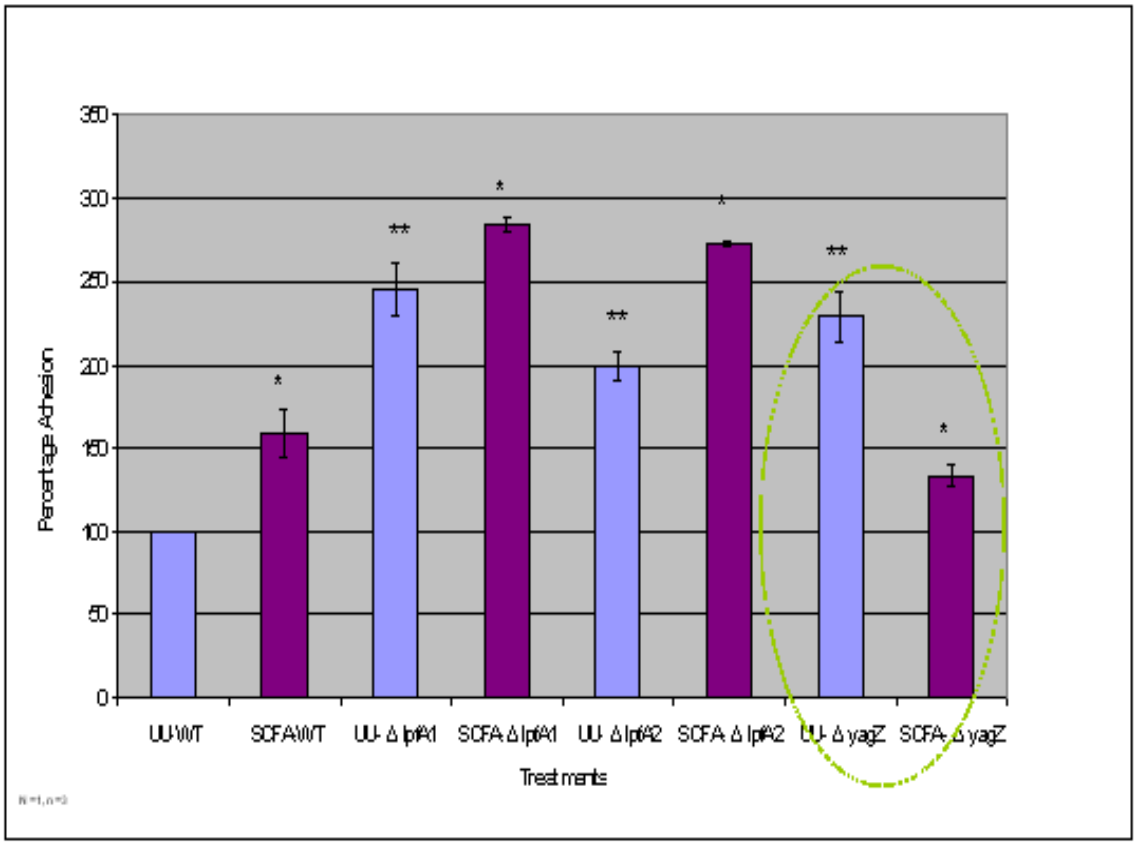


Figure 3.4: Adhesion of SCFA-stressed EHEC 8624 and mutants to HEp-2 Cells. Effect of SCFA stress on *E. coli* O157:H7 in adhesion to HEp-2 cells was assessed by plate count assay (N=1, n= 3). Results representative of 2 independent experiments are shown and the values are means ± standard deviations. Bacteria were grown overnight in 90mM SCFA and stressed in DMEM supplemented with 90 mM SCFA at 37°C, 5% CO_2. Controls were adapted as well stressed at pH 7.4 37°C, 5% CO_2. HEp-2 cells were infected with bacteria for 3-4 hrs to determine adhesion at 37°C, 5% CO_2. Adhesion to Hep-2 has been normalized to the unstressed wild-type. Results representative of 2 independent experiments are shown and the values are means ± standard deviations. UU: unstressed; SCFA: SCFA stressed. WT: wild type; ΔyagZ: *yagZ* mutant; ΔlpfA1 - *lpfA1* mutant; ΔlpfA2: *lpfA2* mutant. *SCFA significantly different from corresponding control UU (P≤0.10); ** significantly different from UU wild type (P<0.005)

3.1.5 Viability of EHEC 8624 and complemented mutants after acid adapted acid stress

Since the complemented mutant of *yagZ* had vector, wild type EHEC 8624,Δ *yagZ* were transformed with pSE380 vector and all the strains were tested for viability. There was a significant decrease in the viability of 8624 wild-type following acid adapted acid stress. A similar pattern was seen with thΔ *yagZ*, comp-*yagZ* mutants acid stress treatments. Although there was a reduction in the viability of stressed bacteria following acid adapted acid stress, there was a considerable amount of stress-exposed viable bacteria (Figure 3.5).

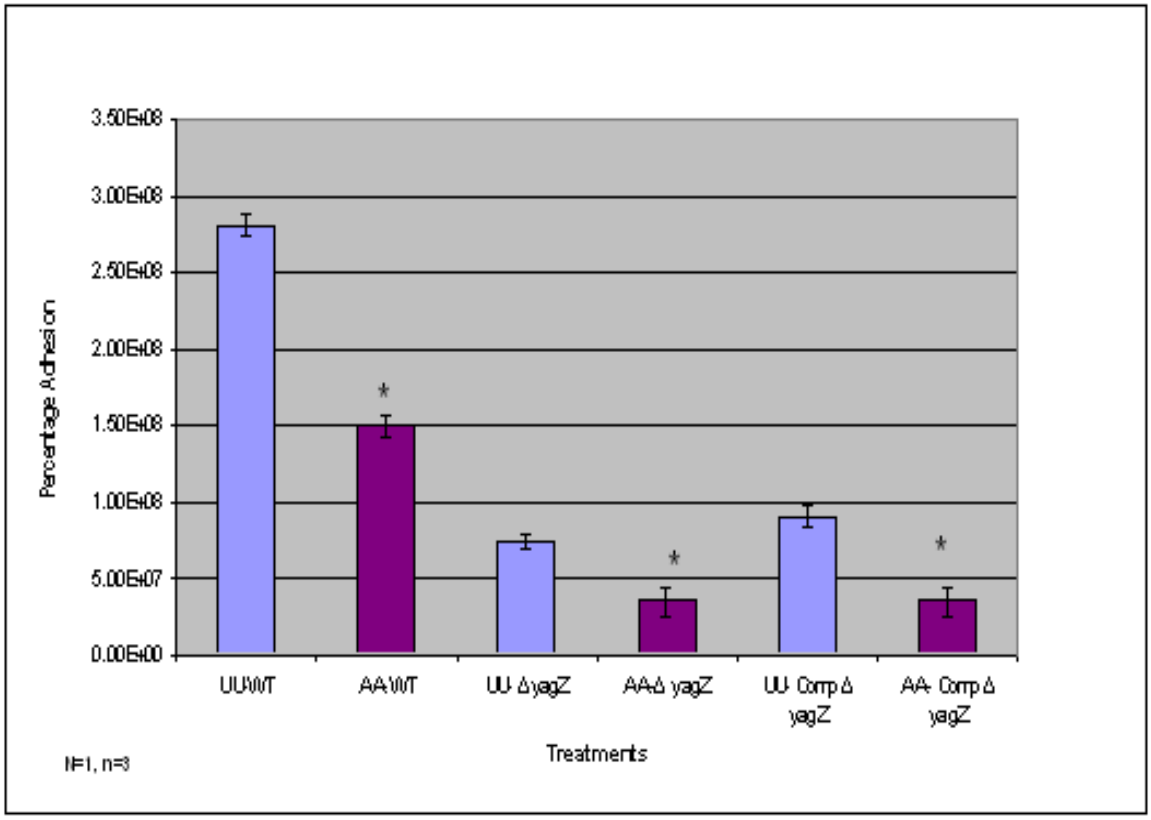


Figure 3.5: Viability of EHEC 8624 strain 8624 and complemented mutants of *yagZ* following acid adapted acid stress. Effect of acid adapted acid stress on viability was assessed by a plate count assay (N (number of biological sample) =1, n (number of counts per biological sample) =3). Result representative of 2 independent experiments. Values are means ± standard deviations. Bacteria were adapted for 1 hr. at pH5.0 and shocked for 30 min at pH 3.0. Controls were adapted as well stressed at pH 7.4. Result representative of 2 independent experiments. Values are means ± standard deviations. UU: unadapted/unstressed; AA: acid adapted/acid stressed. WT: wild type; ΔyagZ: *yagZ* mutant; comp-*yagZ*- complemented *yagZ* mutant. *Significantly different from corresponding control (P≤0.10)

3.1.6 Adhesion of EHEC 8624 and complemented mutants to human epithelial cells after acid adapted acid stress

The adhesion of the Δ *yagZ* mutant was significantly decreased compared to wild-type cells following acid stress treatment (Figure 3.6). However, the enhanced adhesion of acid-induced complemented strain was only partially restored, possibly because all the genes regulated by a common promoter were not cloned back in the complemented strain. The complemented strain had *yagZ* and *matC* restored in frame, both of which have been reported to be under the control of the promoter of *yagZ*. Whether other genes downstream of *matC, yagX, yagW* and *yagV,* are also regulated by the same promoter is yet unknown (see Figure 1.3).

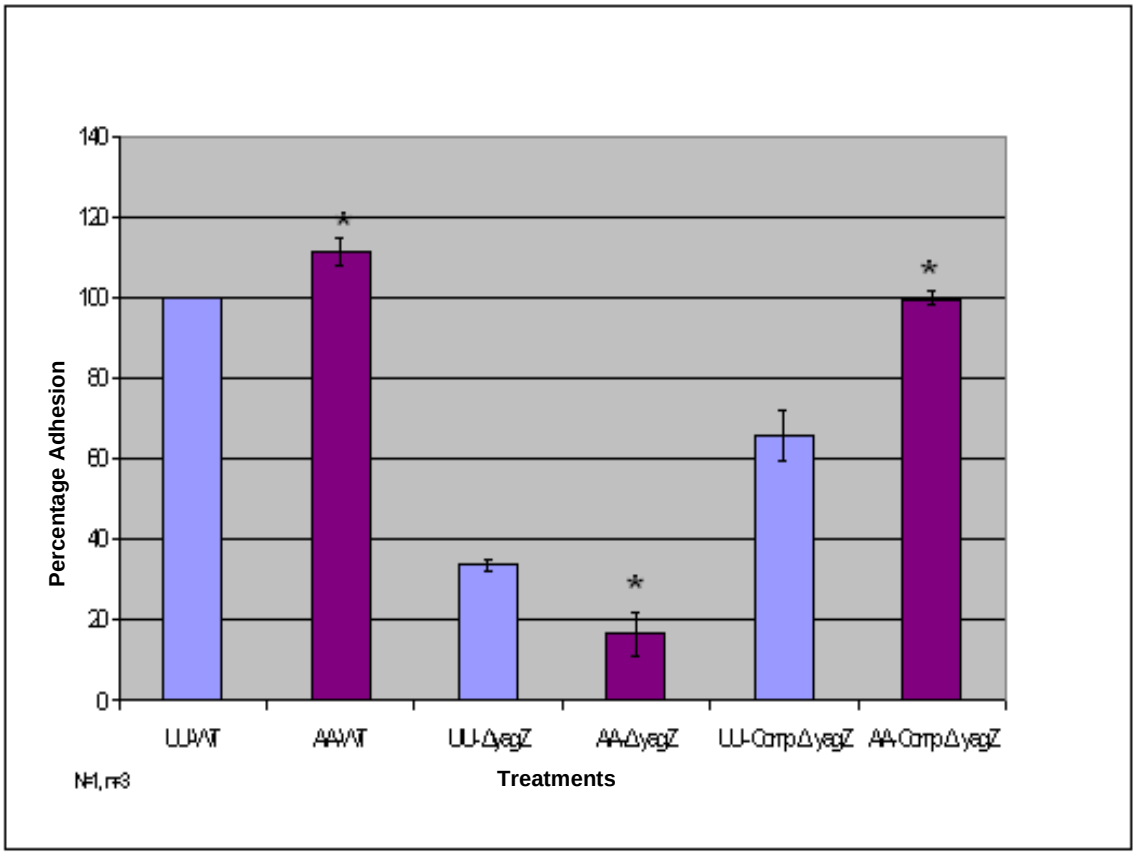


Figure 3.6: Complementation of *yagZ* partially restores acid adapted acid-induced adhesion. Effect of acid adapted acid stress on *E. coli* O157:H7 in adhesion to HEp-2 cells was assessed by plate count assay (N=1, n=3). Results representative of 2 independent experiments are shown and the values are means ± standard deviations. Bacteria were adapted for 1 hr at pH 5.0 and stresses for 30 min at pH 3.0 at 37°C, 5% CO_2. Controls were adapted as well stressed at pH 7.4, 37°C, 5% CO_2. HEp-2 cells were infected with bacteria for 3-4 hrs to determine adhesion at 37°C, 5% CO_2. Adhesion to Hep-2 has been normalized to the unadapted, unstressed wild-type. Results representative of 2 independent experiments are shown and the values are means ± standard deviations. UU: unadapted/unstressed; AA: acid adapted/acid stressed. WT: wild type; ΔyagZ: *yagZ* mutant; comp-yagZ: complemented mutant of *yagZ*.*Significantly different from corresponding control (P≤0.10)

3.1.7 Viability of EHEC 8624 and complemented mutants after short chain fatty acid stress

There was a significant decrease in the viability of 8624 wild-type following SCFA stress. A similar pattern was seen with the Δ*yagZ* and complemented mutant of *yagZ* after SCFA stress treatments. Although there was a reduction in the viability of stressed bacteria following short chain fatty acid stress, there was a considerable amount of stress-exposed viable bacteria (Figure 3.7).

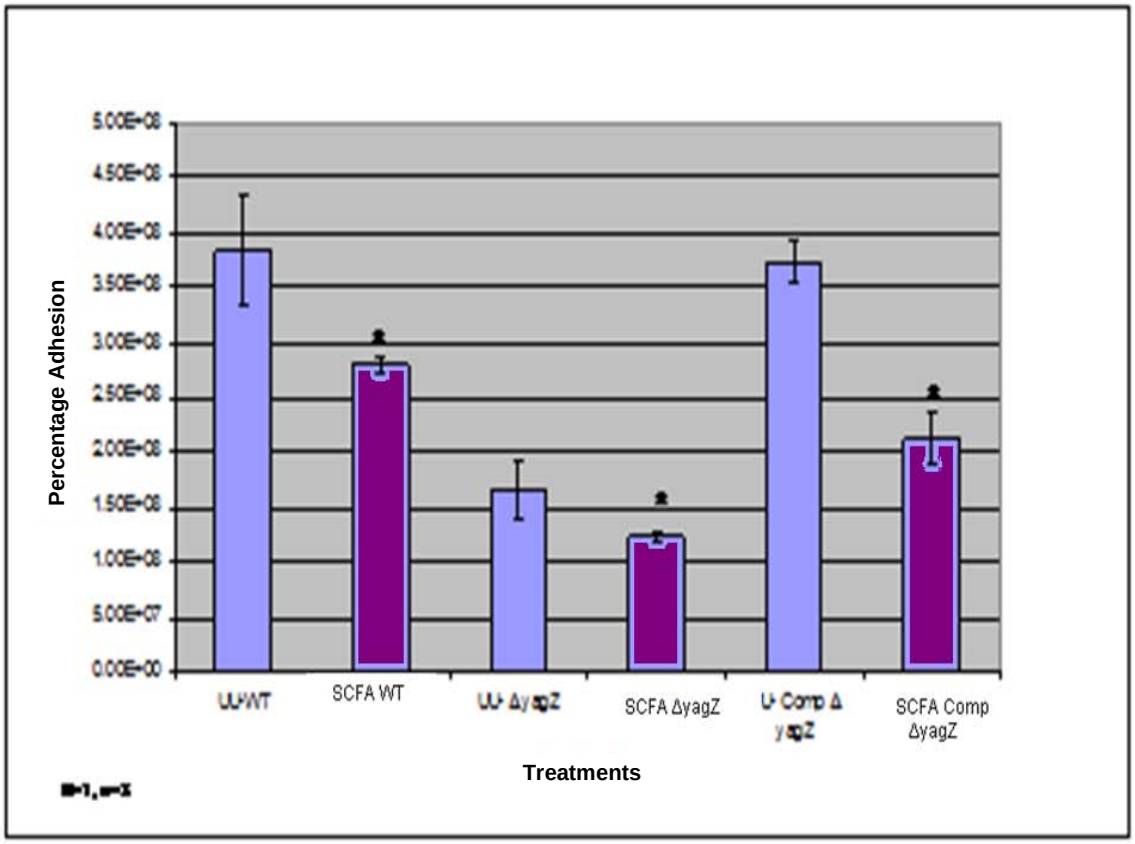


Figure 3.7: Viability of EHEC 8624 strain 8624 and complemented mutants of *yagZ* following SCFA stress. Effect of acid adapted acid stress on viability was assessed by a plate count assay (N=1, n=3). Result representative of 2 independent experiments. Values are means ± standard deviations. Bacteria were adapted for 1 hr. at pH 5.0 and shocked for 30 min at pH 3.0. Controls were adapted as well stressed at pH 7.4. Result representative of 2 independent experiments. Values are means ± standard deviations. UU: unstressed; SCFA: SCFA stressed. WT: wild type;ΔyagZ: *yagZ* mutant; comp-yagZ: complemented mutant of *yagZ*.*Significantly different from corresponding control (P≤0.10)

3.1.8 Adhesion of EHEC 8624 and complemented mutants to human epithelial cells after SCFA stress

The adhesion of 8624 wild-type, complemented mutants of *yagZ* to HEp2-cells increased after acid-adapted acid stress (Figure 3.8). The adhesion of the Δ*yagZ* mutant was significantly decreased compared to wild-type cells following acid stress treatment. However, the enhanced adhesion of acid-induced complemented strain was only partially restored, possibly because all the genes regulated by a common promoter were not cloned back in the complemented strain.

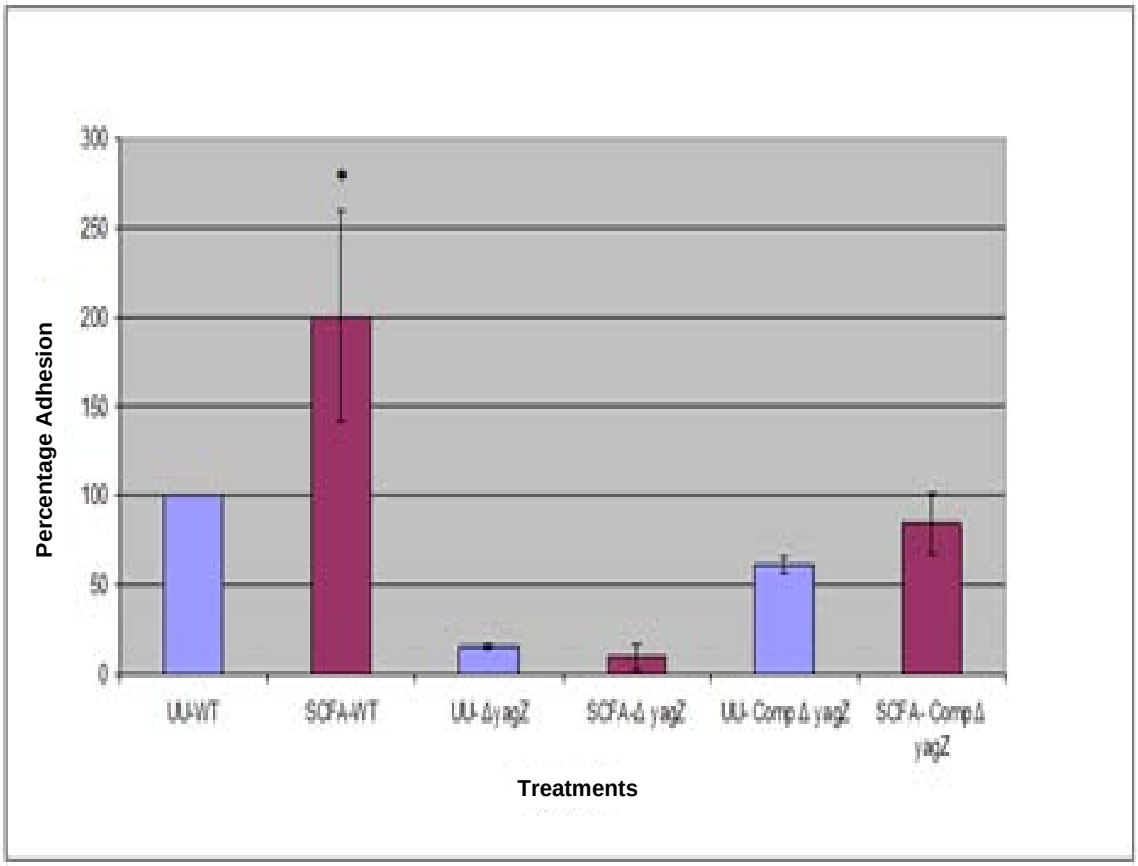


Figure 3.8: Complementation of *yagZ* partially restores SCFA-induced adhesion. Effect of SCFA stress on *E. coli* O157:H7 in adhesion to HEp-2 cells was assessed by plate count assay (N=1, n= 3). Results representative of 2 independent experiments are shown and the values are means ± standard deviations. Bacteria were grown overnight in 90 mM SCFA and stressed in DMEM supplemented with 90 mM SCFA at 37°C, 5% CO_2. Controls were adapted as well stressed at pH 7.4 37°C, 5% CO_2. HEp-2 cells were infected with bacteria for 3-4 hrs to determine adhesion at 37°C, 5% CO_2. Adhesion to HEp-2 has been normalized to the unstressed wild-type. Results representative of 2 independent experiments are shown and the values are means ± standard deviations. UU: unstressed; SCFA: SCFA stressed. WT: wild type; ΔyagZ: *yagZ* mutant; comp-yagZ: complemented mutant of *yagZ*.*significantly different from corresponding control ($P \leq 0.10$)

3.2 Promoter activity of *yagZ* after various stress treatments

The promoter activity for *yagZ* was low following stress treatments. However, there was significant increase in promoter activity compared to controls, when EHEC-stressed bacteria were exposed to conditioned media from EHEC-infected HEp-2 cells.

3.2.1 Promoter activity of *yagZ* following acid-adapted acid stress

The activity of PMatB was extremely low compared to its corresponding control PacrA under both the shocked and unshocked conditions (Figure 3.9). These results are suggesting that *matB* (*yagZ*) is minimally expressed under both unshocked and acid-adapted acid treatments. The activity levels of PacrA are considerably higher and confirm that the assay conditions and detection are functional.

Treatment	Promoter activity	St Dev
UU30 PacrA	2851.65	15.78
UU30 PMatB	37.95	3.10
AA30 PacrA	3350.79	48.08
AA30 PMatB	30.14	3.66

Fig 3.9: Promoter activity of EHEC 8624-PZ and 8624-PA after acid adapted acid stress. Isolated colonies of 8624 PZ and PA were grown overnight and acid adapted for an hr in pH 5.0 followed by acid stress at pH 3.0 at 37ºC , 5% CO_2. The bacteria were then resuspended in PBS and assessed for promoter activity by β-galactosidase assay (N=1, n=4). Results representative of 2 experiment and values are mean ± standard deviations. PacrA: promoter of *acrA;* PMatB: promoter of *matB (yagZ/ecpA*). UU: Unadapted/unstressed; AA: acid adapted/acid stressed.

3.2.2 Promoter activity of yagZ following short chain fatty acid stress.

The activity of PMatB was extremely low compared to its counterpart control PacrA under both the shocked and unshocked conditions (Figure 3.10). *yagZ* is likely to be under the control of a low activity promoter, indicating that yagZ is minimally expressed under unstressed as well as short chain fatty acid stressed conditions. The high activity of PacrA shows that the assay conditions and detection are functional.

Treatment	Promoter activity	St Dev
UU PacrA	1784.42	36.41
UU PMatB	23.91	1.81
SCFA PacrA	806.12	6.41
SCFA PMatB	7.08	1.56

N=1, n=4

Fig 3.10: Promoter activity of EHEC 8624 PZ after 90 mM SCFA stress. Isolated colonies of 8624-PZ and 8624-PA were grown in 90 mM SCFA for 16-18 hrs and then cultivated in DMEM with 90mM SCFA for 2 hrs at 37ºC , 5% CO_2. The bacteria were then resuspended in PBS and assessed for promoter activity of *yagZ* by β-galactosidase assay (N=1, n=4). Results representative of 2 experiments and values are mean ± standard deviations. UU: Unshocked; SCFA: SCFA stressed. PacrA: promoter of *acrA;* PMatB: promoter of *matB (yagZ/ecpA*). UU: unshocked; SCFA: SCFA stressed.

3.2.3 Promoter activity of EHEC 8624 P, PZ and PA after acid-adapted acid stress in conditioned media

The promoter activity of *yagZ* was low following acid-adapted acid stress (Figure 3.9). In order to address the possibility that factors from HEp-2 cells or (EHEC) infected HEp-2 cells could enhance expression of promoter activity; acid-stressed bacteria were exposed to conditioned media from uninfected as well as infected HEp-2 cells. However, the activity of PMatB was still extremely low compared to its counterpart control, PacrA, under both the shocked and unshocked conditions (Figure 3.11a, 3.11b). *yagZ* is likely to be under the control of a low activity promoter. Although the level of promoter activity of *yagZ* was low under all conditions, there was a significant increase in promoter activity when exposed to conditioned media from infected HEp-2 cells (3.11b).

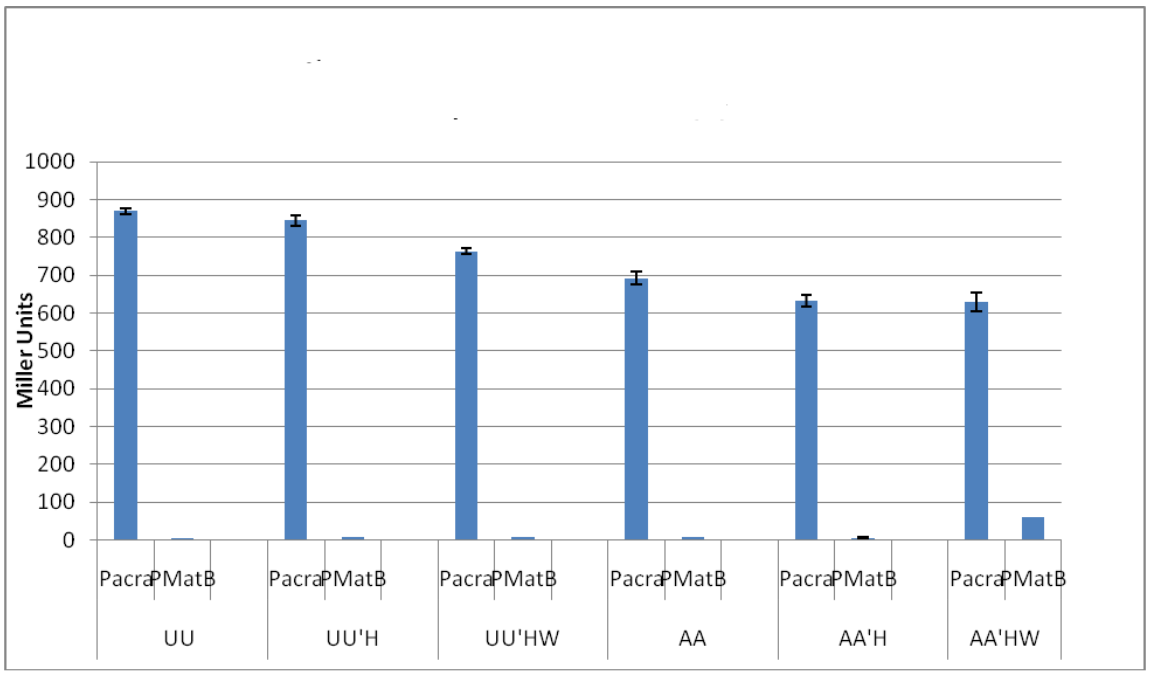


Figure 3.11a: Promoter activity of EHEC 8624 P, PZ and PA after acid adapted acid stress in conditioned media. Isolated colonies of 8624 P and 8624-PA were grown overnight and acid adapted for an hr in pH 5.0 followed by acid stress in conditioned media at pH 3.0 at 37ºC , 5% CO_2. The bacteria were then incubated in conditioned media for 3 hrs; Conditioned media: media used for maintenance of Hep-2 cells. The bacteria were then resuspended in PBS and assessed for promoter activity by β-galactosidase assay (N=1, n=4). Results representative of 2 experiment and values are mean ± standard deviations. PacrA: promoter of *acrA;* PMatB: promoter of *matB (yagZ/ecpA*). UU: unadapted unshocked ; UU'H: unadapted unshocked/conditioned media of Hep-2 cells; UU'HW: unadapted unshocked/conditioned media of Hep-2 cells infected with EHEC 8624; AA: acid adapted acid shocked; AA'H: acid adapted acid shocked/conditioned media of Hep-2 cells; AA'HW: acid adapted acid shocked/conditioned media of Hep-2 cells infected with EHEC 8624

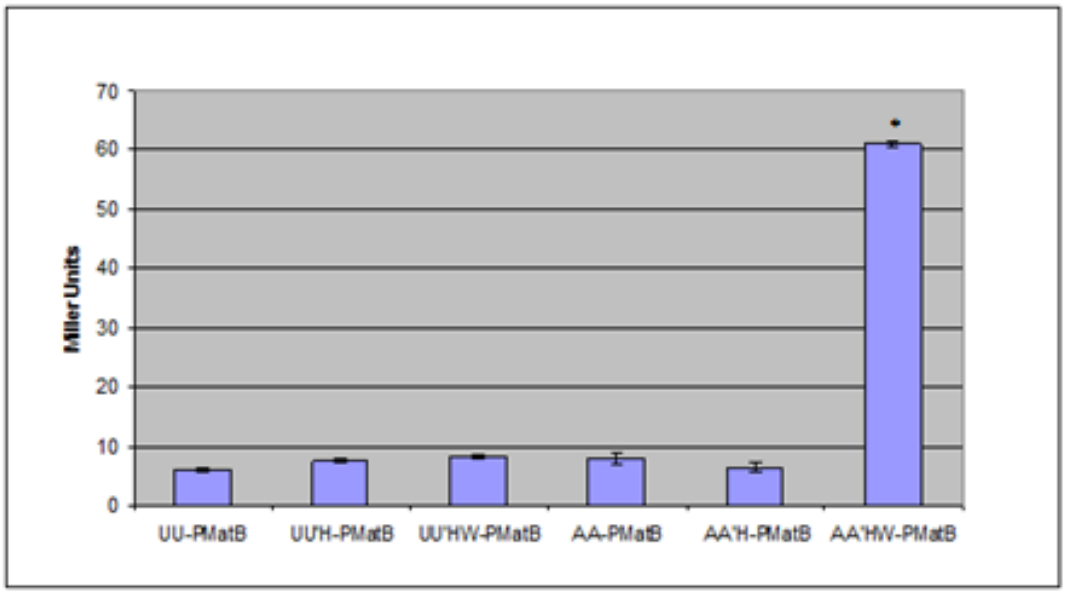


Figure 3.11b: Relative promoter activity of *matB/yagZ* promoter after various acid stress treatments. Promoter activity of PMatB alone from Figure 3.12a shown here. Isolated colonies of 8624 P were grown overnight and acid adapted for an hr in pH 5.0 followed by acid stress in conditioned media at pH 3.0 at 37ºC , 5% CO_2. The bacteria were then incubated in conditioned media for 3 hrs; Conditioned media: media used for maintenance of Hep-2 cells. The bacteria were then resuspended in PBS and assessed for promoter activity by β-galactosidase assay (N=1, n=4). Results representative of 2 experiment and values are mean ± standard deviations. PMatB: promoter of *matB (yagZ/ecpA*). UU: unadapted unshocked ; UU'H: unadapted unshocked/conditioned media of Hep-2 cells; UU'HW: unadapted unshocked/conditioned media of Hep-2 cells infected with EHEC 8624; AA: acid adapted acid shocked; AA'H: acid adapted acid shocked/conditioned media of Hep-2 cells; AA'HW: acid adapted acid shocked/conditioned media of Hep-2 cells infected with EHEC 8624. * Significantly different from corresponding control ($P \leq 0.0001$)

3.2.4 Promoter activity of EHEC 8624 PZ after 90mM SCFA stress in conditioned media.

The promoter activity of *yagZ* was low previously found to be low following exposure to 90 mM short chain fatty acid (Figure 3.10). Short chain fatty acid-stressed EHEC was, therefore, exposed to (EHEC) infected as well as uninfected conditioned media from HEp-2 cells, in order to determine if other factors during the infection period could play a role in increasing the expression levels of *yagZ.* The activity of PMatB was still extremely low compared to its counterpart control PacrA under both the shocked and unshocked conditions (Figure 3.12a, 3.12b). *yagZ* is likely to be under the control of a low activity promoter. Even though the level of expression for PMatB was low, there was significant increase in expression following exposure to conditioned media from infected HEp-2 cells (Figure 3.12b).

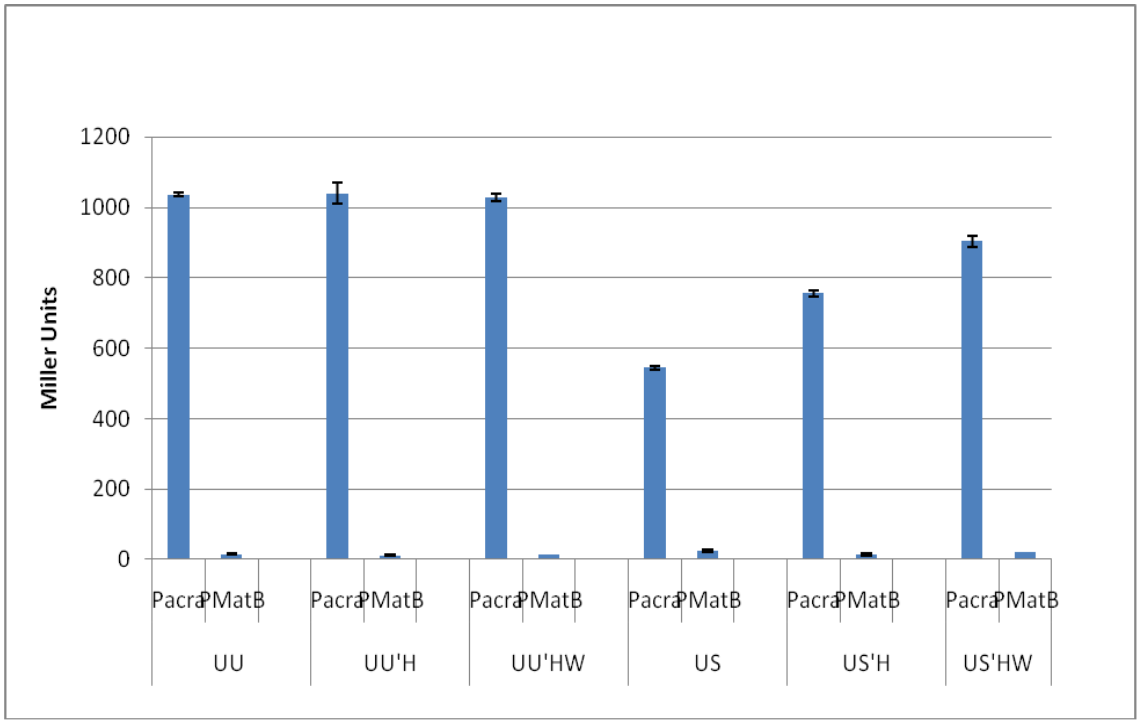


Figure 3.12a: Promoter activity of EHEC 8624 PZ after 90mM SCFA stress in conditioned media. Isolated colonies of 8624 PZ and PA were grown in 90mM SCFA for 16-18 hrs and then cultivated in conditioned DMEM supplemented with 90mM SCFA for 2 hrs at 37ºC , 5% CO_2. The bacteria were then incubated in conditioned media for 3 hrs; Conditioned media: media used for maintenance of Hep-2 cells. The bacteria were then resuspended in PBS and assessed for promoter activity by β-galactosidase assay (N=1, n=4). Results representative of 2 experiment and values are mean ± standard deviations. PacrA: promoter of *acrA;* PMatB: promoter of *matB (yagZ/ecpA*). UU: unshocked; UU'H: unshocked/conditioned media of Hep-2 cells; UU'HW: unshocked/conditioned media of Hep-2 cells infected with EHEC 8624; US: SCFA shocked; US'H: SCFA shocked/conditioned media of Hep-2 cells; US'HW: SCFA shocked/conditioned media of Hep-2 cells infected with EHEC 8624.

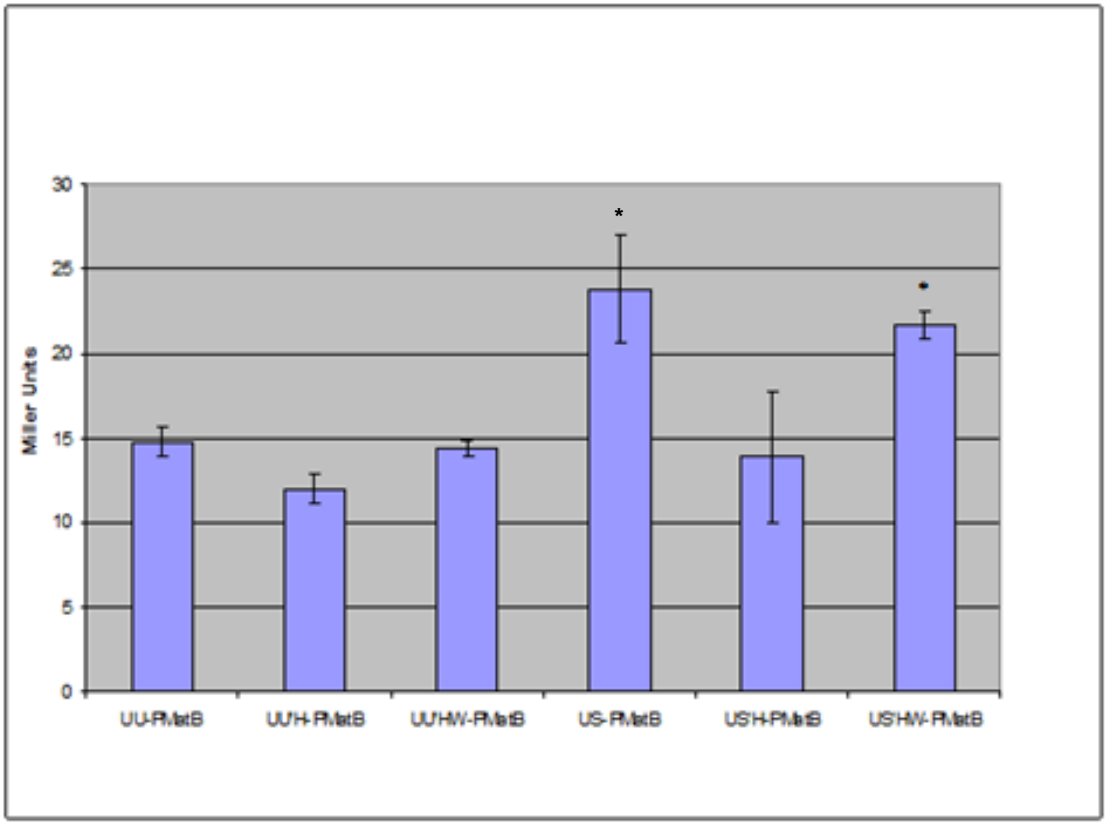


Figure 3.12b: Relative promoter activity of *matB/yagZ* promoter after various SCFA stress treatments. Promoter activity of PMatB alone from Figure 3.13a shown here. Isolated colonies of 8624 PZ were grown in 90mM SCFA for 16-18 hrs and then cultivated in conditioned DMEM supplemented with 90mM SCFA for 2 hrs at 37ºC, 5% CO_2. The bacteria were then incubated in conditioned media for 3 hrs; Conditioned media: media used for maintenance of Hep-2 cells. The bacteria were then resuspended in PBS and assessed for promoter activity by β-galactosidase assay (N=1, n=4). Results representative of 2 experiment and values are mean ± standard deviations. PMatB: promoter of *matB (yagZ/ecpA*). UU: unshocked; UU'H: unshocked/conditioned media of Hep-2 cells; UU'HW: unshocked/conditioned media of Hep-2 cells infected with EHEC 8624; US: SCFA shocked; US'H: SCFA shocked/conditioned media of Hep-2 cells; US'HW: SCFA shocked/conditioned media of Hep-2 cells infected with EHEC 8624. *Significantly different from corresponding control ($P \leq 0.005$)

Discussion

Gastrointestinal pathogens must overcome the acute acid conditions of the stomach and bile stress in the small intestine to be able to successfully colonize and cause illness in their victims in the large intestine where they must also survive the short chain fatty acid stress. Several of these microorganisms including the major human pathogen enterohemorrhagic *Escherichia coli* O157:H7, have developed ways to adapt and survive against acid stress even in the envrionment. EHEC possesses several sophisticated acid-response systems that allows it to tolerate acid-challenged media *in vitro* and *in vivo.* Whole genome transcriptional analyses also reveal that certain virulent genes are highly expressed in acid induced EHEC cells. These genes can promote the bacteria's pathogenicity, including its adherence to host tissue cells (28).

This study identifies a putative fimbrial adhesin gene, *yagZ,* that appears to play a crucial role in EHEC pathogenesis. Microarray genome profiling has revealed that acid-induced EHEC had significantly upregulated levels of flagella and adhesions (28). In the same transcriptional study, *yagZ* was found to be upregulated in acid-induced EHEC (28). Subsequent data mining lead to the question of whether the upregulation in *yagZ* would contribute to the enhanced adhesion phenotype of EHEC following either acid-adapted acid or short chain fatty acid treatment.

The adhesion of the *yagZ* isogenic mutant significantly differed from the adhesion of the wild-type parent following stress treatments. The Δ*yagZ* mutant did not show the stress-induced adhesion increase observed with the isogenic parent strain. Under either acid

adapted-acid stress or short chain fatty acid stress, the Δ*yagZ* mutant showed no increase in adhesion to HEp-2 cells suggesting that is important for bacteria-host interaction in acid-adapted acid and short chain fatty acid-induced conditions. To confirm whether or not the decreased adhesion in the Δ*yagZ* was due to the disruption of the *yagZ* locus, the Δ*yagZ* complemented strain was assayed for host adhesion under similar conditions. As expected, either stress treatment induced increased adhesion to HEp-2 cells was observed in the complemented strain suggesting that *yagZ* contributes to stress-induced conditions under conditions examined. However, restoration of adhesion of the complemented *yagZ* mutant was not equal to that of the wild type (Figure 3.6 and 3.8). A possible reason could be that the complement did not have the entire gene cluster restored and hence the partial restoration of the enhanced adherent phenotype following stress treatments.

The finding that YagZ (MatB) fimbrial protein in pathogenic EHEC can enhance host adhesion under either acid and SCFA conditions considerably broadens our understanding of both the environmental and genetic factors that might promote its virulence within the host system. In fact, the idea that gastric acid serves as a defense barrier against food-borne pathogens in humans is now open to arguement; we have now established that acid-adapted acid stressed EHEC adhere more effectively to host cells suggesting that they become more virulent upon exposure to the acute acid stress, typically experienced in the stomach.

In order to confirm that the acid or short chain fatty acid-induced upregulation of EHEC under acid or short chain fatty acid conditions, the activity of the yagZ promoter was

assayed using a β-galactosidase assays. The results indicate the promoter activity is extremely low for *yagZ* in both the acid-adapted acid and short chain fatty acid treatments. Previous research also reported low promoter activity of the Mat operon (39). On the contrary, one research group were able to show increased expression of YagZ under culture conditions in DMEM and 5% CO_2 (56). The difference in this interpretation could be a result of difference in technique employed, since immunoblotting and reverse transcriptase PCR was used to determine the level of expression in that study (56). On the other hand, the promoter activity of *yagZ* in this project resembles levels shown (39), under various conditions where the β-galactosidase assay have been used in a manner similar to this study. They have identified the promoter of *yagZ* as one of the low activity promoter which is in agreement with the findings of this project (39). Moreover, *yagZ* may be responsive to short chain fatty acids as well as quorum signals from commensal bacteria, both of which would be present in the large intestine. EHEC uses Ecp to mimic commensal strains which may indicate why *yagZ* would be upregulated in the large intestine where it completes with commensal *E. coli* for colonization sites. In this study, there is a increase in promoter activity of *yagZ* after exposure to acid adapted acid as well as short chain fatty acid in combination with filtered media from EHEC-infected HEp-2 cells (Figure 3.11b and 3.12b). This is interesting because the results indicate that during infection of HEp-2 cells, the bacteria might have secreted soluble mediators that now help in expression of *yagZ.*

The microarray data mining of acid-induced EHEC had also shown that *lpfA1* and *lpfA2* were upregulated upon acid adapted acid stress. So, the mutant strains of *lpfA1* and *lpfA2*

were assayed for host adhesion in a manner similar to that of the *yagZ* mutant. On contrary to the findings with Δ *yagZ*, the Δ *lpfA1* and Δ *lpfA2* were hyperadherant upon exposure to either acid adapted acid or short chain fatty acid stress. The hyperadherance phenotype of the *lpfA1* and *lpfA2* mutants were also reported upon contact with intestinal tissues (20). They found that mutations in the long polar fimbriae rather than decreasing the FAE increased the FAE adhesion to the small intestine. Another study also showed that none of the mutations in either *lpfA1* or *lpfA2* showed any effect on adherence or formation of AE lesions to HeLa cells. The role *in vivo* of the EHEC O157:H7 long polar fimbriae 1 and 2 have also been investigated in the pig and sheep models. They also showed that there was not any significant difference in the fecal shedding between wild type and lpfA1 mutants in *in vivo* models (74). This altered tropism is indicating opposed actions of long polar fimbriae in the colonization of EHEC O157:H7 probably as result of expression of other fimbriae which might extend and facilitate adhesion to small intestinal regions of the host. The hyperadherant trait was also found in EHEC with mutations in both *lpf* operons, although no additional effect was evident in a double mutant of *lpf1* and *lpf2* (20). As indicated by some research groups, the deletion of long polar fimbriae, seems not have any potential effect on the adherence of EHEC to HEp-2 cells suggesting that the overall EHEC adhesion is under the control of multiple adhesins which may independently regulate expression of long polar fimbraie genes (74).

In general, the knowledge from this study provides us with new insights on EHEC O157:H7 pathogenesis that can be used to investigate both preventive and therapeutic measures for the management of EHEC infection in the future.

Summary

The results of this study confirm that acid stress enhances adhesion of EHEC O157:H7 to human epithelial cells under acid adapted-acid stress and short chain fatty acid stress. y*agZ* plays a role in the acid-induced adhesion to host cells. Deletion of *lpfA1* and *lpfA2* render EHEC its hyperadherant phenotype possibly by triggering other virulence factors. Additionally, the results strongly suggest that acid stress, which is a part of the host's natural assault to resist invasion by foreign organisms, may actually regulate factors responsible for enhanced bacteria-host attachment, resulting in increased EHEC virulence and hence ability to cause disease.

Future Work

This study has revealed that *yagZ* plays a role in adhesion of EHEC to Hep-2 cells. However, the promoter activity of *yagZ* was extremely low following acid-adapted acid or short chain fatty acid stress. Although, low levels of activity have been previously reported for *yagZ* under several conditions, it is important to show, even at this low level of promoter activity, *yagZ* plays a significant role in adhesion to host cells. In order to prove the fact, analysis of YagZ expression prior to and following infection of HEp-2 cells of acid-adapted acid-induced and short chain fatty acid-induced EHEC can be carried out by immunoblotting experiments.

Findings from this study and other groups (20) have opened up an interesting trait of

EHEC adherence in the absence of *lpfA1* and *lpfA2*. This thesis study has also shown that deletion of either *lpfA1* or *lpfA2* confers hyperadherence of the mutant strains following acid-adapted acid stress and short chain fatty acid stress. On the other hand, previous studt has shown that with increasing infection periods, the mutants tend to become increasingly hyperadherent to tissue culture cells (20). This suggests that disruption of *lpfA1* and *lpfA2* may unmask other adhesins or upregulate expression of other adhesins that can more than compensate for the loss of *lpfA1* and *lpfA2*. It would, therefore, be very interesting to unveil the other "unmasked" adhesins or upregulated adhesin genes in these mutants, possibly by doing microarray profiling during infection of tissue culture cells at several time points, following acid-adapted acid stress or short chain fatty acid.

References

1. **Aldick T, Bielaszewska M, Zhang W et. al, J.** 2007. Hemolysin from Shiga toxin-negative E.scherichia coli O26 strains injures microvascular endothelium. Microbes Infect. **9:**282-290. doi: 10.1016/j.micinf.2006.12.001.

2. **AudiaJP, Webb CC, Foster JW.** 2001. Breaking through the acid barrier: an orchestrated response to proton stress by enteric bacteria. Int. J. Med. Microbiol. **291:**97-106.

3. **Bansal T, Englert D, Lee J et. al.** 2007. Differential Effects of Epinephrine, Norepinephrine, and Indole on *Escherichia coli* O157:H7 Chemotaxis, Colonization, and Gene Expression. Infect. Immun. **75:**4597-4607.

4. **Barlow RS, Gobius KS, Desmarchelier PM.** 2006. Shiga toxin-producing Escherichia coli in ground beef and lamb cuts: results of a one-year study. Int. J. Food Microbiol. **111:**1-5. doi: 10.1016/j.ijfoodmicro.2006.04.039.

5. **Bearson S, Bearson B, and Foster JW.** 1997. Acid stress responses in enterobacteria. FEMS Microbiol. Lett. **147:**173-180.

6. **Berryman M, Franck Z, Bretscher A.** 1993. Ezrin is concentrated in the apical microvilli of a wide variety of epithelial cells whereas moesin is found primarily in endothelial cells. J Cell Sci. **105:**1025-1043.

7. **Bielaszewska M, Kock R, Friedrich AW et. al.** 2007. Shiga toxin-mediated hemolytic uremic syndrome: time to change the diagnostic paradigm? PLoS One. **2:**e1024. doi: 10.1371/journal.pone.0001024.

8. **Blackburn D, Husband A, Saldana Z et. Al.** 2009. Distribution of the Escherichia coli common pilus among diverse strains of human enterotoxigenic E. coli. J. Clin. Microbiol. **47:**1781-1784. doi: 10.1128/JCM.00260-09.

9. **Brando RJ, Miliwebsky E, Bentancor L et. al.** 2008. Renal damage and death in weaned mice after oral infection with Shiga toxin 2-producing Escherichia coli strains. Clin. Exp. Immunol. **153:**297-306. doi: 10.1111/j.1365-2249.2008.03698.x.

10. **Burland V, Shao Y, Perna N et. al.** 1998. The complete DNA sequence and analysis of the large virulence plasmid of Escherichia coli O157:H7. Nucleic Acids Res. **26:**4196-4204.

11. **Capriolo A et. al.** 2005. Enterohaemorrhagic Escherichia coli: emerging issues on virulence and modes of transmission. Vet Research. **36:**289-311.

12. **Carey CM, Kostrzynska M, Ojha S et. al.** 2008. The effect of probiotics and organic acids on Shiga toxin 2 gene expression in enterohemorrhagic *Escherichia coli* O157:H7. J. Microbiol. Methods. **73:**125-132.

13. **Castanie-Cornet MP, Cam K, Bastiat B et. al.** 2010. Acid stress response in Escherichia coli: mechanism of regulation of gadA transcription by RcsB and GadE. Nucleic Acids Res. **38:**3546-3554. doi: 10.1093/nar/gkq097.

14. **Ceponis PJ, Riff JD, Sherman PM.** 2005. Epithelial cell signaling responses to enterohemorrhagic Escherichia coli infection. Mem. Inst. Oswaldo Cruz. **100 Suppl 1:**199-203. doi: /S0074-02762005000900034.

15. **Chingcuanco F, Levesque C, Foster DB.** May 17-21, 2009. Role of a Novel Fimbrial Adhesin in Acid-induced Host Adhesion of Escherichia coli O157:H7. American Society for Microbiology 109th General Meeting, Philadelphia. .

16. **Dean-Nystrom EA, Bosworth BT, Cray WC et. al.** 1997. Pathogenicity of Escherichia coli O157:H7 in the intestines of neonatal calves. Infect. Immun. **65:**1842-1848.
17. **Diez-Gonzalez F, and Y. Karaibrahimoglu.** 2004. Comparison of the glutamate-, arginine- and lysine-dependent acid resistance systems in Escherichia coli O157:H7. J. Appl. Microbiol. **96:**1237-1244. doi: 10.1111/j.1365-2672.2004.02251.x.
18. **Donnenberg, M. S.** 1999. Interactions between enteropathogenic Escherichia coli and epithelial cells. Clin. Infect. Dis. **28:**451-455. doi: 10.1086/515159.
19. **Dziva F, Vlisidou VF, Crepin VF et. al.** 2007. Vaccination of calves with EspA, a key colonisation factor of Escherichia coli O157:H7, induces antigenspecific humoral responses but does not confer protection against intestinal colonisation. Vet. Microbiol. **123:**254-261.
20. **Fitzhenry R, Dahan S, Torres AG et. al.** 2006. Long polar fimbriae and tissue tropism in Escherichia coli O157:H7. Microbes Infect. **8:**1741-1749. doi: 10.1016/j.micinf.2006.02.012.
21. **Foster JW.** 2001. Acid Stress Responses of *Salmonella* and *E. coli* : Survival Mechanisms, Regulation, and Implications for Pathogenesis. J Microbiol. **39:**89-94.
22. **Fraser ME, Fujinaga M, Cherney AR et. al.** 2004. Structure of shiga toxin type 2 (Stx2) from Escherichia coli O157:H7. J Biol Cherm. **279:**27511-27517.
23. **Garmendia J, Frankel G and Crepin AF.** 2005. Enteropathogenic and enterohaemorrhagic Escherichia coli infections: Translocation, Translocation , Translocation. Infect. Immun. **2573:**2585.
24. **Gu L, Wang H, Guo LO et. al.** 2008. Heparin blocks the adhesion of E. coli O157:H7 to human colonic epithelial cells. Biochem. Biophys. Res. Commun. **369:**1061-1064. doi: 10.1016/j.bbrc.2008.02.160.
25. **Herold S, Karch H, Schmidt H.** 2004. Shiga toxin-encoding bacteriophages-Genomes in motion. Int J Med Microbiol. **294:**115-121.
26. **Ho TD, Davis BM, Ritchie JM et. al.** 2008. Type 2 secretion promotes enterohemorrhagic Escherichia coli adherence and intestinal colonization. Infect. Immun. **76:**1858-1865. doi: 10.1128/IAI.01688-07.
27. **Hoey DE. Sharp L, Currie C et. al.** 2003. Verotoxin 1 binding to intestinal crypt epithelial cells results in localization to lysosomes and abrogation of toxicity. Cell. Microbiol. **5:**85-97.
28. **House B, Kus JV, Prayitno N et. al.** 2009. Acid-stress-induced changes in enterohaemorrhagic Escherichia coli O157 : H7 virulence. Microbiology. **155:**2907-2918. doi: 10.1099/mic.0.025171-0.
29. **Kaper JB and Brien ADO.** 1998. Escherichia coli 0157:H7 and other shiga toxin-producing E. coli strains. ASM Press, Washington, DC.
30. **Kenny B.** 1999. Phosphorylation of tyrosine 474 of the enteropathogenic Escherichia coli (EPEC) Tir receptor molecule is essential for actin nucleating activity and is preceded by additional host modifications. Mol Microbiol. **31:**1229-1241.
30a. **Lawhon SD, Maurer R, Suyemoto M et. al.** 2002 Intestinal short-chain fatty acids alter *Salmonella typhimurium* invasion gene expression and virulence through BarA/SirA. Mol Microbiol. **45:** 1451–1464
31. **Lee JH.** 2008. Antimicrobial resistance of Echerichia coli O26 and O111 isolates from cattle and their characteristics. Vet. Microbiol. **30:**135-401.

32. **Leenanon B and Drake MA.** 2001. Acid stress, starvation, and cold stress effect poststress nehavior of *Escherichia coli* O157:H7 and nonpathogenic *Escherichia coli*. Food Prot. **64:**970-974.
33. **Leotta GA, Miliwebsky ES, Chinen I et. al.** 2008. Characterisation of Shiga toxin-producing Escherichia coli O157 strains isolated from humans in Argentina, Australia and New Zealand. BMC Microbiol. **8:**46. doi: 10.1186/1471-2180-8-46.
34. **Leyer GJ, Wang LL, Johnson EA.** 1995. Acid adaptation of Escherichia coli O157:H7 increases survival in acidic foods. Appl. Environ. Microbiol. **61:**3752-3755.
35. **Lim JI, Sheng H, Seo K et. al.** 2007. Characterization of an *Escherichia coli* O157:H7 Plasmid O157 Deletion Mutant and Its Survival and Persistence in Cattle. Applied and Environmental Microbiology. **73:**2037-2047.
36. **Lin J, Smith MP, Chapin KC et. al.** 1996. Mechanism of acid resistance in entrohaemorrhagic Escherichia coli. Appl. Environ. Microbiol. **62:**3094-3100.
37. **Lousie CB and Obrig TG.** 1995. Specific interaction of Escherichia coli O157:H7-derived Shiga-like toxin II with human renal endothelial cells. J. Infect. Dis. **172:**1397-1401.
38. **Low AS., Dziva F, Torres AG et. al.** 2006. Cloning, expression, and characterization of fimbrial operon F9 from enterohemorrhagic Escherichia coli O157:H7. Infect. Immun. **74:**2233-2244. doi: 10.1128/IAI.74.4.2233-2244.2006.
39. **Low AS, Holden N, Rosser T et. al.** 2006. Analysis of fimbrial gene clusters and their expression in enterohaemorrhagic Escherichia coli O157:H7. Environ. Microbiol. **8:**1033-1047. doi: 10.1111/j.1462-2920.2006.00995.x.
40. **Ma Y, Mao X, Li J et. al.** 2008. Engineering an anti-Stx2 antibody to control severe infections of EHEC O157:H7. Immunol. Lett. **121:**110-115. doi: 10.1016/j.imlet.2008.09.008.
41. **Macfarlane S and Macfarlane GT.** 2003. Regulation of short-chain fatty acid production. Proc. Nutr. Soc. **62:**67-72. doi: 10.1079/PNS2002207.
42. **Manjarrez-Hernandez HA, Baldwin TJ, Williams PH et. al.** 1996. Phosphorylation of myosin light chain at distinct sites and its association with the cytoskelton during enteropathogenic Escherichia coli infection. Infect. Immun. **64:**2368-2370.
43. **Marcato P, Griener TP, Mulvey GL et. al.** 2005. Recombinant Shiga toxin B-subunit-keyhole limpet hemocyanin conjugate vaccine protects mice from Shigatoxemia. Infect. Immun. **73:**6523-6529. doi: 10.1128/IAI.73.10.6523-6529.2005.
44. **McKee Ml and O'Brien AD.** 1995. Investigation of enterohemorrhagic Escherichia coli O157:H7 adherance characteristics and invasion potential reveals a new attachment pattern shared by intestinal E coli. Infect. Immun. **63:**2070-2074.
45. **Nataro JP and Kaper JB.** 1998. Diarrheagenic Escherichia coli. Clin Microbiol Rev. **11:**142-201.
46. **Neri P, Nagano SI, Yokoyama S et. al.** 2007. Neutralizing activity of polyvalent Gb3, Gb2 and galacto-trehalose models against Shiga toxins. Microbiol. Immunol. **51:**581-592.
47. **Oh M, Rohlin L, Kao KC et. al.** 2002. Global Expression Profiling of Acetate-grown *Escherichia coli*. J Biol Chem. **277:**13175-13183.
48. **Orth D, Grif K, Zimmerhackl LB et. al.** 2008. Prevention and treatment of enterohaemorrhagic Escherichia coli infections in humans. Expert Rev Anti-Infect Ther.

6:101-108.
49. **Orth D, Khan AB, Naim A et. al.** 2009. Shiga Toxin Activates Complement and Binds Factor H: Evidence for an Active Role of Complement in Hemolytic Uremic Syndrome. J Immunol. **182:**1394-1400.
50. **Ostroff SM, Griffin PM, Tauxe RV et. al.** A statewide outbreak of escherichia coli infections in Washington state. American J Epidemiol. **132:**239-247.
51. **Paton AW and Paton JC.** 1998. Detection and characterization of Shiga toxigenic Escherichia coli using muliplex PCR assays for stx1, stx2, eaeA, enterohemorrhagic E coli hlyA, rfbO111 and rfbO157. J Clin Microbiol. **36:**598-602.
52. **Perna NT, Mayhew GF, Posfai G. et. al.** 1998. Molecular evolution of a pathogenicity island from enterohemorrhagic Escherichia coli O157:H7. Infect. Immun. **66:**3810-3817.
53. **Pouttu R, Westerlund-Wikstrom B, Lang H et. al.** 2001. matB, a common fimbrillin gene of Escherichia coli, expressed in a genetically conserved, virulent clonal group. J. Bacteriol. **183:**4727-4736. doi: 10.1128/JB.183.16.4727-4736.2001.
54. **R. L. Buchanan, Edelson-Mammel SG, Boyd G et. al.** 1990. Serotypes of Escherichia coli That Hybridized with DNA Probes for Genes Encoding Shiga-Like Toxin 1, Shiga-Like Toxin II, and Serogroup 0157 Enterohemorrhagic E. coli Fimbriae Isolated from
Adults with Diarrhea in Thailand. J. Clin. Microbiol. 28:293-295.
55. **Razzaq S.** 2006. Hemolytic uremic syndrome: an emerging health risk. Am Fam Phys. **74:**991-996.
56. **Rendon MA, Saldana Z, Erdem AL et. al.** 2007. Commensal and pathogenic Escherichia coli use a common pilus adherence factor for epithelial cell colonization. Proc. Natl. Acad. Sci. U. S. A. **104:**10637-10642. doi: 10.1073/pnas.0704104104.
57. **Robinson CM, Sinclair JF, Smith MJ et. al.** 2006. Shiga toxin of enterohemorrhagic Escherichia coli type O157:H7 promotes intestinal colonization. Proc. Natl. Acad. Sci. U. S. A. **103:**9667-9672. doi: 10.1073/pnas.0602359103.
58. **Saldana Z, Erdem AL, Schuller S et. al.** 2009. The Escherichia coli common pilus and the bundle-forming pilus act in concert during the formation of localized adherence by enteropathogenic E. coli. J. Bacteriol. **191:**3451-3461. doi: 10.1128/JB.01539-08.
59. **Sandbig K , Spilsberg B, Lauvrak ML et. al.** 2004. PAthways followed by protien toxins into cells. Int J Med Microbiol. **293:**483-490.
60. **Sandvig K and van Deurs B.** 2002. Membrane trafffic exploited by protein toxins. Annu Rev Dev Biol. **18:**1-24.
61. **Scheiring J, Andreoli SP, Zimmerhackl LB.** 2008. Treatment and outcome of Shiga-toxin-associated hemolytic uremic syndrome (HUS). Pediatr. Nephrol. **23:**1749-1760. doi: 10.1007/s00467-008-0935-6.
62. **Schmidtn H, Henkel B, Karch H.** 1997. A gene cluster closely related to type II secretion pathway operons of gram-negative bacteria is located on the large plasmid of enterhemorrhagic Escherichia cole O157 strains. FEMS Microbiol. Lett. **48:**265-272.
63. **Serbanescu MA, Prayytino NA, and Tsui E.** 2008. Host Ingestion Stress Cues Virulence Changes in E coli O157:H7. .
64. **Shin R, Suzuki M, Morishita Y.** 2002. Influence of intestinal anaerobes and organic acids on the growth of enterohaemorrhagic *Escherichia coli* O157:H7. J Med Microbiol. **51:**201-206.

65. **Spears KJ, Roe AJ, Gally DL.** 2006. A comparison of enteropathogenic and enterohaemorrhagic Escherichia coli pathogenesis. FEMS Microbiol. Lett. **255:**187-202. doi: 10.1111/j.1574-6968.2006.00119.x.
66. **Srinivas CN, Pratap B, Abraham G et. al.** 2007. Post-renal transplant hemolytic uremic syndrome following combination therapy with tacrolimus and everolimus, Saudi J Kidney Dis Transplant. **18:**609-612.
67. **Tatsuno I, Horie M, Abe H et. al.** 2001. toxB gene on pO157 of enterohemorrhagic Escherichia coli O157:H7 is required for full epithelial cell adherence phenotype. Infect. Immun. **69:**6660-6669.
68. **Taylor M.** 2008. Enterohaemorrhagic Escherichia coli and Shigella dysenteriae type 1-induces haemolytic ureaemic syndrome. C. Pediatr Nephrol. **23:**1425-1431.
69. **Todar K.** Todar's online textbook of bateriology. http://www.textbookofbacteriology.net/e.coli_4.html. .
70. **Todar, K.** Shigella and Shigellosis. **2009**
71. **Toma C, Martinez Espinosa E, Song T et. al.** 2004. Distribution of putative adhesins in different seropathotypes of Shiga toxin-producing Escherichia coli. J. Clin. Microbiol. **42:**4937-4946. doi: 10.1128/JCM.42.11.4937-4946.2004.
72. **Torres AG, Milflores-Flores L, Garcia-Gallegos JG et. al.** 2007. Environmental regulation and colonization attributes of the long polar fimbriae (LPF) of Escherichia coli O157:H7. Int J Med Microbiol. **297:**177-185.
73. **Torres AG, Giron JA, Perna NT et. al.** 2002. Identification and characterization of lpfABCC'DE, a fimbrial operon of enterohemorrhagic Escherichia coli O157:H7. Infect. Immun. **70:**5416-5427.
74. **Torres AG, Kanack KJ, Tutt CB et. al.** 2004. Characterization of the second long polar (LP) fimbriae of Escherichia coli O157:H7 and distribution of LP fimbriae in other pathogenic E. coli strains. FEMS Microbiol. Lett. **238:**333-344. doi: 10.1016/j.femsle.2004.07.053.
75. **Tu X, Nisan I, Yano C et. al.** 2003. EspH, a new cytoskeleton-modulating effector of enterohaemorrhagic and enteropathogenic Escherichia coli. Mol Microbiol. **47:**595-606.
76. **Welinder-Olsson C & Kaijser B.** 2005. Enterohemorrhagic Escherichia coli (EHEC). Scandinavian Journal of Infectious Diseases,. **37:**405-416.
77. **Whale AD, Hernandes RT , Ooka T et. al.** 2007. TccpP2-mediated subversion of actin dynamics by EPEC 2-a distinct evolutionary lineage of enteropathogenic Escherichia coli. Microbiology. **153:**1743-1755.
78. **Yoon, J. W., and C. J. Hovde.** 2008. All blood, no stool: enterohemorrhagic Escherichia coli O157:H7 infection. J. Vet. Sci. **9:**219-231.