CHARACTERIZATION OF *ESCHERICHIA COLI* ENTEROHEMOLYSIN 1

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A Thesis
Submitted to the Faculty of Graduate Studies
In Partial Fulfillment of the Requirements
For the Degree of

Master of Science

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ACKNOWLEDGEMENTS

This study would not have been possible without the assistance of many people.

I would like to thank the members of my committee for their support and advice over the past 5 years. They include Drs. Grant McClarty, Yuewen Gong, and Gehua Wang.

I would like to thank Dr. James B. Kaper from the University of Maryland School of Medicine for supplying me with the vector pRS551. In addition, I would also like to thank Dr. Lothar Beutin from the Robert Koch-Institute in Germany for supplying me with the *E.coli* O26:HNM reference isolate C3888.

I would like to acknowledge the DNA core facility at the Canadian Science Centre for Human and Animal Health for assistance with primer synthesis and DNA sequencing.

In addition, I would like to recognize and thank the University of Manitoba, Department of Medical Microbiology for their support.

Lastly, I would like to express my gratitude to my supervisor Dr. Gehua Wang for his guidance, patience, and encouragement.

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ACP	acyl carrier protein
AMP	ampicillin
BHI	brain heart infusion broth
BP	base pair
cDNA	complementary deoxyribonucleic acid
CFU	colony forming units
DAB	3,3'-diaminobenzidine
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleoside triphosphate
DTT	dithiotretol
EDTA	ethylenediamine-tetraacetic acid
EHEC	Enterohemorrhagic *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
ETEC	Enterotoxigenic *Escherichia coli*
FBS	fetal bovine serum
HCl	hydrochloric acid
HeLa cells	human cervical epithelial cells
HEp-2 cells	human laryngeal carcinoma cells
His	histidine
HRP	horse radish peroxidase
HTE	high concentration tris ethylenediamine-tetraacetic acid buffer
HUS	hemolytic uremic syndrome
IDL	intermediate low-density lipoprotein
INT407 cells	human embryonic intestinal epithelial cells
IPTG	isopropyl b-D-thiogalactoside
KAN	kanamycin
kDA	kilo Dalton
LB medium	Luria-Bertani medium
LDL	low-density lipoprotein
LNA	locked nucleic acid
LPS	lipopolysaccharide
LT	heat-labile *Escherichia coli* enterotoxin
MAC	MaConkey Agar
MCS	multiple cloning site
MEM	minimal essential medium
Min	minutes
mRNA	messenger ribonucleic acid
NC_{AVG}	average optical density of control wells (cytotoxicity assay)
NICU	neonatal intensive care unit

NML	National Microbiology Laboratory
NTA	Ni^{2+}:nitrilotriacetic acid complex
OD	optical density
Ops	operon-polarity suppressor
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PVDF	polyvinylidene fluoride transfer membrane
RBS	ribosome binding site
RNA	ribonucleic acid
RNase	ribonuclease
RPM	rotations per minute
RT	reverse transcription
RTX	repeats in toxin
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sec	seconds
SSC	sodium chloride-sodium citrate buffer
ST	heat-stabile *Escherichia coli* enterotoxin
STEC	Shiga-toxin producing *Escherichia coli*
Stx	Shiga toxin
TBE	tris borate ethylenediamine-tetraacetic acid buffer
TBS	tris buffered saline buffer
TE	tris ethylenediamine-tetraacetic acid buffer
TEG	tris-HCl ethylenediamine-tetraacetic acid glucose
TIR	translational initiation region
UV	ultraviolet
Vero cells	green monkey kidney cells
VLDL	very low-density lipoprotein
VTEC	verotoxigenic *Escherichia coli*
VT1	verotoxin 1
VT2	verotoxin 2
w/v	weight over volume
X-gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside
Y-1 cells	mouse epithelial cells

ABSTRACT

E. coli is the causative agent of many clinical cases of extraintestinal and intestinal infections, urinary tract infections, bacteremia, meningitis, and diarrheal disease (Bopp *et al*., 1999). Additionally, some Enterohemorrhagic *E. coli* (EHEC) isolates can produce complications during infection such as hemorrhagic colitis and Hemolytic Uremic Syndrome (HUS). Certain serotypes of *E. coli* are more invasive than others possibly becoming more pathogenic by the production of specific virulence factors, such as adherence factors, fimbriae, polysaccharide capsules, lipopolysaccharide, hemolysins, and toxins. These virulence factors make *E. coli* an important cause of diarrheal disease in humans and animals. The present study will further provide knowledge of *E. coli* Enterohemolysin 1 (*ehl1*), specifically pertaining to the characterization of *ehl1* as an additional virulence factor and look at the possibility of linking this gene to other virulence factors that are co-expressed with *ehl1*, by using microarray technology, and tissue culture assay.

Initially *ehl1* was assayed in cell culture cytotoxicity assays and determined not to be synergistic with verotoxins 1 and 2. Furthermore, *ehl1*, was not found to be significantly cytotoxic in any of the cell monolayers tested. To further characterize *ehl1* and to determine if *ehl1* is coexpressed with other virulence factors, *ehl1* was cloned in the pQE-60 expression system and tested on an in-house microarray chip specifically focusing on 21 other virulence factor genes. Comparing the net intensities of *ehl1* signal on the microarray to over 21 virulence factors examined resulted in a trend towards an increase in gene expression for genes involved in pili and fimbriae production, adhesion and pore formation, genes involved in invasion, integrase, *Ecs2917* (unknown function),

the gene for *E. coli* serine hydroxymethyltransferase, heat resistant agglutinin, and hemolysin genes (*ehl1*, *hlyA).* Additionally, there was an observed trend towards a decrease in net gene expression for the repeats in toxin gene, intimin, *E. coli* cytolethal distending toxin genes, iron response protein, and EPEC siderphore receptor and urovirulence factor. Thus, it appears that *E. coli ehl1* may be co-expressed with virulence factors that are involved in the attachment and invasion process of infection.

The presented thesis contained four major concept errors, which affected the scientific research and data obtained from the experiments. Thus, Appendix III has been added to address these errors through identification of the flawed experiments, explaining the problems within the experiments and their resulting affect on the data, and clarifying the topics by explaining how the research should have been properly carried out.

INTRODUCTION

1.1 *Escherichia coli*

1.1.1 History

In 1885, the German physician Theodor Escherich discovered *Escherichia* while analyzing the gastrointestinal contents of infants (Janda and Abbott, 1998). Theodor Escherich originally named the bacterium, *Bacterium coli* (Janda and Abbott, 1998). During the period of 1887-1902, it was thought that the causative agent of typhoid fever, *Bacillus typhi* (*Salmonella typhi*) was identical to *Bacterium coli*. The development of new diagnostic tests, including the ability to test for lactose fermentation, separated these two organisms (Janda and Abbott, 1998). In 1919, Castellani and Chalmers recognized that, based upon morphology and biochemical profiles, *Bacterium coli* did not meet the criteria for inclusion within this genus (Bettelheim, 1991). Thus, they proposed the genus be changed to *Escherichia* to honor Theodor Escherich (Janda and Abbott, 1998).

Escherichia coli is a gram-negative bacterium in the family *Enterobacteriaceae*. All *Escherichia* strains ferment D-glucose, produce gas, and can be either non-motile or motile via peritrichious flagella (Bopp *et al*., 1999). In 1958, the Judicial Commission of the International Committee on Bacteriological Nomenclature rendered Opinion No. 15 that stated that the genus *Escherichia* is the type genus for the family *Enterobacteriaceae* and that *Escherichia coli* was the type species (Janda and Abbott, 1998).

1.1.2 Transmission of *E. coli*

E. coli occurs naturally as part of the bowel flora in humans and animals. *E. coli* does not occur as a free-living organism in the environment. However, it is frequently isolated from contaminated food or water (Venkateswaran *et al.*, 1996). The presence of *E. coli* in water is an indicator of fecal contamination (Bopp *et al*., 1999). Transmission of *E. coli* is through the fecal-oral route or through food or water contamination. In Canada and the United States, medical costs have been estimated to be between $47 million and $84 million annually due to food-borne disease caused by *E. coli* O157:H7 (Todd, 1989). *E. coli* is also a major cause of nosocomial infections spread to systemic sites after surgery, catheterization, chemotherapy, and invasive diagnostic procedures (Janda and Abbott, 1998).

1.1.3 Diseases associated with *E. coli*

There are five species of *Escherichia* with *E. coli* being the type species and the species most often isolated from humans (Bopp *et al*., 1999). Even though *E. coli* is part of the natural bowel flora in humans, many pathogenic strains can cause both extraintestinal and intestinal infections (Bopp *et al*., 1999). In addition to extraintestinal and intestinal infections, other clinical symptoms of *E. coli* include: urinary tract infections, bacteremia, meningitis, and diarrheal disease (Nataro *et al*., 1998). Some Enterohemorrhagic *E. coli* isolates can produce additional complications during infection such as hemorrhagic colitis (bloody diarrhea) and Hemolytic Uremic Syndrome, HUS, which is characterized by microangiopathic hemolytic anemia, thrombocytopenia, and acute renal failure (Bopp *et al*., 1999). There are four types of pathogenic *E. coli*:

Enteroinvasive *Escherichia coli* (EIEC), Enterotoxigenic *Escherichia coli* (ETEC), Enterohemorrhagic *Escherichia coli* (EHEC), and Enteropathogenic *Escherichia coli* (EPEC).

1.1.4 Biology of Pathogenic *E. coli*

Enteroinvasive *Escherichia coli* have been associated with infant diarrhea and dysentery-like illness. EIEC do not produce toxins but instead produce adherence factors that aid in its virulence (Levine, 1987). Enteroinvasive *E. coli* have a pathogenic mechanism similar to *Shigella*. They invade colon cells resulting in the production of watery diarrhea but EIEC have also been known to produce bloody diarrhea (Bopp *et al*., 1999). An infectious dose of $\geq 10^8$ colony-forming units, cfu, of EIEC are required to produce infection (Janda and Abbott, 1998). The typical incubation period for EIEC infection is <24 hours and the duration of illness is from 1 to 12 days with the average illness lasting 2 to 4 days (Janda and Abbott, 1998). Enteroinvasive *E. coli* are associated with a few characteristic serotypes (Bopp *et al*., 1999) (Table 1).

Enterotoxigenic *Escherichia coli* are often associated with traveller's diarrhea and have been implicated as an important cause of diarrhea, especially in children (Barry, 1992). ETEC are distributed primarily in subtropical and tropical developing countries (Ewing, W. H., 1986). The clinical symptoms of ETEC illness are: abdominal cramps and diarrhea sometimes accompanied with headache and nausea, but with little fever or vomiting (Cohen *et al.*, 1991). ETEC strains tend to cluster in a few serotypes (Bopp *et al*., 1999) (Table 1). ETEC produce both a heat-labile *E. coli* enterotoxin (LT) and/or a heat-stable *E. coli* enterotoxin (ST) as their main virulence factors. An infectious dose of

10^8 to 10^{10} cfu of ETEC are required to produce infection (Janda and Abbott, 1998). The typical incubation period for ETEC infection is 3 to 166 hours and the duration of illness is from 1 to 53 days with the average illness lasting 4 to 7 days (Janda and Abbott, 1998).

Enterohemorrhagic *Escherichia coli* are also known as Shiga-toxin producing *E. coli* (STEC). EHEC toxins are known as Shiga–like toxins (stx) due to their evolutionary relations to *Shigella dysenteriae* 1. They are also referred to as Verotoxins due to the cytotoxic effect these toxins have on Vero (African green monkey kidney) cells (Janda and Abbott, 1998). The most common virulence factors that EHEC produce are Stx1 and Stx2 or one of the other variant forms of these Shiga toxins. In addition to the Shiga toxins, EHEC also possess the virulence-associated Intimin production that is involved in cell adherence, and enterohemolysin production (Bopp *et al*., 1999). One of the most common serotypes of EHEC found in Canada is O157:H7. The O157:H7 serotype of E. coli was discovered in 1982 as the etiologic agent of hemorrhagic colitis (Riley *et al.*, 1983). *E. coli* O157 infection has a very low infective dose and has a high association with the development of hemorrhagic colitis and haemolytic uremic syndrome (HUS). HUS complicates approximately 10% of *E. coli* O157 infections and has a mortality rate of 2 –10% (Law, 2000). An infectious dose of 50 to 700 cfu of STEC are required to produce infection (Janda and Abbott, 1998). The typical incubation period for STEC infection is 24 –336 hours and the duration of illness is from 6 to 9 days with the average illness lasting 4 to 5 days (Janda and Abbott, 1998). Symptoms of EHEC infection are abdominal cramps, lack of fever, mild nonbloody diarrhea, hemorrhagic colitis, and HUS (Bopp *et al*., 1999). Since O157:H7 is known to colonize cattle, contaminated ground

beef is the most common cause of outbreaks (Griffin, 1995). However, other contaminated food-sources include: roast beef, sausage, raw milk, unchlorinated municipal water, apple cider, salads, raw vegetables, and mayonnaise (Bopp *et al*., 1999). There are currently over 100 serotypes of non-O157 STEC that have been isolated from humans (Bopp *et al*., 1999) (Table 1).

Enteropathogenic *E. coli* have been associated with watery diarrhea and gastroenteritis. EPEC have no enterotoxins and have been known to invade colon cells in a mechanism similar to *Shigella*. EPEC are distributed worldwide and are a common cause of infantile diarrhea in developing countries but EPEC are rare in Canada and the United States (Bopp *et al*., 1999). EPEC have a very high mortality rate and are often associated with outbreaks in hospitals and nurseries in which patient-to-patient spread occurs, frequently through the contaminated hands of medical personnel or relatives (Janda and Abbot, 1998). However, sporadic and community EPEC infections also occur. There are currently about 20 serotypes most common to EPEC infection (Bopp *et al*., 1999) (Table 1). These serotypes have the characteristic EPEC pattern of adherence to HEp-2 and HeLa cells. In addition, these serotypes also show the attaching-and-effacing lesion in vivo (Nataro *et al*., 1998). An infectious dose of 10^6 to 10^{10} cfu, of EPEC is required to produce infection (Janda and Abbott, 1998). The typical incubation period for EPEC infection is 9 to 19 hours and the duration of illness is from 3 to 14 days with the average illness lasting 5 days (Janda and Abbott, 1998). In most infantile cases, the illness progresses with severe prolonged nonbloody diarrhea, vomiting, and fever. This may lead to maladsorption, malnutrition, weight loss, and growth retardation if the patient survives (Bopp *et al*., 1999).

Table 1: Serotypes of *E. coli* isolated from patients with diarrhea

ETEC	EPEC	EIEC	STEC		
O6:NM	O26:NM	O26ac:NM	O1:NM	O73:H34	O119:H5
O6:H16	O26:H11	O29:NM	O1:H1	O75:H5	O119:H6
O8:NM	O55:NM	O112ac:NM	O1:H7	O82:H8	O120:H19
O8:H9	O55:H6	O115:NM	O2:H1	O84:H2	O121:NM
O11:H27	O55:H7	O124:NM	O2:H5	O85:NM	O121:H8
O15:H11	O86:NM	O124:H7	O2:H6	O86:H10	O121:H19
O20:NM	O86:H2	O124:H30	O2:H7	O88:NM	O125:NM
O25:NM	O86:H34	O135:NM	O4:NM	O91:NM	O125:H8
O25:H42	O111ab:NM	O136:NM	O4:H10	O91:H14	O126:NM
O27:NM	O111ab:H2	O143:NM	O5:NM	O91:H21	O126:H8
O27:H7	O111ab:H12	O144:NM	O5:H16	O100:H32	O126:H21
O27:H20	O111ab:H21	O152:NM	O6:NM	O101:H19	O128:NM
O49:NM	O114:NM	O164:NM	O6:H1	O103:H2	O128:H2
O63:H12	O114:H2	O167:NM	O6:H28	O103:H6	O128:H8
O78:H11	O119:H6		O18:NM	O104:NM	O128:H12
O78:H12	O125ac:H21		O18:H7	O104:H21	O128:H25
O85:H7	O126:H27		O22:H8	O105:H18	O132:NM
O114:H21	O127:NM		O22:H16	O110:H19	O133:H53
O115:H21	O127:H6		O23:H7	O111:NM	O141:NM
O126:H9	O127:H9		O23:H16	O111:H2	O145:NM
O128ac:H12	O128ab:H2		O25:NM	O111:H7	O145:H25
O128ac:H21	O142:H6		O26:NM	O111:H8	O146:NM
O128ac:H27	O158:H23		O26:H2	O111:H30	O146:H21
O148:H28			O26:H8	O111:H34	O153:H25
O149:H4			O26:H11	O111:HNT	O157:NM
O153:H45			O26:H32	O112:H21	O157:H7
O159:NM			O38:H21	O113:H2	O163:H19
O159:H4			O39:H4	O113:H7	O165:NM
O159:H20			O45:NM	O113:H21	O165:H10
O166:H27			O45:H2	O113:H53	O165:H19
O167:H5			O48:H21	O114:H4	O165:H25
O169:H41			O50:NM	O114:H8	O166:H12
			O50:NM	O115:H10	O166:H15
			O52:H25	O115:H18	OX3:H21
			O55:NM	O117:H4	O rough:H20[a]
			O55:H7	O118:H12	ONT:NM[b]
			O55:H10	O118:H30	ONT:H1
					ONT:H28

[a] O rough, O antigen rough and serotype not determined.
[b] NT, not typeable.

(modified from Bopp *et al*., 1999)

ETEC, Enterotoxigenic *Escherichia coli*; EPEC, Enteropathogenic *Escherichia coli*; EIEC, Enteroinvasive *Escherichia coli*; STEC, Shiga toxin-producing *Escherichia coli*

1.1.5 Diagnosis and Case Management

Pathogenic *E. coli* are isolated from stool samples from patients with diarrhea (Bopp *et al*., 1999). Most patients will excrete the pathogen for a few days but in some cases it may be fecally shed for up to 30 days or more after the onset of diarrhea (Bopp *et al*., 1999). Identification of *E. coli* involves biochemical identification, serotyping, and virulence testing on a colony isolated from the stool sample. Confirmation of diarrheagenic *E. coli* includes the standard procedure of testing biochemicals in tube or using a commercial screening system that screens for various types of sugar fermentation and uses enough biochemicals to identify the strain as an *E. coli* by ruling out other *Enterobacteriaceae* species such as *Salmonella, Citrobacter,* and other species of *Escherichia* (Bopp *et al*., 1999). The serologic classification of *E. coli* is based on the somatic O antigen and the flagellar H antigen. These antigens are identified through tube agglutination tests with antisera prepared against the different antigenic components (Bopp *et al*., 1999). Testing their group specific virulence factors (characteristic toxins, adherence, or invasiveness) further identifies the four pathogroups of *E. coli*. Bioassays (cell culture), immunologic methods (immunoblotting), or the detection of gene sequences through the use of polymerase chain reaction (PCR) or colony blot hybridization, are some techniques used to test for virulence factors (Bopp *et al*., 1999).

Treatment for *E. coli* infection may include supportive therapies including fluid rehydration for severe diarrhea. However, antimicrobial therapy for O157 STEC infection is not efficacious or safe, except for cases of cystitis and pyelonephritis (Griffin, 1995). The use of antibiotics in STEC infection will kill the bacteria but in doing so also cause the release of the Shiga toxin. In addition, O157 and other non-O157 STEC have

acquired increasing levels of resistance to certain antibiotics (streptomycin, sulfonamides, and tetracycline) over the last 15 years (Bopp *et al*., 1999). The treatment with an appropriate antibiotic in ETEC infection has been associated with a reduction in the severity and duration of infection. However, resistance to tetracycline is on the rise in ETEC isolated from outbreaks in the United States (Dalton *et al*., 1995). In addition, most EPEC strains associated with outbreaks are also resistant to multiple antimicrobial agents (Bopp *et al*., 1999).

Certain serotypes of *E. coli* are more invasive than others and cause extra-intestinal disease when entering sites other than the bowel resulting in symptoms such as bacteremias, urinary tract infections, and meningitis. *E. coli* can become pathogenic by production of certain virulence factors, such as adherence factors, fimbriae, polysaccharide capsules, lipopolysaccharide, hemolysins, and toxins. These virulence factors make *E. coli* an important cause of diarrheal disease in humans and animals.

1.2 *E. coli* Hemolysins

E. coli strains that cause extraintestinal disease have the ability to lyse erythrocytes in different mammals, this is known as hemolysis (Schmidt *et al*., 1995). Hemolysins, also known as cytolysins, are important virulence factors of bacteria that cause extraintestinal diseases, and are active on different cells, such as lymphocytes, granulocytes, renal tubular cells, and erythrocytes (Schmidt *et al*., 1995).

Four major hemolysin groups have been described in *E. coli* from different pathogroups; these are: alpha- hemolysin (α-hly), enterohemorrhagic *E. coli* hemolysin (also known as EHEC-hly or HlyA or Ehx), enterohemolysin 1 (*ehl1*), and

enterohemolysin 2 (*ehl2*). Hemolysins are thought to increase virulence by increasing the availability of iron to the bacteria. This is accomplished through the release of hemoglobin from either formation of a pore or by membrane leakage (Moxley *et al*., 1998). Both EHEC-hly and α-hemolysin are members of the RTX family of toxins. RTX toxins have been highly researched and the representative toxin in this family is *E. coli* α-hemolysin.

1.3 RTX toxins

The family of RTX toxins are more accurately called the Repeats in Toxin family. This is due to the classic nonapeptide Ca^{2+} binding repeats that are consistently present within members of this family. RTX toxins are calcium dependent and the toxin repeats are responsible for the calcium binding. The Repeats in Toxin family is found in a wide variety of gram-negative bacteria. The first members of this group were hemolysins and leukotoxins produced by *E. coli*, *Pasteurella hemolytica*, *Actinobacillus* species, and *Bordetella pertussis* (Goni and Ostolaza, 1998).

The RTX family can be broken down into three different functional groups, which are the leukotoxins, hemolysins, and cytotoxins with mixed cell specificity. Leukotoxins have a narrow cytotoxic spectrum with leukocytes from a close phylogenetic grouping (e.g. ruminants or primates alone) being affected (Taichman *et al*., 1987). Hemolysins have a wider range of specifity. They are active towards many different cell types including erythrocytes and leukocytes from a wide variety of species (Calvalieri and Snyder, 1982). The mixed specifity group of RTX toxins can act against a wider range of cells or species types than the leukotoxins, but they have clear limits in activity that is not

observed for the hemolysins (Bauer and Welch, 1996). In the past few years, there have been two new additions to the Repeats in Toxins family: the plasmid encoded EhxA toxin found in enterohemorrhagic *E. coli* O157:H7 and the RTX determinant chromosomally encoded in epidemic strains of *Vibrio cholerae* (Bauer and Welch, 1996; Lin *et al*., 1999).

As previously mentioned, RTX toxins can act as a secretion system. In the majority of RTX toxins, the final 60 amino acids of the C-terminus contain the extracellular targeting sequence. In contrast, the N-terminal sequence does not have any functional significance and has great divergence among toxins. A common structural characteristic of RTX proteins is the presence of tandem arrays of a nine amino acid repeat with the following consensus sequence: L/I/F-X-G-G-X-G-N/D-D-X (Welch, 1991; Felmee & Welch, 1988). *E. coli* α-hemolysin has 13 tandem repeats of this sequence, while hemolysins from *P. haemolytica* and *A. pleuropneumoniae* have 11 repeats (Schmidt *et al*., 1995). The amino acid residues 200 to 450 have been shown to have a long stretch of hydrophobic amino acids. It was originally thought that these hydrophobic amino acids in this region would form transmembrane α-helices that function as a pore (van der Goot, 2001). It is also possible that the amphipathic α-helices insert into the lipid bilayer but do not transverse it causing the displacement of phospholipids in one layer of the membrane. This would result in destabilization of the bilayer, which causes pseudo-pore like activity (van der Goot, 2001). Both hypotheses result in the cytotoxic activity seen in RTX toxins. In addition to the important cytotoxic effects of RTX toxins, sublytic concentrations of these toxins are thought to modulate the immune response after infection with a given pathogen (Schmidt and Karch, 1996)

RTX toxins characteristically have a C-terminal secretion signal and are known to act as a type I exoprotein secretion system. Another characteristic, as found in *E. coli* α-hemolysin, is the ABC transporter system that involves linked B and D genes and a homologue of TolC (van der Goot, 2001). All RTX toxins are required to have the RTX A protein modified through the RTX C gene product. Lysine is uniquely modified with a fatty acid and a Glycine residue just N-terminal to the lysine is common to all modification sites (van der Goot, 2001). Four acylation independent events occur in RTX toxin biogenesis that result in toxin activity. Calcium binds to the toxin molecule and the toxin is secreted extracellularly. A pore forms in the lipid bilayer and the RTX toxin will associate with the target cells.

It is thought that RTX toxins act as dimers or oligomers. The monomers form an ordered dimeric or oligomeric tertiary structure (Ludwig *et al*., 1993). It is also possible that unordered complexes of toxin protein can produce the cytotoxic activity (van der Goot, 2001). However, there is no direct evidence of a monomeric or polymeric structure for the RTX toxin cell associated lethal activity. An additive, linear dose-response curve is not evident for RTX toxins (HlyA, LktA, and EhxA) (Calvalieri and Snyder, 1982). There is little lysis at low doses (van der Goot, 2001).

1.4 *E. coli* α-hemolysin

E. coli α-hemolysin is one of the best-studied hemolysins. It is the prototype of the RTX family of toxins. It is classified in the RTX family because generally RTX toxins possess the Glycine-rich nonapeptide motif that is repeated in tandem between 9 to 46

times (Goni and Ostolaza, 1998), which acts as a Ca^{2+} binding domain to initiate the lytic activity of HlyA.

The operon responsible for *E. coli* α-hemolysin production is *hly*CABD, which may be located on a pathogenicity island on the chromosome or on a plasmid. There is a promoter upstream of *hlyC* and a rho-independent terminator in the *hlyA – hlyB* intergenic region (Gentschev *et al.*, 2002). RfaH, an elongation protein, and a cis-acting 5' DNA sequence termed the JUMPstart element or ops (operon-polarity suppressor) element can suppress termination. These two factors act together to allow the efficient transcription of the *hly* operon (Gentschev *et al.*, 2002).

The *hlyA* gene encodes for the hemolysin protein. However, this form of hemolysin is not active and is known as Pro-HlyA. HlyC activates pro-HlyA inside the cytoplasm. HlyC is a fatty acid acyltransferase. Maturation of proA requires HlyC to acylate proA via an acyl carrier protein (ACP). This mechanism adds fatty acids via an amide linkage to two specific internal lysine residues (Koronakis and Hughes, 1996). HlyB and HlyD are membrane proteins that act as the secretion apparatus for the hemolysin. HlyB is an ABC transporter (ATP-binding cassette). Its N-terminal region spans the cytoplasmic membrane, and its C-terminal region acts as the ABC domain. HlyB is thought to move the hemolysin through the pore via energy from ATP and the proton motive force. It may function as a dimer in the membrane. HlyD is thought to bring HlyB in contact with the outer membrane channel. Secretion is also aided by TolC, which is not linked to the *hly*CABD operon. TolC is the proposed channel for the *E. coli* α-hemolysin system. The HlyA protein contains a secretion signal at the C terminus (Koronakis and Hughes, 1996) (Figure 1).

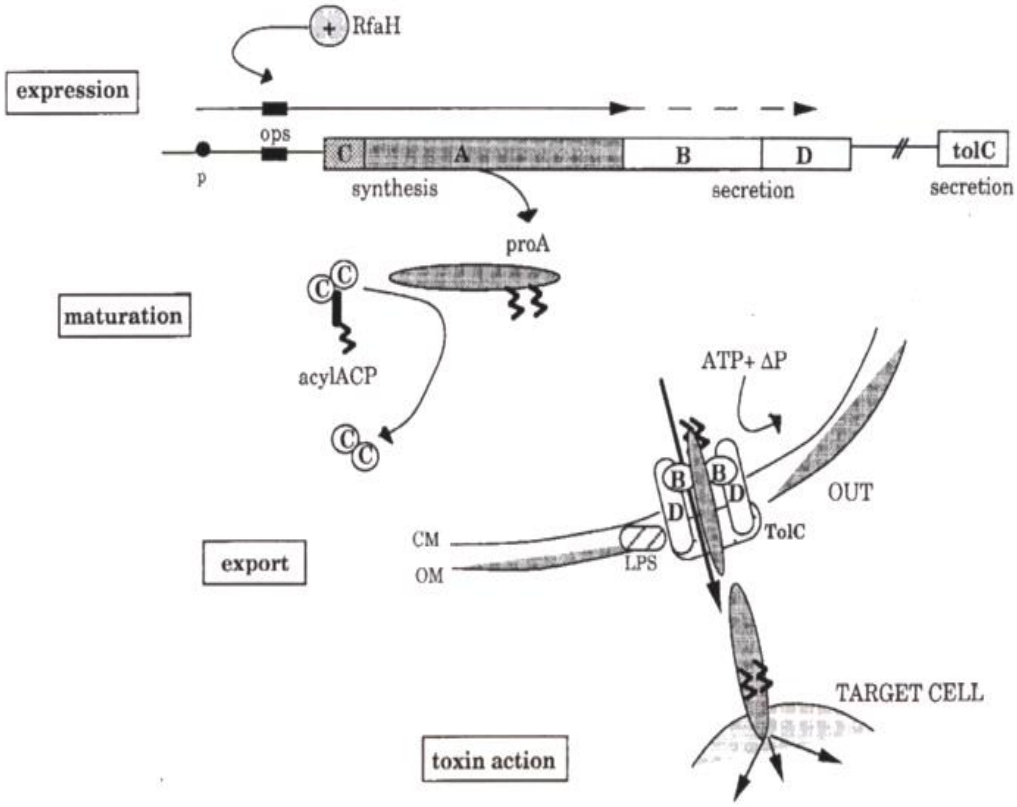

Figure 1: *E. coli* α-hemolysin synthesis, maturation, and export. The *hlyA* gene synthesizes Pro-HlyA, which is not active. Maturation of proA requires HlyC to acylate proA via an acyl carrier protein (ACP). HlyC, a fatty acid acyltransferase, activates pro-HlyA inside the cytoplasm. HlyB and HlyD assist export of HlyA. HlyB and HlyD are membrane proteins that act as the secretion apparatus for the hemolysin. HlyB uses energy from ATP and the proton motive force to move the hemolysin through the pore. TolC is the proposed channel for the *E. coli* α-hemolysin system (Koronakis and Hughes, 1996).

E. coli α-hemolysin has a characteristic phenotype that produces a clear zone of hemolysis on Sheep blood agar plates that can be observed after three hours (Weiler *et al*., 1996). It is a hemolysin that forms pores by the insertion of a single polypeptide chain into its target membrane. *E. coli* α-hemolysin hemolysis is temperature dependent and is inhibited by the addition of 30mM dextran 4 to the extracellular medium. Dextran is an osmotic protectant. Once in the membrane, the α-hemolysin toxin channel is not destroyed by trypsin.

1.5 *E. coli* Ehx Toxin

A recent addition to the RTX family is the Enterohemolysin toxin found in enterohemorrhagic *E. coli* O157:H7. This EHEC-hemolysin is encoded on the large virulence plasmid pO157. Enterohemolysin, Ehx, is possibly important as a virulence factor as it appears in *E. coli* O157 isolates and reacts to sera from patients that have haemolytic uremic syndrome (HUS) (Schmidt *et al*., 1995).

The EHEC operon contains four genes (*ehxA*, *ehxB*, *ehxC*, and *ehxD*) that are found on the pO157 plasmid. These ehx genes are organized in an operon structure similar to that of *E. coli* α-hemolysin (Schmidt *et al*., 1995). In some publications, these four genes have been referred to as: *hlyA, hlyB, hlyC,* and *hlyD,* due to their similarity to the *E. coli* α-hemolysin genes. The *ehxA* gene encodes the EHEC hemolysin, while *ehxC* is responsible for its activation. *EhxB* and *ehxD* encode part of the secretory machinery (Boerlin *et al*., 1998). In STEC strains, the *ehx* operon is highly conserved. This suggests that Ehx is involved in STEC survival and that Ehx is under strong selective pressure. (Law, 2000)

There is about 60% homology between *E. coli* α-hemolysin and EHEC-hemolysin. Both of these hemolysins are members of the RTX family. Ehx is characterized by biphasic hemolysis that produces small turbid zones of haemolysis after 18-24 hours incubation on washed erythrocytes. This is in contrast to *E. coli* α-hemolysin, which produces large clear zones of haemolysis after 4 hours of incubation on either washed or unwashed erythrocytes (Law, 2000). Similar to *E. coli* α-hemolysin, enterohemolysin has 13 tandem repeats of the consensus sequence from amino acids 706-832 in the C-terminal region (Schmidt *et al*., 1995). Ehx also has a stretch of 200 hydrophobic amino acids in the N-terminal region, which is another common structural feature to RTX proteins. This distribution of hydrophobicity is similar to that which occurs in *E. coli* α-hemolysin (Schmidt *et al*., 1995). The amino terminal one-fifth of RTX proteins is important for target cell specifity. EHEC-HlyA has only 40% homology to α-hemolysin, which suggests that there is different target cell specifity between the two hemolysins (Schmidt *et al*., 1995). α-hemolysin is active against human lymphocytes while enterohemolysin is not active against these cells (Law, 2000). Both EHEC-hly and α-hemolysin are pore-forming toxins and their toxicity is the result of their insertion into the cytoplasmic membrane of eukaryotic cell and the resulting permeability changes (Schmidt and Karch, 1996).

Enterohemolysin secretion by *E. coli* O157 strains appears to be defective. This defective secretion results in the small biphasic zones of hemolysis. There also appears to be a lack of hemolytic activity in supernatant fluids (Law, 2000). Transferring the *E. coli* α-hemolysin transport system to *E. coli* O157 can enhance the hemolytic activity of Ehx (Law, 2000). Production of Ehx in vitro is enhanced by mimicking conditions that

are similar to human and animal large bowel (anaerobic conditions and decreased oxygen tension). It is thought that since these enhanced growth conditions increase Ehx production, the toxin is probably produced at a maximum in the large bowel (Law, 2000).

There is a high incidence of EHEC-hemolysin in STEC. Therefore, there is a possible link between these two proteins in the pathogenesis of hemorrhagic colitis and HUS. This may suggest that EHEC-hemolysin is a possible bacterial virulence factor (Schmidt *et al*., 1995).

1.6 *E. coli* Enterohemolysins

Enterohemolysin was first described in 1988 in Enteropathogenic *E. coli* strains (Beutin *et al*., 1988). The hemolytic activity produced by enterohemolysin was found to be phenotypically different from *E. coli* α-hemolysin; *E. coli* enterohemolysin shows a biphasic haemolytic zone that has a clear zone beneath the colony and a small turbid zone surrounding the bacteria. This type of hemolysis occurs after overnight (16 hours) of incubation on sheep blood agar plates that contain washed sheep red blood cells and $CaCl_2$ (Weiler *et al*., 1996). The *E. coli* enterohemolysins that produce this biphasic hemolysis are known as Ehly1 and Ehly2. *E. coli* Hly_{EHEC} (Ehx) is phenotypically indistinguishable from Ehly1 and Ehly2 in that they all display biphasic hemolysis, however, these toxins differ genetically. Temperate bacteriophages are the genetic determinants that encode enterohemolysin production. These bacteriophages were originally isolated from *E. coli* O26 strains (Stroeher *et al*., 1993).

It is thought that lipoproteins present in mammalian serum interact with bacterial toxins, such as endotoxins, to reduce their toxicity. Lipoproteins can be classified into

five major classes according to their densities: very low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL). Enterohemolysin activity is inhibited by normal mammalian serum and by cholesterol in vitro (Figueiredo *et al*., 2003). Lipoproteins are hemolysin inactivators with high-density lipoprotein having the greatest inhibitory effect on enterohemolysin activity (Figueiredo *et al*., 2003). Enterohemolysin disrupts cell membranes by a detergent like mechanism; Interactions between lipids and membrane proteins may further affect the state of the lipids and cause further disruption of the membrane into mixed micelles or even separation of the lipid and protein constituents (Figueiredo *et al*., 2003).

Cholesterol has been found to inhibit the hemolytic activity of enterohemolysin in vitro (Figueiredo *et al*., 2003). Cholesterol is located in the cell membrane and occurs in mammalian sera as a component of lipoproteins, which are responsible for the transportation of lipids in the organism (Figueiredo *et al*., 2003). Lipopolysaccharides, LPS, of bacteria form complexes with lipoproteins, mainly HDL. It has been suggested that phospholipids on the surface of HDL could bind and neutralize the LPS via the leaflet insertion model (Figueiredo *et al*., 2003). Lipoproteins may inhibit enterohemolysin activity in a similar mechanism to the inactivation of LPS but through cholesterol on the surface of HDL and other lipoproteins (Figueiredo *et al*., 2003).

1.7 *E. coli* Ehly2

Enterohemolysin, *ehl2*, is encoded by a temperate bacteriophage isolated from *E. coli*. Ehly2 is completely unrelated to the sequence encoding synthesis of Ehly1 (Beutin

et al., 1993). The enterohemolysin 2 sequence was found to be partially homologous to the bacteriophage lambda. Ehly2 has three regions of very high homology to λ phage (Beutin *et al*., 1993) (Figure 2). These three regions are: the 117 bp Region A that is 93% homologous to λ, the 31 bp Region B that is completely identical to λ, and Region C that is 261 bp and 95% homologous to λ.

Regions A and B of Ehly2 occur within the open reading frame that encodes a 194 amino acid protein that is about 22.1-kDa. This protein was found to be hydrophilic with no evidence of a signal sequence and it also has a hydrophobic stretch that may possibly prevent membrane localization (Beutin *et al*., 1993). Thus, it is thought that this encoded protein is possibly cytoplasmic. The protein encoded by *ehl2* was also shown to have a high degree of homology with Ea22 protein of λ, which has no known function. Region C of Ehly2 is an intergenic region of λ between the gene encoding for another protein of unknown function, Ea8.5, and *xis*, which is involved in excision of the prophage (Beutin *et al*., 1993). There are many open reading frames within the sequence of Ehly2 making it hard to determine which is the actual protein. Due to the homology of the 22.1 kDa protein to Ea22, possibly Ehly2 corresponds to either the 12 or 13.4 kDa protein (Beutin *et al*., 1993). Both of these proteins are hydrophobic which would be expected of a toxin that could damage membranes, and neither of these proteins have a signal peptide. Thus, Ehly2 is not expected to be a typical secreted protein (Beutin *et al*., 1993).

Ehly2 is found within the cell and is not secreted into the medium. Possibly Ehly2 may require a secretion system that is similar to HlyB and HlyD from *E. coli* α-toxin (Beutin *et al*., 1993).

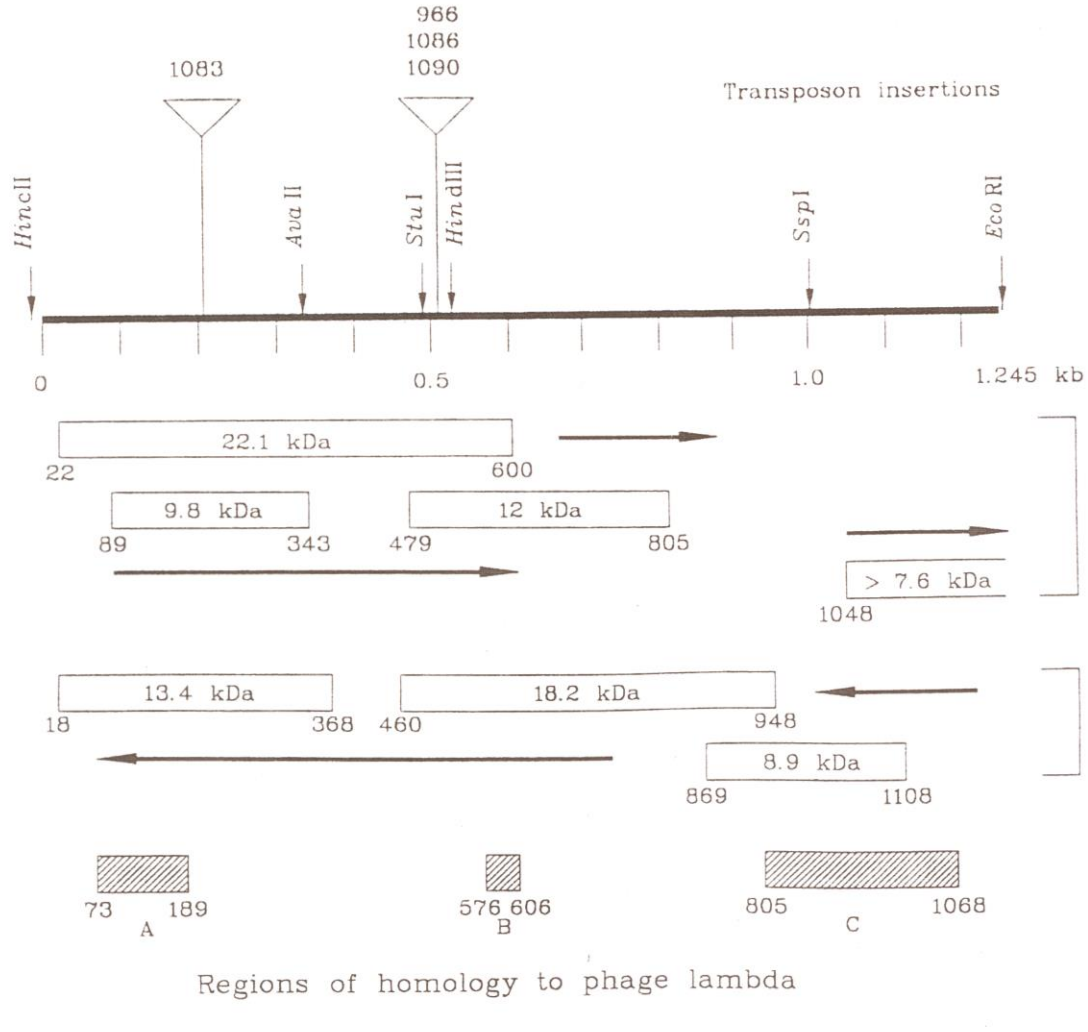

Figure 2: Physical map of the *ehl2* ORFs from the insert in pEO39. The boxes show the ORFs and the proteins that they encode with their respective sizes in kDA. The numbers below the boxes indicate bp. The arrows indicate the directions of transcription. Regions of homology to λ are shown as hatched boxes A, B, and C. Independent Tn*1725* mutations leading to a Hly$^-$ phenotype are indicated above the restriction map of pEO39 (Beutin *et al*., 1993).

1.8 *E. coli* Ehly1

1.8.1 Characteristics of *E. coli* Ehly1

Enterohemolysin 1, Ehly1, is genetically and serologically unrelated to *E. coli* α-hemolysin. Ehly1 does not react to α-hemolysin monoclonal antibodies nor does it hybridize to the α-hemolysin gene probe. Enterohemolysin 1 is frequently expressed by *E. coli* strains producing verotoxins (VTEC) and by verotoxin negative *E. coli* O26 strains (Stroeher *et al*., 1993). Enterohemolysin 1 is a 30-kDa protein. Similar to Ehly2, Ehly1 is encoded by a temperate bacteriophage. It is possible that Ehly1 is post translationally modified by acylation similar to that required for activation of HlyA in *E. coli* (Stroeher *et al*., 1993). This is supported by the fact that some studies have shown that the 29.6 kDA protein sometimes appears to run as a 33-kDa band in Western Blots thus not appearing to run at its true molecular mass (Stroeher *et al*., 1993) (Figure 3). Post-translational modifications occur in prokaryotic proteins (Fischer and Haas, 2004) and frequently in eukaryotic proteins where they modulate the activity of most eukaryotic proteins (Mann and Jensen, 2003). Thus, it is a common phenomenon to have a protein run at a different molecular mass than its true molecular mass due to post-translational modification, whether it be a structural rearrangement, phosphorylation, acylation or one of the other many modifications which could occur.

Ehly1 appears to be a pore forming cytolysin, is not expressed by stationary cultures, and is not secreted into the culture media. It is an intracellular toxin and reaches it maximum intracellular accumulation in late log phase (Jurgens *et al*., 2002). This coincides with cell lysis by a lytic bacteriophage, which may be of interest since Ehly1 is encoded by a bacteriophage. Hemolysin activity is not detected until bacterial cell death

occurs and the hemolysin activity can be increased with the treatment of cells with lysozyme (Stroeher *et al*., 1993).

The susceptibility of red blood cells of various animal species to lysis by enterohemolysin was previously determined by hemolytic titration (Jurgens *et al*., 2002). The erythrocyte spectrum included sheep, rabbit, mouse, rat, cow, guinea pig, human, and horse. Ehly1 exhibits species cell specificity with respect to lytic potential with horse erythrocytes being the most sensitive target (Jurgens *et al*., 2002). The range of erythrocytes lysis by enterohemolysin was: rabbit < mouse < sheep, rat < cow < guinea pig, human < horse (Jurgens *et al*., 2002).

Enterohemolysin 1 is temperature independent and the addition of dextran 4 or dextran 8 will increase erythrocyte lysis in proportion to the concentration ranging from 5 – 30mM. In this case, dextran does not seem to protect against osmotic lysis. Membrane bound enterohemolysin is destroyed by trypsin. This means that after binding to erythrocyte membranes, enterohemolysin is still susceptible to proteolysis.

Analysis of the Ehly1 protein sequence shows that there are many hydrophobic domains but it does not show any evidence of an N-terminal signal sequence (Stroeher *et al*., 1993). This suggests that the Ehly1 protein is localized to the cytoplasmic membrane of *E. coli*. This could also be interpreted as a transport system that allows Ehly1 export in a manner that is similar to the transport seen in HlyA of *E. coli*. Since Ehly1 appears to be encoded by a bacteriophage, a transport system may not have been cotransduced (Stroeher *et al*., 1993). Enterohemolysin 1 may also resemble α-hemolysin and have a secretion signal at the C-terminus.

Figure 3: Restriction map of the 2.1-kb insert in pEO21 showing the 29.6-kDa ORF that encodes *ehl1*. Numbers above the restriction map indicates the Tn*1725* transposon insertions, which inactivate Ehly1. The 464-bp *Eco*RV-*Bam*HI fragment was used as a specific probe (Stroeher *et al*., 1993).

Jurgens *et al*., 2002, found the cell membrane to be the primary site of action of Ehly1 by examining horse erythrocytes through electron microscopy. This study showed the formation of ring structures of approximately 10nm that were found to be irregularly distributed and clustered on the erythrocyte membrane. Jurgens *et al*., 2002, also determined the mode of action of Ehly1 was not enzymatic because proteinase and phosphatase inhibitors failed to inhibit lysis. Since Ehly1 causes rapid destruction of target cell membranes, it is thought to alter and disrupt the cell membrane by a detergent like mechanism (Jurgens *et al*., 2002). The Ehly1 protein has been shown to be a 30 – 33 kDa. However, a 65-kDa form has also been detected. Could this possibly be the result of dimerization of Ehly1 in preparation of pore formation? Antisera from the 30-kDa form will cross-react with the 65-kDa protein from a SDS-PAGE in Western Blot. Antisera raised to both forms of the Ehly1 protein can neutralize the enterohemolysin activity (Stroeher *et al*., 1993). In strain C3888 (Beutin *et al*, 1990), it was found that the 65-kDa form was associated with an increase in enterohemolysin activity while the 30-kDa form was associated with lower levels of enterohemolysin activity.

Sequence analysis of the *ehl1* gene shows that it has a ribosome-binding site before the gene and a strong potential transcriptional terminator after the gene. There are no good –35 and –10 sequences which would correspond to the σ^{70} promoter. There is also a potential Fur-box located about 60 nucleotides upstream from the translational start site and therefore *ehl1* may be regulated by the presence of iron-deprivation (Stroeher *et al*., 1993). Beutin *et al*., 1990, performed homology searches with EMBL and GenBank for both the nucleotide and the predicted amino acid sequence from Ehly1; no significant homology was found to any known DNA or protein sequence.

1.8.2 A Recent Outbreak of *E. coli* Ehly1

The importance of *ehl1* as a virulence factor was noted in 1996 when an outbreak of *E. coli* O18ac:H31 occurred in neonatal nursery ward in Neuquen City, Argentina. These strains of *E. coli* were non-lactose fermenting, non-sorbitol fermenting, negative for the typical virulence factors, and were positive for *ehl1*.

This outbreak occurred from October 9, 1996 to November 12, 1996 and affected 7 premature infants with bloody diarrhea. A prevalence study was performed and stool was obtained from 16 hospitalized neonates and from 33 medical staff members. *E. coli* isolates with identical biochemical characteristics of the outbreak strain were recovered from 11 out of 16 inpatients and from 4 out of 33 staff members. There were 15 isolates in total from the outbreak and the prevalence study. This outbreak occurred despite strict regulations to prevent cross-infection in the hospital (Chinen *et al*., 2002).

It is thought that the premature neonates may have shown clinical symptoms because of their low birth weight, which may be a risk factor for enteric illness (Chinen *et al*., 2002). However, premature neonates are antibody deficient and have an immature immune system, which could also be the main contributing factor to their susceptibility to Ehly1. In a Neonatal Intensive Care unit, NICU, there are many supportive therapies that are used which breach natural barriers to infection. These invasive supportive therapies may negatively affect the ability of a neonate to resist infection. The premature status of the infants may offer an explanation for the outbreak of bloody diarrhea associated with the *E. coli* O18ac:H31 strain that produced *ehl1* (Chinen *et al*., 2002). However, there

were premature infants in the prevalence study that excreted the same strain but did not show any symptoms.

1.8.3 *E. coli* Ehly1 as a Potential Virulence Factor

Originally *ehl1* was not thought of as a significant virulence factor. It was thought to be associated with the verotoxin genes of *E. coli,* which were known virulence factors. This recent outbreak in the Argentinean NICU has brought more attention to *ehl1* as a possible virulence factor. Since e*hl1* is found in *E. coli* isolates that also express verotoxin (Sandhu, *et al*. 1997) it may have a synergistic effect with VT1 or VT2 to increase the virulence of the strain. Thus, it is important to determine how this gene is regulated and to see if any other important virulence factors are co-transduced or co-expressed with Ehly1.

To date, the most consistent factor associated with virulence is serotype (Gyles *et al*., 1998). It is important to produce methods that are easier and quicker in the identification of virulence factors in *E. coli*. Until recently there has been no suitable animal model to test the effects of possible *E. coli* virulence factors in relation to specific virulent *E. coli* strains. Thus limiting the examination of *E. coli*-host interactions. The rabbit ligated ileal loop model was used in the past to examine *E. coli* infection but this model was not practical, as it required surgical alteration of the rabbit (Leitch *et al*., 1966). Recently, a C57BL/6J mouse has been examined as a model of EPEC infection and was determined to be a suitable in vivo model for studying EPEC infection (Savkovic *et al*., 2005).

Ehl1 is found in all of *E. coli* isolates from the Argentina outbreak. However, only some of the neonates showed symptoms of infection. *Ehl1* may only be expressed when another virulence factor is coexpressed or when certain factors are present for induction of *ehl1*. Previous studies have shown that lipoproteins may inhibit enterohemolysin activity (Figueiredo *et al*., 2003). However, since neonates have minimal levels of lipoprotein compared to adults (Behrman *et al*., 2003), this may be a possible explanation as to why only some of the neonates and not the hospital support staff were affected in the Argentinean outbreak.

1.9 Objectives and Hypothesis

The primary objective of this study is to examine *ehl1 c*ytotoxicity on various types of cells in culture and to determine if *E. coli ehl1* has a synergistic effect with *E. coli* VT1 or VT2 cytotoxin that may lead to HUS and other severe complications. This objective will test the hypothesis that e*hl1* may have a synergistic effect with VT1 and VT2 resulting in an increase the virulence of the strain.

The second objective of this study is to examine if *ehl1* is coexpressed with other virulence factors and to determine if *E. coli ehl1* is induced by lipoprotein which may lead to expression of *ehl1* and subsequent symptoms of diarrhea. This objective will test the hypothesis that e*hl1* may only be expressed when another virulence factor is coexpessed or when certain factors are present for induction of *ehl1*.

1.10 Significance of the Study

These studies will provide knowledge of *E. coli* enterohemolysin 1, specifically pertaining to the characterization of Ehly1 as a potential virulence factor. Knowledge of *ehl1* as a virulence factor may lead to using this gene in the genetic screening of isolates from outbreaks. The possibility of linking this gene to other virulence factors that are co-expressed with *ehl1* may offer a plausible explanation for different clinical signs and symptoms, or the lack of signs and symptoms, observed in patients with *E. coli* infections.

2 MATERIALS AND METHODS

2.1 Bacterial Strains and Growth Conditions

The *E. coli* O18ac:H31 strain (NML # 01-1610) containing *ehl1* was obtained from our culture collection here at the National Microbiology Laboratory (NML), Public Health Agency of Canada. This isolate was originally sent to the NML from the Instituto Nacional de Enfermedades Infecciosas in Buenos Aires, Argentina.

E. coli isolate C3888 was used as a positive control for *ehl1* and was kindly provided by Dr. L. Beutin from the *E. coli* Reference Laboratory at the Robert-Koch-Institut in Berlin, Germany.

The other *E. coli* isolates used as controls in this study were also obtained from the NML culture collection.

All *E. coli* strains were streaked onto MaConkey (MAC) agar plates and incubated overnight at 37°C for isolation of single colonies. Single colonies were selected and grown on Nutrient Agar plates at 37°C overnight. Serotyping was performed to confirm the O and H antigens of the isolates. The single colonies of the strains were stored in skim milk medium frozen at -80°C until ready to use in this study.

To recover the *E. coli* isolates from the frozen state, the cells were defrosted at room temperature and then 10 µl was added to 6ml of brain heart infusion (BHI) broth. *E. coli* cultures containing the pRS551 plasmid or the pQE-60 plasmid had 10 µl of culture added to 6ml of BHI broth containing 50 µg/ml ampicillin. The cultures were then incubated in a 37°C incubator, with rotation (150 rpm), overnight.

E. coli XL1-Blue chemically competent cells and *E. coli* BL21-Gold (DE3) competent cells were purchased from Stratagene. *E. coli* M15[pREP4] cells were purchased from Qiagen. These competent cells were stored at -80°C until ready to use.

2.2 Antibodies and Primers

Tetra-His HRP Conjugate Kit (Qiagen) and the Penta-His HRP Conjugate Kit (Qiagen) contained the antibodies used for western blotting. Both of these antibodies, the Tetra-His HRP conjugate and the Penta-His HRP conjugate, consist of mouse monoclonal IgG1 Anti-His Antibodies joined to horseradish peroxidase (HRP). HRP eliminates the need for secondary antibodies and allows for direct detection through chemiluminescent and chromogenic methods.

See Appendix I for a list of primers used. Primers for *E. coli ehl1* are based on Genbank sequence Accession Number X70047. See Appendix II for the nucleotide sequence of *E. coli ehl1* from isolate 01-1610.

2.3 DNA Extraction

DNA extraction was performed to generate a DNA template for PCR. *E. coli* cells were grown on nutrient agar plates over night at 37°C. Isolation of DNA was performed in the following manner: 1) cells were taken directly from the nutrient agar plate and dissolved in HTE Buffer (50 mM Tris-HCl, pH 8.0, 20 mM EDTA); 2) 2% Sarcosyl in HTE Buffer was added and mixed; 3) RNase in TE Buffer was added, mixed, and incubated for 30 minutes at 37°C; 4) Proteinase K (20 mg/ml) was added, mixed, and incubated at 50°C until the solution became clear; 5) phenol chloroform extraction was

performed for separation of DNA from protein through phase separation; 6) DNA was precipitated with isopropyl alcohol; 7) DNA was washed with 70% ethanol and 8) DNA was redissolved in TE buffer (Maniatis, *et al*, 1989).

2.4 Polymerase Chain Reaction (PCR)

PCR was performed using FastStart Taq DNA polymerase (Roche, Canada) in a 2400 DNA Thermocycler from Applied Biosystems. FastStart Taq DNA polymerase was chosen since it has been shown to improve amplification specificity, sensitivity, and product yield. FastStart Taq DNA polymerase is inactive at temperatures below 75°C and is activated at 95°C for 2 to 5 minutes i.e., hot start PCR. This allows for the elimination of setting up the PCR on ice. PCR was performed to amplify the *ehl1* gene for cloning into vectors pRS551 and pQE-60.

A typical 50 µl reaction consisted of: 45 µl of master mix, 5 µl of DNA (1:100 dilution) and 0.5 µl (5 units) of FastStart Taq DNA polymerase.

A typical 1 ml PCR mastermix consisted of: 100 µl of 10x PCR buffer, 8 µl of dNTP (10 mM each), 6.5 µl of 10 µM forward primer, 5.25 µl of 10 µM reverse primer, and 780.25 µl of sterile waster. Amount of sterile water, forward primer, and reverse primer were adjusted accordingly depending on the primers used in the mastermix.

A typical thermocycling protocol for PCR included:

Cycles	Temperature and Time
1x	Initial denaturation 95°C for 8 min
40x	-Denaturation at 95°C for 30 s
	-Annealing at 58°C for 30 s
	-Extension at 72°C for 30 s
1x	Final extension time 72°C for 7 min
∝	4°C

2.4.1 Product Analysis

All amplicons were verified for quality and size by 1% agarose gel electrophoresis, run in Tris/Borate/EDTA (TBE) electrophoresis buffer. Ethidium bromide was used as a stain and added to the melted agarose after which the gels were cast, run at 120 volts for 45 min, and DNA was viewed with an Imagemaster VDS transilluminator (Pharmacia Biotech, Sweden).

2.5 Cloning

Intact fragments containing *ehl1* were generated by PCR using sequence specific primers. The inserts were then verified by 1% agarose gel electrophoresis and DNA sequencing. The amplicons and the appropriate vector were digested in parallel with restriction enzymes *Bcl*I and *Bam*HI (New England Biolabs). The pRS551 vector and the amplicons for insertion into its polylinker were further prepared by PCR polishing (PCR polishing kit, Stratagene). The digested vector and insert were ligated together using 1 unit of T4 DNA ligase [Stratagene, a typical ligation reaction consisted of 1.0 – 10.0 μl of sterile water, 2.0 μl of 10x buffer, 5.0 – 6.0 μl vector, 2.0 – 12.0 μl of insert and 1.0 μl of T4 DNA ligase (for a total volume of 20.0 μl)] and incubated overnight at room temperature. The ligated vector containing pRS551 was transformed into both *E. coli* XL1-Blue competent cells and *E. coli* BL21-Gold (DE3) competent cells. The ligated vector containing pQE-60 was transformed into both *E. coli* XL1-Blue competent cells and *E. coli* M15[pREP4] competent cells, respectively.

2.5.1 Transformation into *E. coli* XL1-Blue competent cells

E. coli XL1-Blue competent cells were transformed by thawing cells on ice, aliquoting 100 μl to individual prechilled tubes, and then adding β-mercaptoethanol to give a final concentration of 25 mM. β-mercaptoethanol increases the transformation efficiency of the cells. The competent cells were then incubated on ice for 10 minutes. Then 1 μl of the ligation reaction (undiluted, 1/5 dilution, or 1/10 dilution) was added to the competent cells. A pUC18 control plasmid was also prepared by adding 1 μl of pUC18 to the competent cells. This was followed by incubation on ice for 30 minutes. Cells were heat shocked at 42°C for 45 sec, and then placed on ice for 2 minutes and SOC medium pre-warmed to 42°C was added. The reaction was incubated at 37°C with shaking for 1 hour. Then 200 μl of the transformation reaction mixture was plated on LB+Amp (100 μg/ml) plates and incubated overnight at 37°C.

2.5.2 Transformation into *E. coli* BL21-Gold (DE3) competent cells

E. coli BL21-Gold (DE3) competent cells were transformed by thawing cells on ice and then aliquoting 100 μl to individual prechilled tubes. Then 1 μl of the ligation reaction (undiluted, 1/5 dilution, or 1/10 dilution) was added to the competent cells. A pUC18 control plasmid was also prepared by adding 1 μl of pUC18 to the competent cells. This was followed by incubation on ice for 30 minutes. Cells were heat shocked at 42°C for 20 sec, and then placed on ice for 2 minutes and SOC medium added. The reaction was incubated at 37°C with shaking for 1 hour, then was plated on LB+Amp (100 μg/ml) plates and incubated overnight at 37°C.

2.5.3 Transformation into *E. coli* M15[pREP4] competent cells

E. coli M15[pREP4] cells were plated on LB+Kan (25 μg/ml) for isolation and grown at 37°C overnight. The cells were made competent by following the protocol recommended in the QIAexpressionist, technical manual 5th edition, 2001. A single colony was selected and grown it on LB+Kan (25 μg/ml) plates at 37°C overnight. These cells were then harvested by washing with 10% glycerol and centrifuged at 4,000g at a temperature of 4°C for 5 minutes, the pellet was kept and the process was repeated three times. The cell concentration was adjusted to 3×10^{10} cells/ml with 10% glycerol. The competent M15[pREP4] cells were then dispensed 100 μl/tube and stored at -80°C until use.

Competent M15[pREP4] cells were transformed by thawing cells on ice and 1 μl of the ligation reaction (undiluted, 1/5 dilution, or 1/10 dilution) was added to the competent cells. The DNA-cell mixture was then transferred to a chilled electroporation cuvette and placed into the electroporation chamber on the Bio-Rad Gene Pulser (Bio-Rad) set at 1.7KV, 200Ω, and 25 μFD on the Bio-Rad Pulse Controller (Bio-Rad). The sample was then pulsed once and then 960 μl of 37°C psi broth was added to resuspend the cells. The reaction was then transferred into 15 ml falcon tubes and incubated at 37°C with shaking for 1 hour. A negative control containing transformed cells with TE buffer was used. This was followed by incubation on ice for 30 minutes. Then 200 μl was plated on LB+Kan (25 μg/ml) plates and incubated overnight at 37°C.

2.5.4 Colony selection and screening

The pRS551 recombinant plasmid contains *ehl1* fused to lacZ; therefore colonies were screened by color selection by adding X-gal and IPTG to the LB+Amp (100 μg/ml) plates, and blue colonies were selected after the overnight incubation at 37°C. In addition, colonies from both the pRS551 and pQE-60 transformations were also screened by restriction enzyme digestion. The selected construct was fully sequenced through the insert region using Dye Terminator sequencing which is based on Sanger *et al*., (1977). The amplicons were analyzed by using an ABI 3100 and ABI 3730 Genetic Analyzer.

2.5.5 pRS551 constructs

The vector pRS551 is a 12.5kb vector and it is derived from pRS415. pRS551 is the pRS415 vector that has been modified with the addition of a *kan* gene (Figure 4) (Simons et al., 1987). In addition, pRS551 has ampicillin and kanamycin selection markers (Simons *et al*., 1987). Cloning into the polylinker of pRS551 results in an operon fusion protein. The order of the cloning site and structure of its flanking region is as follows: *bla*-*kan*-$T1_4$-*Eco*RI-*Sma*I-*Bam*HI-*lac*Z+ (Simons *et al*., 1987). Between *bla* and *tet'* in pRS551, there are four tandem copies of the strong transcriptional terminator, T1, from the *E. coli rrnB* operon, to block transcription from upstream promoters (Simons *et al*., 1987). Hence, pRS551 is a considered a promoterless vector since it has very little promoter activity and allows the recombinant protein to be under the control of its own promoter (Simons *et al*., 1987).

Dr. J. Kaper from the University of Maryland School of Medicine, Department of Microbiology and Immunology in Baltimore, Maryland, kindly provided the pRS551

vector. pRS551 was plated for isolation on LB+Amp (100 μg/ml)+Kan (35 μg/ml) plates and grown at 37ºC overnight. Colonies were selected and grown in 10 ml of Brain Heart Infusion (BHI) broth supplemented with ampicillin (100 μg/ml) and kanamycin (35 μg/ml) and grown at 37ºC overnight with shaking. Then an alkaline plasmid extraction was performed using a StrataPrep Plasmid Miniprep Kit (StrataPrep Plasmid Miniprep Kit Instruction Manual, Stratagene, 2002) to recover the pRS551 DNA. pRS551 plasmid DNA was diluted 1/10 with distilled water for use in cloning.

Sequence specific primers containing *Sac*I, *Pst*I and *Hind*III restriction sites (Appendix IA) were used to amplify the *E. coli ehl1* coding sequence by PCR. The pRS551 vector was digested with *Pst*I and *Hind*III restriction enzymes. Both the PCR inserts and the pRS551 vector digested by *Pst*I and *Hind*III were PCR polished and ligated with the PCR fragment.

2.5.6 pQE-60 expression system

The QIAexpress System (Qiagen) contains many vectors including the pQE-60 vector (Figure 5A) (The QIAexpressionist, technical manual 5th edition, 2001). QIAexpress pQE vectors belong to the pDS family of plasmids and were derived from plasmids pDS56/RBSII and pDS781/RBSII-DHFRS (The QIAexpressionist, technical manual 5th edition, 2001). pQE plasmids are low-copy plasmids and are based on the T5 promoter transcription-translation system. These plasmids have various features that make them ideal for expression studies including a 6xHis-tag coding sequence either 5' or 3' to the cloning region. In the case of pQE-60, the recombinant construct will have the 6xHis-tag at the C-terminus of the protein of interest.

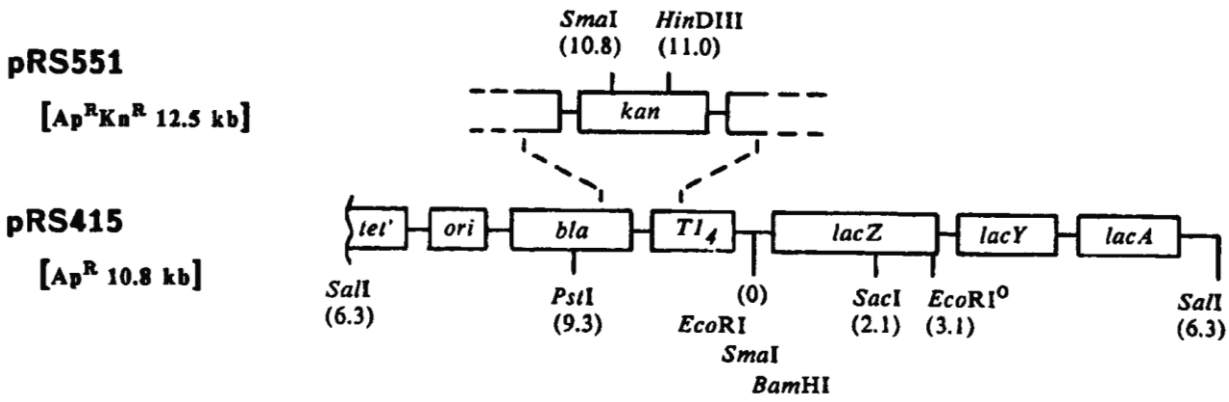

Figure 4: Operon fusion vectors pRS551 and pRS415. The vector pRS551 is derived form pRS415 with the only difference being the addition of the *kan* gene (Simons *et al*., 1987).

In pQE-60, high levels of the *lac* repressor protein are necessary to regulate and repress the high transcription rate of the T5 promoter. This expression system uses *E. coli* host strains that use the *lac* repressor gene either in *trans* or *cis* to the gene to be expressed. The pQE-60 expression vector uses *trans*-Repression when *E. coli* host strains containing the pREP4 plasmid, such as *E. coli* M15[pREP4], are used. In the *trans* system, the host strains contain the low-copy plasmid pREP4 (Figure 5B) that confers kanamycin resistance and constitutively expresses the *lac* repressor protein encoded by the *lac* I gene (The QIAexpressionist, technical manual 5th edition, 2001). The host cells contain numerous copies of pREP4 and thus numerous copies of the *lac* repressor protein. This ensures that the *lac* repressor protein will bind to the operator sequence and tightly regulate recombinant protein expression (Figure 6) (The QIAexpressionist, technical manual 5th edition, 2001). The pREP4 plasmid is maintained in the presence of kanamycin (25 µg/ml). Adding isopropyl-β-D-thiogalactoside (IPTG) will rapidly induce the recombinant protein since IPTG will bind the *lac* repressor protein and inactivate it. Once the *lac* repressor is inactivated, the host cell's RNA polymerase can transcribe the sequences downstream from the promoter; transcripts are produce and then translated into the recombinant protein (The QIAexpressionist, technical manual 5th edition, 2001).

The pQE-60 expression vector uses *cis*-Repression when *E. coli* strains that have the *lac*I^{q} mutation, such as *E. coli* XL1-Blue, are used. The *E. coli* XL1-Blue strain makes enough *lac* repressor to block transcription but expression is regulated less tightly than in strains containing the pREP4 plasmid.

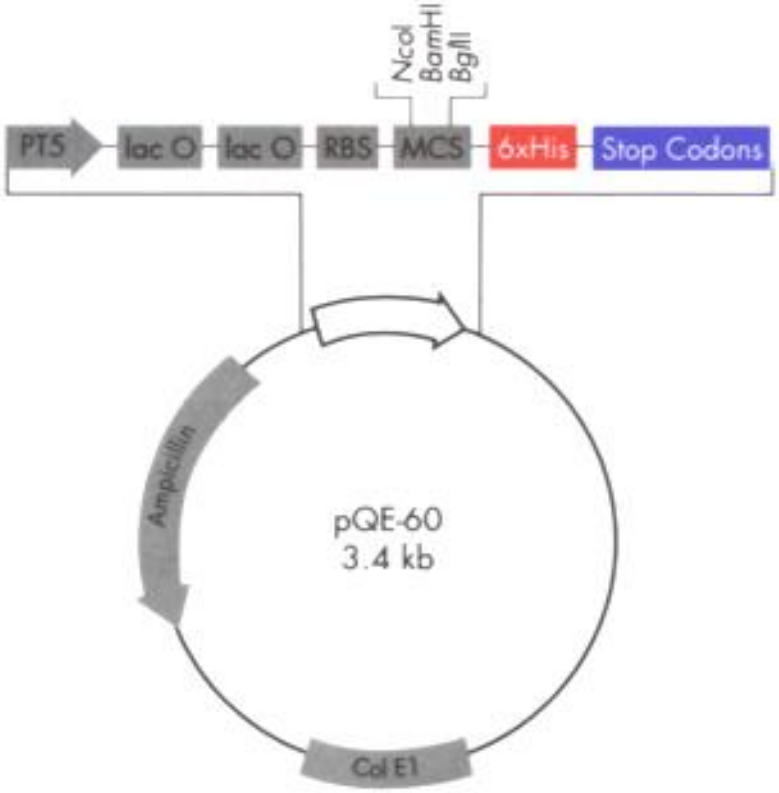

Figure 5A: Vector map of pQE-60 (Qiagen). PT5: T5 promoter, lac O: lac operator, RBS: ribosome binding site, ATG: start codon, 6xHis: 6xHis tag sequence, MCS: multiple cloning site containing the *Nco*I, *Bam*HI, and *Bgl*II restriction sites, Stop Codons: stop codons in all three reading frames, ColE1: Col EI origin of replication, Ampicillin: ampicillin resistance gene (The QIAexpressionist, technical manual 5th edition, 2001).

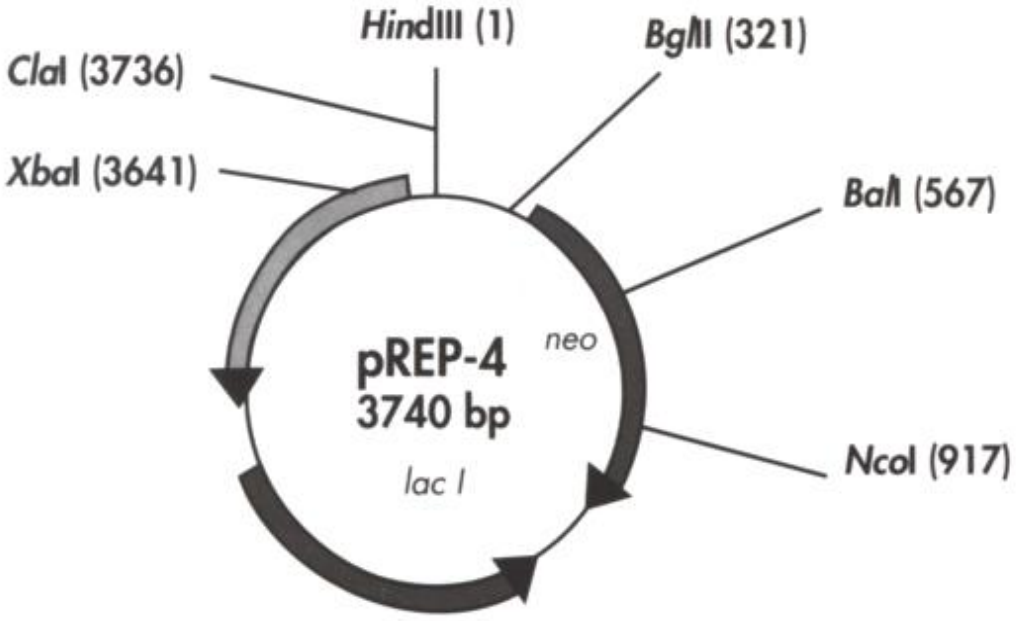

Figure 5B: Restriction map of pREP4 (The QIAexpressionist, technical manual 5th edition, 2001).

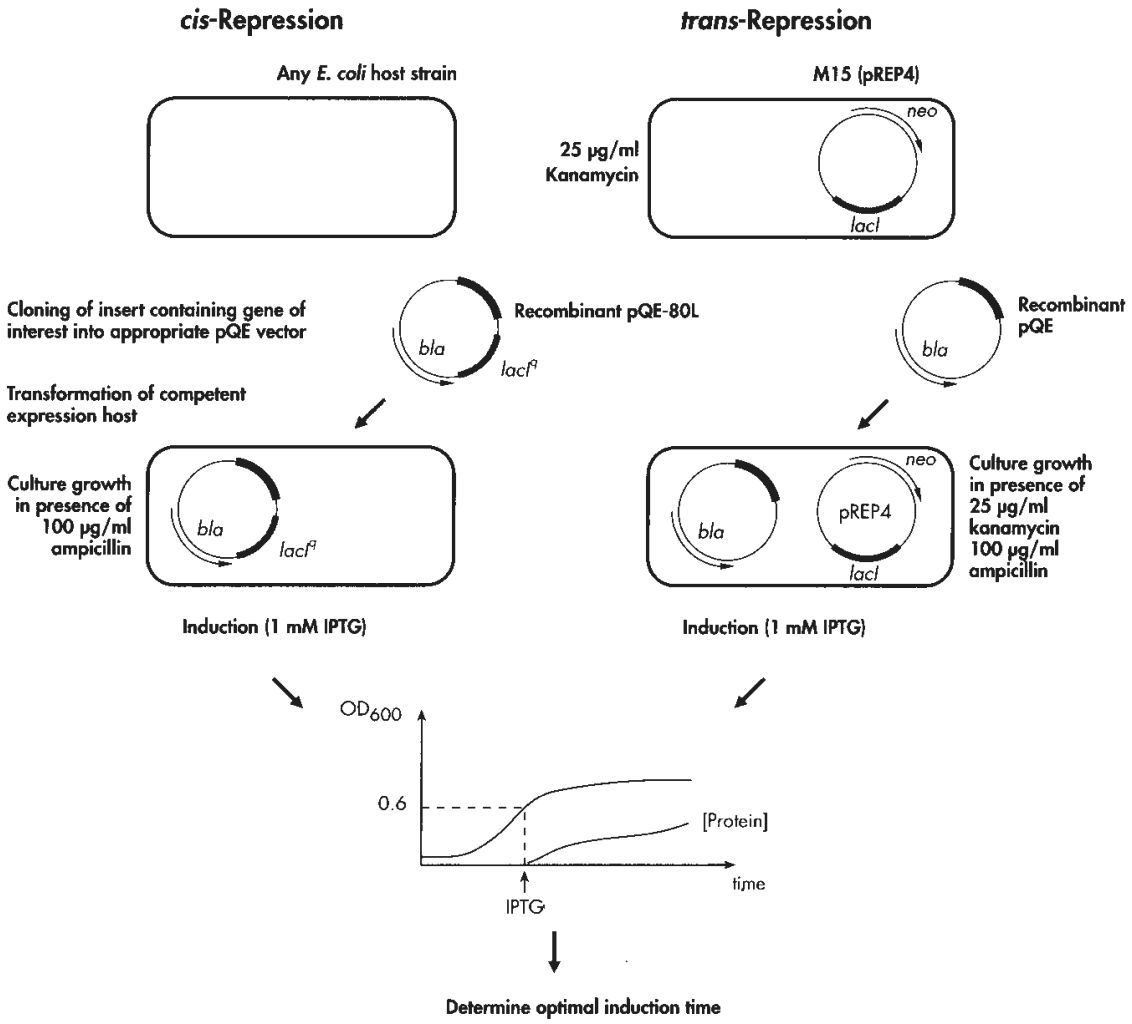

Figure 6: Expression of 6xHis-tagged proteins with the QIAexpress System (The QIAexpressionist, technical manual 5th edition, 2001). In the *trans* system, the host strains used are M15[pREP4]. pREP4 confers kanamycin resistance and constitutively expresses the *lac* repressor protein encoded by the *lac* I gene. Adding IPTG will induce the recombinant protein since IPTG binds the *lac* repressor protein and inactivates it. Once the *lac* repressor is inactivated, the host cell's RNA polymerase can transcribe the sequences downstream from the promoter; transcripts are produce and then translated into the recombinant protein. In the *cis* system, any *E. coli* host strain that contains the *lac*I^q mutation can be used. These cells would make enough lac repressor to efficiently block transcription but expression is regulated less tightly than in strains containing the pREP4 plasmid.

2.6 Alkaline Plasmid Extraction

All steps of the alkaline plasmid extraction were performed on ice. Briefly, 20 ml broths of plasmid (pRS551 or pQE-60) were placed into a 50 ml Falcon tube and centrifuged at 5,000 rpm at 4ºC for 15 minutes. The supernatant was decanted and 1 ml of TEG buffer (25 mM Tris-HCl, pH8.0, 10 mM EDTA, 50 mM glucose) was added to resuspend the pellet. 5 ml of 0.4 M NaOH (4 ml of 5 M NaOH and 46 ml of sterile water) was mixed with 5 ml of 1% SDS. 2 ml of this mixture was added to the plasmid extraction and it was then incubated on ice for 5 minutes. 1.5 ml of 3 M sodium acetate was added to the plasmid extraction and it was again incubated on ice for another 5 minutes. The extraction mixture was then centrifuged at 5,000 rpm at 4ºC for 5 minutes, the supernatant was placed into 15 ml falcon tubes, and double the volume of isopropanol was added. The solution was mixed by inversion, and the centrifuged at 5,000 rpm at 4ºC for 10 minutes. The supernatant was discarded, and 500 µl of sterile water was added to dissolve the pellet. The dissolved pellet was transferred to a smaller 1.5 ml tube and then centrifuged at 14,000 rpm at room temperature for 5 minutes. The supernatant was transferred to a fresh 1.5 ml tube and 2.5x the volume of 100% ethanol was added. This was left in a -20ºC freezer overnight. The following day, the plasmid extraction was centrifuged at 14,000 rpm for 5 minutes, the ethanol was decanted, and then the pellet was dried in a vacuum for approximately 5 minutes. The pellet was resuspended in 50 µl of sterile water and stored at 4ºC until use.

2.7 Cell Culture and Cytotoxicity Assays

2.7.1 Cell Cultures

Green monkey kidney (Vero) cells, ATCC No. CCL-81, were cultured in Minimal Essential Medium (MEM) (Invitrogen, Gibco) with 10% heat inactivated fetal bovine serum (FBS) (Sigma), 1% MEM non-essential amino acid solution (Invitrogen, Gibco), 1% L-Glutamine (Invitrogen, Gibco), 0.4% penicillin-streptomycin (Invitrogen, Gibco), and 0.4% gentamicin (10 mg/ml) (Invitrogen, Gibco).

Human cervical epithelial (HeLa) cells, ATCC No. CCL-2, were cultured in MEM with 10% heat inactivated FBS, 1% MEM non-essential amino acid solution (Invitrogen, Gibco), 1% L-Glutamine (Invitrogen, Gibco), 0.4% penicillin-streptomycin (Invitrogen, Gibco), and 0.4% gentamicin (10 mg/ml) (Invitrogen, Gibco).

Human epithelial (HEp-2) cells, ATCC No. CCL-23, were cultured in MEM with 10% heat inactivated FBS, 1% MEM non-essential amino acid solution (Invitrogen, Gibco), 1% L-Glutamine (Invitrogen, Gibco), 0.4% penicillin-streptomycin (Invitrogen, Gibco), and 0.4% gentamicin (10 mg/ml) (Invitrogen, Gibco).

Mouse epithelial (Y-1) cells, ATCC No. CCL-79, were cultured in Ham's F10 Nutrient Mixture (Invitrogen, Gibco) with 10% heat inactivated FBS, 0.4% penicillin-streptomycin (Invitrogen, Gibco), and 0.4% gentamicin (10 mg/ml) (Invitrogen, Gibco).

Human embryonic intestinal epithelial (INT407) cells, ATCC No. CCL-6, were cultured in MEM with 10% heat inactivated FBS, 1% MEM non-essential amino acid solution (Invitrogen, Gibco), 1% L-Glutamine (Invitrogen, Gibco), 0.4% penicillin-streptomycin (Invitrogen, Gibco), and 0.4% gentamicin (10 mg/ml) (Invitrogen, Gibco).

All cell lines were incubated in the presence of 5% CO_2 at 37°C.

2.7.2 Preparation of Culture Supernatants

After transformation into XL1-Blue competent cells and inducing with IPTG, six colonies were selected which were:

- colony 1 – selected for blue color, named as pRS551-1
- colony 2 – selected for blue color, named as pRS551-2
- colony 3 – selected for blue color, named as pRS551-3
- colony 4 – selected for white color, named as pRS551-4
- colony 5 – selected for white color, named as pRS551-5
- colony 6 – selected for white color, named as pRS551-6

Culture supernatants were prepared from the following isolates: Colonies pRS551-1 and pRS551-2, which were both blue colonies (positive for *ehl1*) from cloning into pRS551. Colony pRS551-5, which was a white colony (negative for *ehl1*) from cloning into pRS551. Strain H19, which is an *E. coli* reference strain positive for VT1. Strain 90-2380, which is an *E. coli* reference strain positive for VT2.

6 ml of Brain Heart Infusion (BHI) broth was supplemented with 6 µl ampicillin and 6 µl of kanamycin, and was inoculated with the isolates that contain the pRS551 vector (pRS551-1, pRS551-2, pRS5515, and pRS551). BHI broth without antibiotics was inoculated with the VT1 and VT2 reference strains, H19 and 90-2380 respectively. BHI broth without antibiotics was double inoculated with combinations of the pRS551-1, pRS551-2, and pRS551-5 isolates and either H19 or 90-2380. The broths were incubated at 37ºC overnight on a Cell-Production Roller Drum (Bellco Glass, Inc.) at 150 rpm.

After incubation, the cultures were centrifuged at 14,000 rpm for 5 minutes. The culture supernatant was filtered using 0.2 µm filters (Millipore) into sterile tubes.

Dilution plates of the filtered culture supernatants were prepared. 100 µl of sterile phosphate buffered saline (PBS) was added to each well of a 96-well tissue culture plate. The top row and left columns were left blank. The left column will be the media control in the 96-well tissue culture plate containing the monolayer of cells. To the top row, 200 µl of each culture filtrate was added. A two fold serial dilution was performed.

2.7.3 Cytotoxicity Assays

Cells were previously grown to a confluent monolayer in an 80-cm^2 flask.

The same day that the bacteria were set up for overnight growth, 96-well tissue culture plates were set up to maintain a monolayer the following day. The cells were washed with Dulbecco's PBS (Gibco), and digested with 5 ml of prewarmed 1X Trypsin (37ºC). The cells were resuspended in 6 ml of media. Combining 0.8 ml of PBS, 0.1 ml of Trypan Blue (Gibco), and 0.1 ml of the suspended cells made a 1/10 dilution of the cells suspension. Cells were mixed and 30 µl was removed and dropped and pulled (by capillary action) into the counting chamber of the hemocytometer. The 4 outer large squares, containing 16 smaller squares per large outer square, of the chamber grid of the hemocytometer were counted by viewing under the inverted microscope. The number of cells per ml was calculated by the following formula:

$$\text{Cells / ml} = \frac{\text{Count} \times 10^4 \text{ (chamber correction)} \times 10 \text{ (dilution)}}{4 \text{ (\# of squares)}}$$

The required cell concentration was calculated using the following formula:

$$\text{Dilution factor} = \frac{\text{Cells / ml}}{2 \times 10^5 \text{ cells/ml}}$$

For each 96-well tissue culture plate, 22 ml of media with the required cell concentration is used to fill the wells. Then 200 µl of the cells, mixed with fresh media, were added to each well of a 96-well tissue culture plate. The plates were incubated at 37ºC with 5% CO_2 overnight for obtaining a monolayer.

After 24 hours, the monolayers were confirmed under the inverted microscope for uniformity. Then, 20 µl of the diluted supernants (pRS551, pRS551-1, pRS551-2, pRS551-5, pRS551 + 90-2380, pRS551-1 + 90-2380, pRS551-2 + 90-2380, pRS551-5 + 90-2380, pRS551 + H19, pRS551-1 + H19, pRS551-2 + H19, and pRS551-5 + H19) were added to the monolayer plates. The plates were then incubated at 37ºC with 5% CO_2.

After 24 hours the plates were read under the inverted microscope and the percent of killed cells was estimated. The percentage value corresponds to the amount of cytotoxicity in each well, i.e., the approximate percentage of cells that are dead. The plates were incubated at 37ºC with 5% CO_2 overnight. After 48 hours, the plates were again read under the inverted microscope and the percent of killed cells were estimated as follows: The media was dumped off the plates into waste and the plates were tapped gently upside-down on paper towel to remove any excess liquid. Then, 100 µl of 2% Crystal violet stain (Sigma) in 95% Ethanol was diluted to 1/80 in 10% Buffered formalin (Fisher) and then was added to each culture well and the plates sat for 30 minutes. The crystal violet stain was dumped off and the plates were rinsed with a light stream of tap water to remove the excess stain and detached cells. The stained live cells were solubilized by adding 100 µl of Solubilization buffer (0.3% Acetic Acid and 0.5% SDS solution) to each well and the plates were placed on the microtitre plate shaker for 20

minutes. During this time, the Dynatech MRX plate reader (Dynex Technologies, Chantilly, Va.), which is used to read optical density of the plates, was turned on to allow for stabilization. The plates were read using the Dynatech MRX plate reader at 620 nm by using Relevation software. Taking the OD values for the control wells that added only PBS and comparing them to the test wells determined the cytotoxicities. The percent cytotoxicity was determined as follows (where OD = the optical density and NC_{AVG} = average OD of the control wells):

$$\% \text{ Cytotoxicity} = 100 - [(OD/NC_{AVG}) \times 100]$$

2.8 Protein Purification

Protein purification of Ehly1 in pQE-60 was performed through both denaturing conditions and native conditions according the protocols supplied in The QIAexpressionist handbook (The QIAexpressionist, technical manual 5th edition, 2001). Qiagen Ni-NTA Spin Columns (Qiagen) were used to purify the protein.

After transformation into XL1-Blue competent cells and inducing with IPTG, six colonies were selected which were:

- colony 1 – selected for blue color, named as pQE60-1
- colony 2 – selected for blue color, named as pQE60-2
- colony 3 – selected for blue color, named as pQE60-3
- colony 4 – selected for white color, named as pQE60-4
- colony 5 – selected for white color, named as pQE60-5
- colony 6 – selected for white color, named as pQE60-6

Cells were prepared by growing in BHI broth containing 50 µg/ml ampicillin at 37ºC overnight with rotation. The protein was either induced for 5 hours with IPTG or lipoprotein, or left uninduced. After 24 hour culture, the cells were harvested by centrifugation at 4,000x g for 15 minutes, and the pellet was processed immediately.

2.8.1 Protein purification by denaturing conditions

Protein purification of Ehly1 in pQE-60 was performed by denaturing conditions according the protocols supplied in The QIAexpressionist handbook (The QIAexpressionist, technical manual 5th edition, 2001).

The pellets from pQE60-1, pQE60-2, and pQE60-5 containing the protein were resuspended in 1 ml of Buffer B (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-HCl pH 8.0), and incubated with shaking for 1 hour at room temperature. The solutions became translucent when the lysis was complete. The lysates were centrifuged at 10,000g for 30 minutes to pellet and cellular debris, and the supernatants were collected. The Ni-NTA spin column was equilibrated with 600 µl of Buffer B and then centrifuged at 2,000 rpm for 2 minutes. The cleared supernatant lysates, which contain the 6x HIS-tagged protein, were loaded into the equilibrated Ni-NTA spin column, centrifuged for 2 minutes at 2,000 rpm, and the flow through was discarded. The Ni-NTA spin columns were washed with 600 µl of Buffer C (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-HCl pH 6.3), centrifuged at 2,000 rpm for 2 minutes, and the flow throughs were discarded. The wash with Buffer C was repeated and again the flow throughs were discarded again. The protein was eluted by adding 200 µl of Buffer E (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-HCl pH 4.5), centrifuging at 2,000 rpm for 2 minutes, and collecting the eluate. This

final step of eluting the protein was repeated twice since most of the protein would elute in the first 200 µl but any remaining protein would elute in the second 200 µl. 20 µl of the first protein elute was mixed with 20 µl of SDS buffer/dye and heat at 100ºC for 10 minutes. The purified protein was then run on an SDS-PAGE.

2.8.2 Protein purification by native conditions

Protein purification of Ehly1 in pQE-60 was performed with native conditions according the protocols supplied in The QIAexpressionist handbook (The QIAexpressionist, technical manual 5th edition, 2001).

The pellets from pQE60-1, pQE60-2, and pQE60-5, containing the protein were resuspended in 1 ml of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imadazole, pH 8.0). Lysozyme was added to 1 mg/ml and the solution was incubated on ice for 30 minutes. To lyse the cells, the solution was sonicated six times for a duration of 10 sec with 5 sec pulses between. The lysate was centrifuged at 10,000 g for 30 minutes at 4ºC, and the supernatant was collected. The Ni-NTA spin column was equilibrated with 600 µL of lysis buffer and centrifuged at 2,000 rpm for 2 minutes. The cleared supernatant lysate, which contains the 6x HIS-tagged protein, was loaded into the equilibrated Ni-NTA spin column, centrifuged for 2 minutes at 2,000 rpm, and the flow through was discarded. The Ni-NTA spin column was washed twice with 600 µL of wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imadazole, pH 8.0), centrifuged at 2,000 rpm for 2 minutes, and the flow through was discarded. The protein was eluted by adding 200 µl of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imadazole, pH 8.0), centrifuging at 2,000 rpm for 2 minutes, and collecting the eluate. This final step of

eluting the protein was repeated twice since most of the protein would elute in the first 200 µl but any remaining protein would elute in the second 200 µl. 20 µl of the first protein elute was mixed with 20 µl of SDS buffer/dye and heat at 100ºC for 10 minutes. The purified protein was then run on an SDS-PAGE.

2.9 SDS-PAGE and Semi-dry transfer

Colonies were grown overnight at 37ºC with rotation in 2 ml of BHI and 4 µl of ampicillin. The following morning, the broths were induced for 5 hours with either 2 µl IPTG (1 mM), 2 µl lipoprotein (1 mM), 10 µl lipoprotein, or left uninduced. Then the broths were spun at 10,000 rpm for 3 minutes at room temperature. The supernatant was discarded and the pellet was resuspended in 60 µl of Protein Sample-SDS Loading Buffer (10 mM Tris, 1 mM EDTA, 4% SDS, 10% Betamercaptoethanol, 0.002% Bromophenol Blue). This solution was heated at 100ºC for 10 minutes. The samples were then ready to load onto the SDS-PAGE. A Low Range Prestained SDS-PAGE Standard (Bio-Rad, USA) was run as a marker. In the gels to be used for the semi-dry transfer and subsequent immunoblot, a 6xHis Protein ladder (Qiagen) was run as an additional marker. In gels to be used for staining with the InVision His-tag In-gel Stain (Invitrogen), a BenchMark His-tagged Protein standard (Invitrogen) was used as an additional marker.

Proteins were electrophoresed (150 volts for 45 – 90 minutes) on 10 cm (l) x 7.5 cm (h) (thickness 1.0 mm) SDS mini-gels, containing 14% resolving gel and 5% stacking gel, or on precast Ready Gel Tris-HCl Gels (Bio-Rad) containing 12% resolving gel and 4% stacking gel, using 1x SDS running buffer.

The SDS-PAGE gels were stained with Coomassie blue, and de-stained overnight in 10% Acetic Acid. The gels were then dried using the Dry Ease Mini-Gel Drying System (Invitrogen), and photographed, or the gels were then transferred from the SDS gel to PVDF transfer membrane (Amersham Pharmacia Biotech, USA) using a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad).

Semi-dry transfer for the mini-gel was performed according to: Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell Instruction Manual (Bio-Rad). The transfer of the mini-gel was run at 160 mA for 90 minutes.

2.10 Immunoblot

The protocol for the immunoblot was followed from the QIAexpress Detection and Assay Handbook (Qiagen).

Following transfer, the membrane was washed twice for 10 minutes per wash in TBS buffer (1.576 g of 10 mM Tris-HCl, 9.766 g of 150 mM NaCl, and 1000 ml of sterilized water; pH at 7.5). Anti-His HRP Conjugate blocking buffer was prepared by adding 0.1 g of Blocking reagent to 20 ml of 1x Blocking reagent Buffer prewarmed to 70ºC, it was stirred until dissolved and then 200 µl of 10% (v/v) Tween 20 was added. The blocking buffer was allowed to cool to room temperature before use. The membrane was then incubated in blocking buffer for 1 hour with rocking at room temperature. The membrane was washed twice for 10 minutes per was in TBS-Tween/Triton buffer (3.151 g of 20 mM Tris-HCl, 29.22 g of 500 mM NaCl, 0.5 ml of 0.05% Tween 20, 2 ml of 0.2% Triton X-100, and 1000 ml of sterilized water; pH 7.5). The membrane was then washed again in TBS buffer for 10 minutes. The membrane was then incubated in

freshly made anti-His HRP conjugate solution (20 µl HRP conjugate and 20 ml blocking buffer) for 1 hour at room temperature with rocking. The blot was again washed in TBS-Tween/Triton buffer two times, followed by another wash in TBS buffer. The blot was then stained with fresh HRP staining solution (0.5 g 3,3'-diaminobenzidine (DAB), Bio-Rad) in 100 ml TBS, and 10 µl 30% H_2O_2) for 5 to 15 minutes or until the signal was clearly visible. Immersing the membrane in sterilized water for 10 minutes stopped the color development. The membrane was dried and then photographed.

2.11 InVision His-tag In-gel Stain

The InVision His-tag In-gel Stain (Invitrogen) is a fluorescent stain that is used for specific and sensitive staining of His-tagged fusion proteins. It consists of a fluorescent dye conjugated to Ni^{2+}:nitrilotriacetic acid (NTA) complex. The Ni^{2+} binds specifically to the oligohistidine domain of the His-tagged fusion protein allowing specific detection of His-tagged fusion proteins from a mixture of endogenous proteins (InVision his-tag In-gel Stain Instruction Manual, Invitrogen, 2004). The fluorescent dye has a maximum excitation at 560 nm and a maximum emission at 590 nm but the dye can also be excited at 300 nm with a lower efficiency (InVision his-tag In-gel Stain Instruction Manual, Invitrogen, 2004). This allows the signal to be detected using a laser-based scanner or a UV transilluminator equipped with a camera. This stain has the capability to detect N-terminal, C-terminal, and internal His-tagged fusion proteins. It allows for direct detection of His-tagged fusion proteins in the gel without the need for western blotting and detection (InVision his-tag In-gel Stain Instruction Manual, Invitrogen, 2004).

The SDS mini gel containing the BenchMark his-tagged Protein Standard (Invitrogen) as a positive control, were electrophoreses and the fixed in Fixing Solution (100 ml ethanol or methanol, 20 ml acetic acid, ultrapure water up to 200 ml) overnight at room temperature with gently rocking. The following day, the gel was washed twice in ultra pure water for 10 minutes each wash. The mini-gel was then incubated in the ready to use solution of His-tag In-gel Stain (Invitrogen) overnight at room temperature with gentle rocking. The following day, the mini-gel was washed twice with 20 mM phosphate buffer, pH7.8, for 10 minutes each wash. The gel was then immediately viewed and photographed using an Imagemaster VDS transilluminator (Pharmacia Biotech).

2.12 RNA Extraction

RNA extraction was performed to generate RNA template for preparation of cDNA through reverse transcription. Total RNA was extracted using an RNeasy Kit (Qiagen). Briefly, isolation of total RNA was performed in the following manner: Bacteria were harvested by centrifugation at 5,000 x g for 5 minutes at 4ºC. The pellet of bacteria was resuspended in 100 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA), pH 8.0, containing 400 µg/ml lysozyme (20 mg/ml). The mixture was then incubated at room temperature for 5 minutes. 350 µl of buffer RLT (containing 10 µl of β-mercaptoethanol per 1 ml of buffer RLT) was added to the sample and mixed vigorously by vortexing. 250 µl of 96 – 100% ethanol was added to the lysate and it was mixed by pipetting. The sample was then applied to an RNeasy mini-column and then placed in a 2 ml collection kit (both supplied with the kit). The sample was then centrifuged for 15 sec at 10,000 rpm and the

flow through was discarded. The column was washed by adding 700 µl of buffer RW1 to the RNeasy column, it was centrifuged again for 15 sec at 10,000 rpm, and flow through and the collection tube were discarded. The RNeasy column was transferred into a new collection tube and 500 µl of Buffer RPE (with ethanol added) was added to the column. The column was centrifuged for 15 sec at 10,000 rpm and flow-through discarded. Another 500 µl of buffer RPE was added to the column and it was centrifuged for 2 minutes at 10,000 rpm to dry the column. Buffer RPE may interfere with downstream reaction so the column was again centrifuged for 1 minute at full speed to eliminate any chance of buffer RPE carryover. To elute the RNA, the column was transferred to a new 1.5 ml collection tube and 40 µl of RNase free water was pipetted directly onto the RNeasy silica-gel membrane. It was then centrifuged for 1 minute at 10,000 rpm to elute the RNA. RNA was stored at 4ºC until ready for use.

The optical densities of the RNA samples were read using a spectrophotometer at 260 nm and the samples were diluted accordingly. The diluted RNA was treated with DNase at room temperature for 15 minutes and then the DNase was heat-inactivated at 95ºC for 10 minutes. The RNA samples were run on a 1% agarose gel to confirm the RNA content.

2.13 Microarray

The microarray work was performed using the 3DNA Array 350RP Expression Array Detection Kit for Microarrays (Genisphere). This kit has a special labelling system that results in a more predictable and consistent signal than direct or indirect dye incorporation (3DNA Array 350RP handbook, Genisphere). In the 3DNA Array 350RP

kit, the fluorescent dye (Cy5) does not need to be incorporated during cDNA preparation because is already a part of the 3DNA reagent. This avoids inefficient hybridization of cDNA to the array that results from the incorporation of fluorescent dye nucleotide conjugates into the reverse transcript (3DNA Array 350RP handbook, Genisphere). Another reason for the predictable and consistent signal produced is because each 3DNA molecule has hundreds of fluorescent dyes (Cy5), and a single 3DNA molecule will detect each bound cDNA fragment. Thus, the signal that is generated from each message will be mostly independent of base composition (3DNA Array 350RP handbook, Genisphere). This is in contrast to microarray using dye incorporation where the signal generated will vary depending on the base composition of the message.

Briefly, the microarray labelling process is as follows: RNA is reverse transcribed using a special Random RT primer provided with the kit to perform reverse transcription on total RNA. The random primed cDNA is purified and ligated to the dendrimer-specific capture oligo. Hybridization is a two-step process in which the cDNA is hybridized to the array first, followed by hybridization of fluorescent 3DNA reagent. The fluorescent 3DNA reagent will hybridize to the cDNA because it has a special capture sequence that is complementary to the sequence ligated to the end of the random primer (3DNA Array 350RP handbook, Genisphere) (Figure 7).

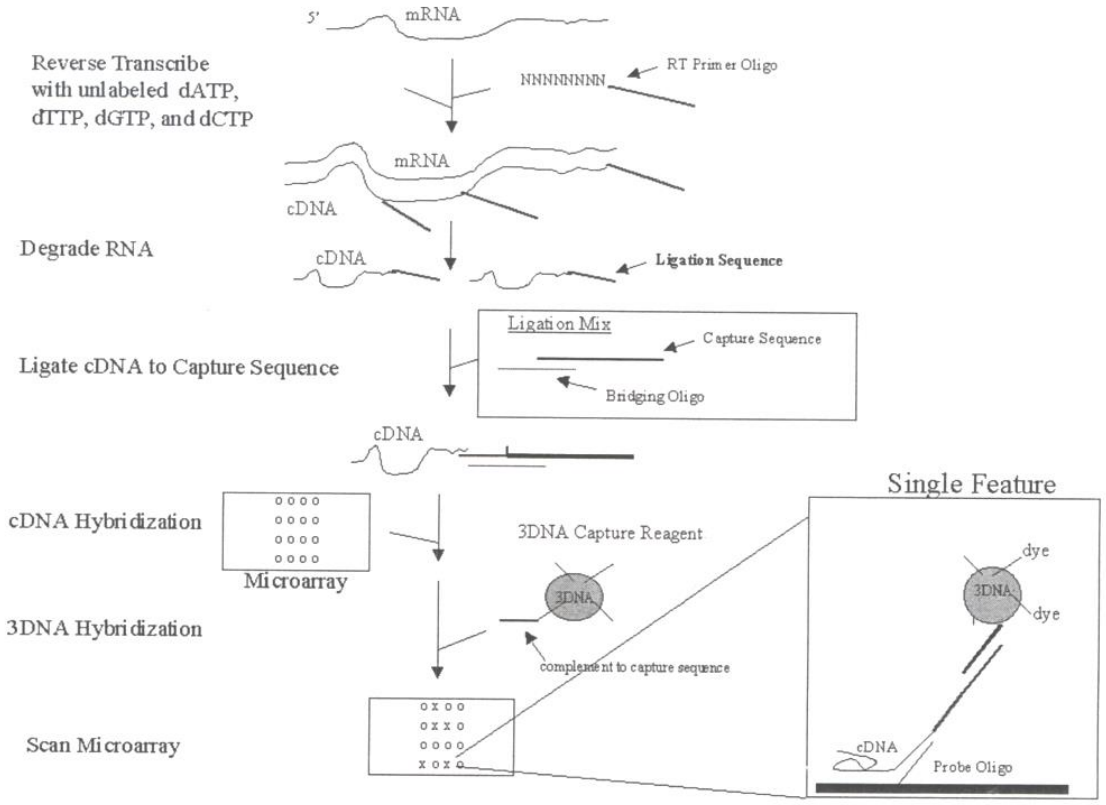

Figure 7: 3DNA Array 350RP Protocol Summary. RNA is reverse transcribed to make cDNA. The cDNA is ligated to the capture sequence and then hybridized to the microarray. This is followed by 3DNA hybridization to the microarray and then signal detection (3DNA Array 350RP handbook, Genisphere).

2.13.1 Preparation of cDNA

cDNA was prepared through reverse transcription using the 3DNA Array 350RP Kit (Genisphere).

The RNA-RT primer mix, containing: 5 µl (1 µg) of total RNA, 1 µl random RT primer (1 µg/ml) (vial 2, 3DNA Array 350RP Kit, Genisphere), and 5 µl nuclease free water (vial 10, 3DNA Array 350RP Kit, Genisphere), was prepared. The RNA-RT primer mix was then heated to 80ºC for 10 minutes, followed by immediate transfer to ice for 2 minutes. Then 1 µl of Superase-In RNase inhibitor (vial 4, 3DNA Array 350RP Kit, Genisphere) was added to the RNA-RT primer mix.

The Reaction mix, containing: 4 µl 5x SuperScript II First Strand Buffer (Gibco), 1 µl dNTP mix (10 mM of each dATP, dCTP, dGTP, and dTTP) (vial 3, 3DNA Array 350RP Kit, Genisphere), 2 µl 0.1 M dithiotretol (DTT) (Gibco), and 1 µl SuperScript II enzyme (200 units) (Gibco), was prepared in a separated microtube on ice.

The RNA-RT primer mix and the reaction mix were gently mixed together and incubated at 42ºC for 90 minutes. Following incubation, the reaction was stopped by adding 3.5 µl of 0.5 M NaOH/50 mM EDTA. To denature any DNA/RNA hybrids, the mixture was incubated at 65ºC for 10 minutes. The reaction was then neutralized by adding 5 µl of 1 M Tris-HCl, pH 7.5. Finally, 21.5 µl of 1x TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA) was added for a total volume of 50 µl. The cDNA mixture was stored at 4ºC overnight.

Each sample of cDNA was purified separately using the QIAquick PCR Purification Kit (Qiagen) with a few adaptations as recommended by the 3DNA Array 350RP–Cy5 Kit (Genisphere). Briefly, 250 µl of Buffer PB was added to the 50 µl

cDNA sample and mixed. The cDNA mixture was applied to a QIAquick column, inside a collection tube, and centrifuged for 1 minute at 13,000 rpm. The flow through was discarded. 750 µl of Buffer PE was added to the QIAquick column and it was centrifuged again for 1 minute. The flow through was again discarded. The centrifugation was repeated again for another minute to remove and residual ethanol. The QIAquick column was then placed into a sterile 1.5 ml tube. Adding 50 µl of Buffer EB to the center of the column membrane, incubation for 2 minutes at room temperature, and then centrifuging the column for 2 minutes to elute the cDNA.

The eluted cDNA was then denatured at 95ºC for 10 minutes and placed on ice immediately for 2 minutes.

The purified cDNA was then ligated to the 3DNA-Specific Capture Sequence. The ligation reaction was prepared as follows: 50 µl cDNA in Elution Buffer, 10 µl of 6x Ligation Mix (vial 11, 3DNA Array 350RP Kit, Genisphere), and 2.5 µl of T4 DNA ligase (vial 12, 3DNA Array 350RP Kit, Genisphere). The ligation reaction was gently mixed and incubated at room temperature for 90 minutes, following which 7 µl of 0.5 M EDTA was added, mixed, and incubated at 65ºC for 10 minutes to stop the ligation reaction. 80 µl of 1x TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA) was added for a total volume of 150 µl.

During the pre-hybridization of the microarray incubation period, the cDNA was concentrated using Millipore Micron YM-30 Microconcentrators (Millipore). The microconcentration of the cDNA reduced the volume of the cDNA from 150 µl to 3 –10 µl in a short duration of time (6-8 minutes). Briefly, the microcon sample reservoir was placed over a 1.5 ml collection tube and the reservoir membrane in the sample reservoir

was pre-washed with 100 µl of TE buffer, pH 8.0. It was then centrifuged for 3 minutes at 10,000 rpm and the flow through was discarded. The 150 µl cDNA reaction was added to the sample reservoir and it was centrifuged at 10,000 rpm for 7 minutes. The sample reservoir assembly was removed, 5 µl of 1x TE buffer (10 mM Tris-HCl pH8.0, 1 mM EDTA) was added to the reservoir membrane, and the concentrator was tapped gently to promote mixing. The reservoir was placed upside down on a sterile collection tube and centrifuged a maximum speed for 2 minutes, resulting in 3-10 µl of concentrated cDNA (3DNA Array 350RP Kit Instruction manual, Genisphere).

2.13.2 Hybridization of cDNA

The next step in the microarray protocol was to successively hybridize the cDNA and the 3DNA to the microarray (3DNA Array 350RP Kit Instruction manual, Genisphere).

The array was prehybridized to reduce background signal. Briefly, the microarray was prewarmed to 50ºC for 10 minutes while the prehybridization mixture was prepared. The prehybridization mixture contained 25 µl of 2x Formamide-Based Hybridization Buffer (50% Formamide, 8x SSC, 1% SDS, and 4x Denhardt's Solution) (vial 7, 3DNA Array 350RP Kit, Genisphere) and 25 µl of nuclease free water. It was heated at 85ºC for 10 minutes and then applied to the prewarmed microarray, which was covered with a 24 x 60 mm coverslip. The microarray was then incubated at 50ºC for 2 hours. During this incubation period, the 2x SSC, 0.2% SDS buffer (2 g SDS, 8.82 g sodium citrate, 17.53 g NaCl, and 1,000 ml sterile water) for washing the microarray was prewarmed to 65ºC.

Following incubation, the microarray was washed with 2x SSC, 0.2% SDS buffer for 15 minutes at 65ºC, then washed with 2x SSC buffer (8.82 g sodium citrate, 17.53 g NaCl, and 1,000 ml sterile water) for 10 minutes at room temperature, and then washed with 0.2x SSC buffer (0.882 g sodium citrate, 1.753 g NaCl, and 1,000 ml sterile water) for 10 minutes at room temperature. The microarray slide was dried by immediately transferring the slide to a dry 50 ml centrifuge tube and centrifuged for 2 minutes at 800 rpm.

The cDNA was hybridized to the microarray according to the 3DNA Array 350RP Kit Instruction manual (Genisphere), as follows: To reduce any potential array artifacts caused by debris, all the glass coverslips were washed in 0.2% SDS, rinsed, and allowed to air dry. The 2x Formamide-Based Hybridization Buffer was thawed and resuspended by heating to 55ºC for 10 minutes, and then it was microfuged for 1 minute. Nuclease free water was added to the cDNA to bring the total volume up to 10 µl. The Hybridization mix was prepared to a final volume of 35 µl as follows: 10 µl of concentrated cDNA, 17.5 µl of 2x Formamide-Based Hybridization Buffer, 2 µl of Array 350RP dT Blocker (containing LNA dT Blocker) (vial 9, 3DNA Array 350RP Kit, Genisphere), and 5.5 µl of nuclease free water. The LNA dT Blocker contained in the Array 350RP dT Blocker is a new, high performance poly T based blocking reagent designed to completely block all the poly A sequence present in array spots. It contains Locked Nucleic Acid (LNA) nucleotides at key positions within the poly dT synthetic strand, and the presence of these modified nucleotides stabilizes the hybridization between the complementary strands of nucleic acids (3DNA Array 350RP handbook, Genisphere). The hybridization mix was gently vortexed and microfuged briefly, and

then incubated first at 80ºC for 10 minutes, and then at 50ºC for 20 minutes. The hybridization mix was added to the coverslip and through capillary action to the microarray (prewarmed to 45ºC) and then incubated overnight in a dark humidified chamber at 45ºC.

After hybridization, the microarray slides were washed to remove unbound cDNA molecules. Briefly, coverslips were removed by immersing the array in 2x SSC, 0.2% SDS buffer at room temperature for 5 minutes or until the coverslips floated off. The microarray slides were then washed for 15 minutes at 65ºC in 2x SSC, 0.2% SDS buffer, next they were washed for 15 minutes in 2x SSC buffer at room temperature, and then washed again for 15 minutes in 0.2x SSC buffer at room temperature. The slides were placed in 95% ethanol for 2 minutes at room temperature to fix the cDNA molecules to the probes. The slides were immediately transferred to dry 50 ml centrifuge tubes, and centrifuged for 2 minutes at 800 rpm to dry the microarray slides.

The following steps for the 3DNA hybridization were performed in low-light conditions to avoid fading of the fluorescent dyes. Briefly, the coverslips were prepared by a quick wash in 0.2% SDS, rinsing in sterile water, and 70% ethanol was used to remove spots. The 2x Formamide-Based Hybridization buffer was thawed and resuspended by heating at 55ºC for 10 minutes and it was microfuged for 1 minute. A stock solution of the anti-fade reagent (vial 8, 3DNA Array 350RP Kit, Genisphere), which reduces fading of the fluorescent dyes after hybridization, was prepared by adding 1 µl of anti-fade reagent to 100 µl of 2x hybridization buffer. The 3DNA Array 350RP Capture Reagent (vial 1, 3DNA Array 350RP Kit, Genisphere) was wrapped in foil to provide a darkened atmosphere and thawed at room temperature for 20 minutes. To

break up any aggregates formed during freezing, the capture reagent was vortexed briefly, and then microfuged briefly. The capture reagent was then incubated at 55ºC for 10 minutes, vortexed briefly, and then microfuged briefly again. A 35 µl volume of the Hybridization mix was prepared as follows: 17.5 µl of the stock solution of anti-fade solution and 2x hybridization buffer, 2.5 µl of the 3DNA capture reagent, and 15 µl of nuclease free water. The hybridization mix was incubated first at 80ºC for 10 minutes and then at 55ºC for 20 minutes. Extra hybridization buffer was prepared and added to the bottom of the microarray chamber to prevent the microarray from drying during incubation, and the microarrays were prewarmed to 55ºC for 15 minutes in the hybridization incubator. The microarrays were removed form the incubator, hybridization mix was added, and the coverslips applied to the array slide. The microarrays were incubated in the dark and humidified microarray chamber at 50ºC for 2 hours.

After hybridization, the microarray slides were washed several times to remove unbound 3DNA molecules. To prevent photobleaching and fading of the fluorescent dyes, the washes were performed in the dark by wrapping the wash box in tin foil. Briefly, coverslips were removed by immersing the array in 2x SSC, 0.2% SDS buffer at room temperature for 5 minutes or until the coverslips floated off. The microarray slides were then washed for 15 minutes at 65ºC in 2x SSC, 0.2% SDS buffer, next they were washed for 15 minutes in 2x SSC buffer at room temperature, and then finally washed again for 15 minutes in 0.2x SSC buffer at room temperature. Immediately transferring the slides to dry 50 ml centrifuge tubes and centrifuging for 2 minutes at 800 rpm dried the microarray slides.

2.13.3 Signal Detection

Due to oxidation, the fluorescent dye, Cy5, of the 3DNA reagent will diminish rapidly in light. Thus, the microarray slides were stored in a light proof box and signal detection was performed immediately.

The microarrays were scanned with the Virtek Chip Reader using chipreader 3.0 software. The microarray signals were analyzed using Array Pro Analyzer software.

3 RESULTS

3.1 Cytotoxicity of *E. coli* Enterohemolysin 1

3.1.1 Confirmation of cloning *ehl*1 and its upstream region into pRS551

E. coli enterohemolysin 1 and its upstream region were cloned into the promoterless vector pRS551. pRS551 will allow any potential promoters in this upstream region to influence expression of *ehl1*. Cloning of *ehl1* and its upstream region into pRS551 was confirmed through PCR screening, restriction enzyme digest with *Bcl*I, and DNA sequencing. The products from the PCR screening and restriction enzyme digest with *Bcl*I were run on a 1% agarose gel stained with ethidium bromide and photographed using a UV transilluminator (Figure 8). Standardized 100 bp ladder was used for band size reference (Figure 8, lanes 1 & 6). pRS551 was transfected and after color selection a white colony was picked to be used a negative control. It did not show any bands after PCR screening with A1 primers (Figure 8, lane 2). The PCR screening on the selected blue colony pRS551-1 was performed using A1 primers and resulted in the correct band size of 827 bp (Figure 8, lane 3). The restriction enzyme digest of the A1 PCR of blue colony pRS551-1 resulted in the appropriate band sizes of 250 bp and 577 bp (Figure 8, lane 4). A restriction enzyme digest of the A1 PCR of blue colony pRS551-2 was also performed but resulted in no visible bands on the agarose gel (Figure 8, lane 5).

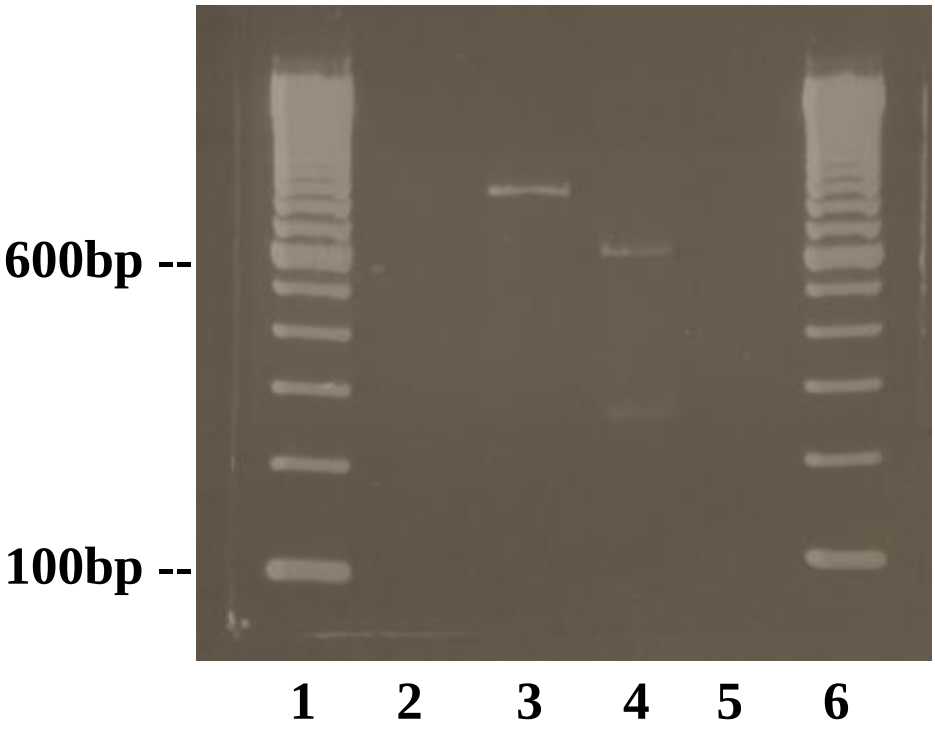

Figure 8: Cloning of *E. coli ehl1* into pRS551 confirmed by PCR screening and restriction enzyme digestion with *Bcl*I. Lanes 1 & 6: 100 bp ladder, Lane 2: negative control (white colony, pRS551-5), Lane 3: PCR of blue colony, pRS551-1 (827 bp), Lane 4: *Bcl*I digest of blue colony, pRS551-1 (250 bp & 577 bp), Lane 5: *Bcl*I digest of blue colony, pRS551-2 (no visible bands).

3.1.2 Synergistic Effects of *ehl1* and Verotoxin

pRS551-1, pRS551-2, and pRS551-5 were grown in BHI broth 37ºC overnight alone and/or in combination with either VT1 or VT2 to test synergistic effects. The overnight broths were centrifuged and the supernatant was filtered with a .20 μm filter. The supernatant was added to cell monolayers previously prepared in 96 well microtitre plates (Vero cells, INT-407 cells, Y-1 cells, and HEp-2 cells). Cellular morphological changes and cell death were observed at 24 and 48 hours (Table 2).

There were no cell changes or cell death in *ehl1* in pRS551 (pRS551-1 and pRS551-2) in any of the different cell lines. Significant cell death was observed from both strains H19 (VT1 producing isolate) and 90-2380 (VT2 producing isolate) in Vero Cells. This was as expected since this assay is designed to detect *E. coli* verotoxins. However, when *ehl1* was grown in combination with either strain H19 or 90-2380, there was no noticeable change in cell morphology or the amount of cell death compared to the observations of H19 or 90-2380 alone.

The microtitre plates were stained with crystal violet, washed using a solubilization buffer, and absorbance was read at 620 nm using a microtitre plate reader that was programmed to calculate the percent cytotoxicity of each well. The cytotoxicity values for each of the monolayer plates were recorded (Table 3).

Overall, *ehl1* alone has little to no cytotoxic effect on Vero cells. *ehl1* does not have a synergistic effect on Vero cells with either VT1 or VT2. Cloned *ehl1* has no detectable cytotoxic effect on INT-407 and HEp-2 cells. In Y-1 cells, *E. coli* isolate 01-1610 has an average cytotoxicity of 15.768%.

Table 2A. Cytotoxicity Assay - Observations of Cell Death at 48 hours for Ehly1 and VT1 on Vero Cells
(Mean Dead Cells ± standard deviation)

Dilution	Media Only	BHI	BHI + AMP + KAN	pRS551-1	pRS551-2	pRS551-5	pRS551	H19	pRS551-1 + H19	pRS551-2 + H19	pRS551-5 + H19	pRS551 + H19
Undiluted	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
1/2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
1/4	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
1/8	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
1/16	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
1/32	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
1/64	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
1/128	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
1/256	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	90.0 ± 0.0	90.0 ± 0.0	90.0 ± 0.0	90.0 ± 0.0	95.0 ± 4.08
1/512	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	75.0 ± 0.0	76.7 ± 2.36	76.7 ± 2.36	76.7 ± 2.36	85.0 ± 4.08
1/1024	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	53.3 ± 4.71	50.0 ± 0.0	46.7 ± 4.71	51.7 ± 2.36	66.7 ± 9.43
1/2048	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	28.3 ± 8.50	26.7 ± 4.71	25.0 ± 4.08	25.0 ± 4.08	50.0 ± 16.33
1/4096	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	6.70 ± 2.36	4.0 ± 1.41	3.7 ± 1.89	5.0 ± 0.0	40.0 ± 14.14
1/8192	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.33 ± 0.471	28.3 ± 16.5
1/16384	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	21.7 ± 14.34
1/32768	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	15.0 ± 12.25

BHI = Brain Heart Infusion Broth; AMP = Ampicillin; KAN = Kanamycin
pRS551-1 = *ehl1* in pRS551; pRS551-2 = *ehl1* in pRS551; pRS551-5 = Negative control (no *ehl1*; cloned in pRS551)
H19 = VT1 positive *E. coli* strain

Table 2B. Cytotoxicity Assay - Observations of Cell Death at 48 hours for Ehly1 and VT2 on Vero Cells
(Mean Dead Cells ± standard deviation)

Dilution	Media Only	BHI	BHI + AMP + KAN	pRS551- 1	pRS551-2	pRS551-5	pRS551	90-2380	pRS551-1 + 90-2380	pRS551-2 + 90-2380	pRS551-5 + 90-2380	pRS551 + 90-2380
Undiluted	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
1/2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
1/4	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
1/8	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
1/16	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
1/32	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
1/64	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
1/128	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
1/256	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
1/512	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
1/1024	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
1/2048	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
1/4096	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	90.0 ± 0.0	90.0 ± 0.0	90.0 ± 0.0	90.0 ± 0.0	90.0 ± 0.0
1/8192	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	83.3 ± 4.71	83.3 ± 4.71	83.3 ± 4.71	83.3 ± 4.71	83.3 ± 4.71
1/16384	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	76.7 ± 2.36	78.3 ± 4.71	76.7 ± 2.36	76.7 ± 2.36	76.7 ± 2.36
1/32768	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	71.7 ± 2.36	75.0 ± 4.08	71.7 ± 2.36	71.7 ± 2.36	71.7 ± 2.36

BHI = Brain Heart Infusion Broth; AMP = Ampicillin; KAN = Kanamycin
pRS551-1 = *ehl1* in pRS551; pRS551-2 = *ehl1* in pRS551; pRS551-5 = Negative control (no *ehl1*; cloned in pRS551)
90-2380 = VT2 positive *E. coli* Reference strain

Table 2C. Cytotoxicity Assay - Observations of Cell Death at 48 hours for Ehly1 on HEp-2, INT-407, and Y-1 Cells
(Mean Dead Cells ± standard deviation)

Cell Type (Undiluted)	**Media**	**BHI**	**BHI + AMP + KAN**	**pRS551-1**	**C3888**	**H19**	**90-2380**	**01-1610**
HEp-2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	10.0 ± 0.0	10.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	10.0 ± 0.0
INT-407	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	28.3 ± 2.357	28.3 ± 2.357	0.0 ± 0.0
Y-1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	10.0 ± 0.0	0.0 ± 0.0	30.0 ± 0.0	30.0 ± 0.0	0.0 ± 0.0

BHI = Brain Heart Infusion Broth; AMP = Ampicillin; KAN = Kanamycin
pRS551-1 = *ehl1* in pRS551
H19 = VT1 positive *E. coli* strain
90-2380 = VT2 positive *E. coli* Reference strain
01-1610 = *E. coli ehl1* positive strain from Argentina (not cloned)
C3888 = *E. coli ehl1* positive strain from Germany (not cloned)

Table 3A. Cytotoxicity of Enterohemolysin 1 - Ehly1 and VT1 on Vero Cells
(Mean Dead Cells ± standard deviation)

Dilution	BHI	BHI + AMP + KAN	pRS551-1	pRS551-2	pRS551-5	pRS551	H19	pRS551-1 + H19	pRS551-2 + H19	pRS551-5 + H19	pRS551 + H19
Undiluted	-1.824 ± 22.909	0.738 ± 0.102	16.241 ± 5.931	10.523 ± 5.970	10.207 ± 6.317	6.678 ± 4.431	80.783 ± 3.192	49.219 ± 51.543	84.790 ± 3.254	42.650 ± 59.484	80.517 ± 1.764
1/2	-3.534 ± 8.649	0.695 ± 0.077	3.561 ± 0.690	-4.524 ± 11.595	-5.737 ± 3.411	6.155 ± 3.188	88.154 ± 2.069	85.649 ± 3.168	82.663 ± 2.901	81.052 ± 1.252	78.301 ± 2.194
1/4	-12.016 ± 13.055	0.680 ± 0.076	1.019 ± 9.174	-3.173 ± 12.150	4.173 ± 11.332	0.394 ± 5.226	83.627 ± 2.729	80.246 ± 2.436	73.759 ± 2.239	80.004 ± 5.018	72.729 ± 3.287
1/8	-6.216 ± 4.847	0.702 ± 0.083	8.923 ± 1.999	-1.856 ± 4.638	-0.430 ± 5.018	-5.051 ± 10.164	75.831 ± 2.325	71.980 ± 0.635	69.458 ± 4.077	70.233 ± 3.138	65.712 ± 1.516
1/16	-4.401 ± 8.715	0.676 ± 0.039	-0.112 ± 7.014	-3.741 ± 11.993	4.132 ± 8.652	-3.713 ± 8.450	62.789 ± 8.137	60.013 ± 8.321	51.704 ± 10.557	53.432 ± 11.1015	50.029 ± 1.632
1/32	-9.139 ± 9.088	0.723 ± 0.055	2.044 ± 2.216	1.981 ± 1.614	4.479 ± 10.753	5.137 ± 1.481	55.239 ± 4.588	56.738 ± 7.032	48.144 ± 9.455	48.926 ± 4.059	47.011 ± 3.886
1/64	-6.449 ± 7.606	0.749 ± 0.119	2.733 ± 10.391	0.679 ± 3.006	-4.017 ± 13.476	-9.604 ± 10.656	59.352 ± 11.300	59.554 ± 12.153	53.669 ± 9.818	52.178 ± 10.123	44.307 ± 3.021
1/128	-0.593 ± 3.555	0.780 ± 0.012	1.220 ± 4.912	0.247 ± 7.982	-10.191 ± 2.813	-2.900 ± 8.315	55.669 ± 5.291	55.158 ± 13.565	51.253 ± 18.211	48.303 ± 10.840	44.867 ± 6.191
1/256	-26.361 ± 31.448	0.841 ± 0.110	3.444 ± 3.279	-6.980 ± 6.451	0.104 ± 7.559	0.849 ± 6.025	56.171 ± 3.952	48.845 ± 0.715	44.258 ± 4.727	39.811 ± 7.727	43.726 ± 9.943
1/512	-3.584 ± 5.700	0.827 ± 0.042	0.380 ± 8.366	-1.383 ± 4.424	-4.954 ± 8.246	-2.069 ± 12.707	34.172 ± 2.769	32.251 ± 3.500	27.142 ± 7.491	28.641 ± 4.115	38.710 ± 7.317
1/1024	-2.087 ± 10.298	0.741 ± 0.020	1.332 ± 7.661	-3.232 ± 10.660	3.798 ± 10.744	3.498 ± 16.650	17.878 ± 8.623	9.680 ± 8.340	12.886 ± 4.469	13.510 ± 1.370	24.726 ± 5.946
1/2048	-8.921 ± 14.437	0.739 ± 0.036	4.049 ± 13.978	0.703 ± 11.866	0.218 ± 11.573	7.981 ± 10.256	10.077 ± 10.942	1.346 ± 7.198	4.616 ± 7.956	7.856 ± 4.892	19.805 ± 8.925
1/4096	-11.033 ± 4.169	0.734 ± 0.073	0.639 ± 9.743	2.278 ± 8.347	2.749 ± 5.763	-3.067 ± 2.933	0.392 ± 5.804	1.848 ± 6.038	-0.906 ± 4.871	6.259 ± 6.548	16.344 ± 14.118
1/8192	-3.423 ± 6.500	0.803 ± 0.073	-2.785 ± 12.507	-1.504 ± 16.668	0.773 ± 12.231	5.932 ± 6.126	10.615 ± 6.562	-3.996 ± 5.281	1.955 ± 9.397	-1.186 ± 11.188	19.048 ± 16.652
1/16384	3.575 ± 12.726	0.851 ± 0.088	9.852 ± 5.673	-1.211 ± 15.879	-2.080 ± 14.141	0.515 ± 8.651	7.492 ± 11.603	1.534 ± 5.317	2.869 ± 13.199	6.981 ± 8.072	17.929 ± 10.029
1/32768	-2.058 ± 17.555	0.789 ± 0.092	1.007 ± 14.209	-7.675 ± 28.713	-8.996 ± 16.341	-13.350 ± 28.565	3.740 ± 12.127	-3.766 ± 2.688	-7.570 ± 9.441	-12.766 ± 5.878	1.580 ± 6.398

BHI = Brain Heart Infusion Broth; AMP = Ampicillin; KAN = Kanamycin
pRS551-1 = *ehl1* in pRS551; pRS551-2 = *ehl1* in pRS551; pRS551-5 = Negative control (no *ehl1*; cloned in pRS551)
H19 = VT1 positive *E. coli* Reference strain

Table 3B. Cytotoxicity of Enterohemolysin 1 - Ehly1 and VT2 on Vero Cells
(Mean Dead Cells ± standard deviation)

Dilution	BHI	BHI + AMP + KAN	pRS551-1	pRS551-2	pRS551-5	pRS551	90-2380	pRS551-1 + 90-2380	pRS551-2 + 90-2380	pRS551-5 + 90-2380	pRS551 + 90-2380
Undiluted	2.348 ± 6.226	0.537 ± 0.048	-16.170 ± 16.701	-4.813 ± 4.211	1.571 ± 2.827	-1.768 ± 3.899	88.250 ± 4.695	87.217 ± 1.011	85.379 ± 3.118	87.087 ± 3.363	75.049 ± 5.723
1/2	-14.392 ± 6.018	0.552 ± 0.074	3.902 ± 7.690	-3.379 ± 5.485	-0.870 ± 8.897	6.027 ± 3.832	82.146 ± 5.153	79.869 ± 4.617	85.125 ± 2.149	81.694 ± 3.774	71.086 ± 3.650
1/4	-2.892 ± 2.329	0.569 ± 0.066	7.713 ± 3.384	1.163 ± 4.589	12.583 ± 8.273	10.454 ± 6.767	74.195 ± 7.301	74.110 ± 5.010	74.340 ± 3.267	74.957 ± 6.878	63.670 ± 6.599
1/8	-9.487 ± 6.169	0.555 ± 0.667	-2.720 ± 2.886	-6.247 ± 0.524	-5.036 ± 9.349	-5.337 ± 9.590	66.306 ± 3.007	69.816 ± 5.898	60.344 ± 18.609	70.350 ± 5.253	58.185 ± 10.164
1/16	-0.906 ± 9.443	0.597 ± 0.061	-1.395 ± 6.554	-0.739 ± 12.882	2.695 ± 17.073	5.964 ± 10.021	64.351 ± 7.825	62.579 ± 3.787	64.060 ± 2.461	64.155 ± 5.425	60.247 ± 2.234
1/32	-10.659 ± 8.164	0.579 ± 0.086	-5.389 ± 6.413	-12.675 ± 8.085	-3.946 ± 11.910	-3.167 ± 19.207	57.811 ± 6.355	56.070 ± 4.539	53.854 ± 4.761	56.298 ± 6.135	55.647 ± 5.190
1/64	2.880 ± 3.835	0.670 ± 0.106	8.176 ± 8.529	-1.946 ± 6.902	1.343 ± 7.648	1.750 ± 8.869	58.256 ± 7.649	60.486 ± 5.597	60.113 ± 1.042	58.780 ± 4.654	53.751 ± 3.513
1/128	-1.928 ± 2.251	0.642 ± 0.102	6.302 ± 4.354	0.066 ± 4.992	1.309 ± 6.666	4.934 ± 6.044	52.700 ± 4.509	54.046 ± 3.898	53.460 ± 4.205	52.675 ± 6.850	47.838 ± 7.254
1/256	4.376 ± 6.176	0.621 ± 0.353	6.465 ± 9.084	-8.570 ± 10.165	-10.706 ± 21.445	-5.491 ± 18.011	51.134 ± 9.850	51.531 ± 7.767	47.834 ± 11.186	48.945 ± 9.004	41.979 ± 6.646
1/512	5.461 ± 11.549	0.652 ± 0.046	-4.016 ± 11.837	-13.065 ± 22.652	0.661 ± 13.557	-2.648 ± 4.586	45.452 ± 5.758	38.079 ± 4.129	40.733 ± 1.122	35.192 ± 8.487	31.867 ± 9.440
1/1024	-6.862 ± 9.834	0.617 ± 0.060	-1.428 ± 11.207	-6.653 ± 22.274	8.309 ± 14.510	-4.433 ± 7.059	34.708 ± 5.954	40.209 ± 2.192	33.001 ± 8.823	28.865 ± 11.234	27.789 ± 12.019
1/2048	-3.399 ± 9.061	0.596 ± 0.065	3.760 ± 8.037	-6.043 ± 13.187	1.793 ± 17.314	-2.310 ± 18.001	31.189 ± 3.238	30.475 ± 6.850	32.937 ± 6.706	23.478 ± 16.905	13.543 ± 18.124
1/4096	4.672 ± 8.721	0.647 ± 0.068	8.017 ± 7.601	11.234 ± 8.677	10.745 ± 12.594	7.440 ± 8.420	29.234 ± 5.325	32.415 ± 5.531	29.082 ± 4.587	23.566 ± 9.510	21.938 ± 13.471
1/8192	-2.034 ± 4.903	0.608 ± 0.051	6.194 ± 7.106	-0.068 ± 12.113	5.467 ± 12.932	-8.389 ± 1.408	24.046 ± 8.455	27.438 ± 8.400	14.552 ± 3.236	10.008 ± 14.951	5.877 ± 21.064
1/16384	1.874 ± 2.484	0.648 ± 0.093	6.775 ± 7.288	8.751 ± 11.477	3.322 ± 12.798	-9.742 ± 7.116	23.019 ± 3.958	24.589 ± 9.053	12.709 ± 5.912	14.160 ± 18.064	5.991 ± 22.968
1/32768	-3.470 ± 15.887	0.587 ± 0.098	-1.845 ± 9.760	-9.000 ± 7.606	-18.824 ± 21.510	-14.953 ± 6.981	20.692 ± 1.761	17.251 ± 0.946	-0.853 ± 7.691	4.005 ± 24.252	2.116 ± 22.475

BHI = Brain Heart Infusion Broth; AMP = Ampicillin; KAN = Kanamycin
pRS551-1 = *ehl1* in pRS551; pRS551-2 = *ehl1* in pRS551; pRS551-5 = Negative control (no *ehl1*; cloned in pRS551)
90-2380 = VT2 positive *E. coli* Reference strain

Table 3C. Cytotoxicity of Enterohemolysin 1 - Ehly1 on HEp-2, INT-407, and Y-1 Cells
(Mean Dead Cells ± standard deviation)

Cell Type (Undiluted)	BHI	BHI + AMP + KAN	pRS551-1	C3888	H19	90-2380	01-1610
HEp-2	5.744 ± 5.997	0.790 ± 0.016	6.898 ± 1.113	0.835 ± 1.703	20.106 ± 2.082	27.574 ± 2.901	-1.223 ± 1.926
INT-407	-0.631 ± 2.107	0.694 ± 0.023	0.893 ± 4.263	7.626 ± 15.809	-4.903 ± 1.410	-0.339 ± 3.443	0.654 ± 4.107
Y-1	3.715 ± 0.474	0.549 ± 0.004	-1.434 ± 3.955	13.272 ± 1.559	19.818 ± 2.082	27.574 ± 4.326	15.768 ± 0.496

BHI = Brain Heart Infusion Broth; AMP = Ampicillin; KAN = Kanamycin
pRS551-1 = *ehl1* in pRS551
H19 = VT1 positive *E. coli* strain
90-2380 = VT2 positive *E. coli* Reference strain
01-1610 = *E. coli ehl1* positive strain from Argentina
C3888 = *E. coli ehl1* positive strain from Germany

3.2 Expression of *E. coli ehl1* in Microarray

3.2.1 Confirmation of cloning and expression of *ehl*1 in pQE-60

E. coli ehl1 was cloned into the expression vector pQE-60 by designing primers to insert *ehl1* into the pQE-60 multiple cloning site (MCS). The forward primer, ehl1prlesf8, was designed to incorporate the *Nco*I restriction site upstream to the ATG start codon of *ehl1*. The translational vector, pQE-60, should then translate the Ehly1 protein at the ATG start codon and read through to the C-terminus 6xHis tag. The pQE-60::*ehl1* recombinant construct has a 6xHis-tag at the C-terminus; in addition, it uses *E. coli* host strains that use the *lac* repressor system either in *trans* or *cis* to *ehl1*. The recombinant construct was transformed into *E. coli* M15[pREP4] cells (*trans*-Repression) but the transformation was not successful upon numerous attempts. Unfortunately, pQE-40 should have been used as a positive control, but this was overlooked at the time of cloning. It is possible that the pREP4 repressor plasmid containing the $lacI^q$ gene may have been lost during the freeze-thaw process or cell propagation. The recombinant construct was then transformed into *E. coli* XL1-Blue cells (*cis*-Repression) and was successful.

Cloning and expression were confirmed through PCR screening and restriction enzyme digest with *Bcl*I and *Bam*HI, and DNA sequencing (Figures 9 and 10, respectively). Standardized 100 bp ladder was used for band size reference (Figure 9, lanes 1; Figure 10, lane 1). pQE-60 was transfected, and after color selection blue colonies pQE60-2 and pQE60-3 were chosen to be representative of pQE-60::*ehl1* construct. After PCR screening with A8 primers, blue colonies pQE60-2 and pQE60-3 showed the appropriate band size of 966 bp (Figure 9, lanes 2 and 3). The *BclI* restriction

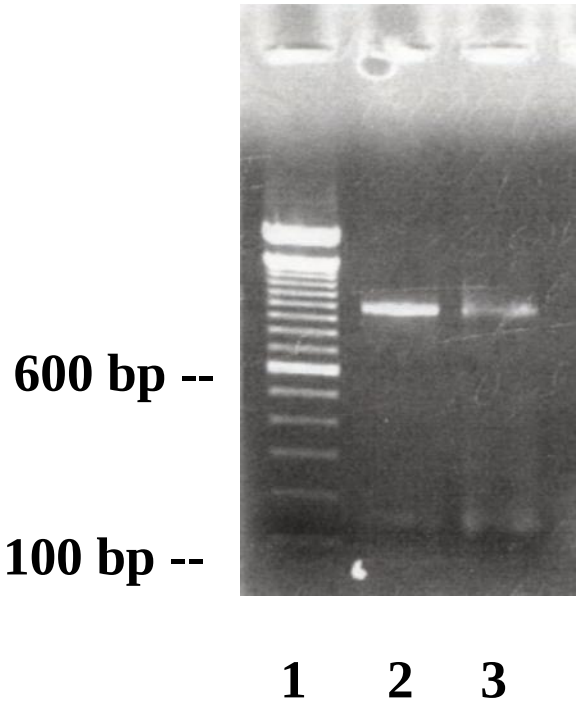

Figure 9: *E. coli ehl1* PCR screening with A8 primers before cloning into pQE-60. Lane 1: 100 bp ladder, Lanes 2 & 3: PCR of *E. coli ehl1* (966 bp).

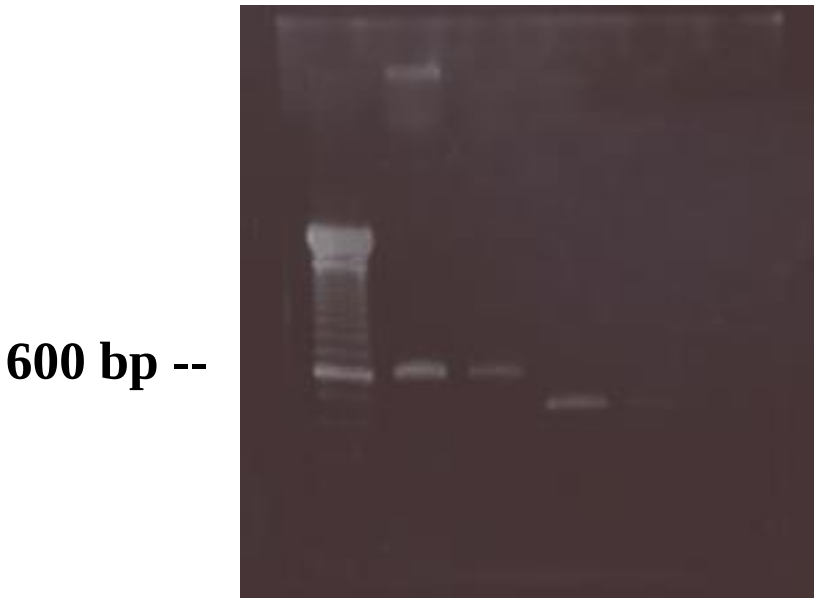

Figure 10: Cloning of *E. coli ehl1* into pQE-60 confirmed by PCR screening with A1 primers and restriction enzyme digestion with *Bcl*I and *Bam*HI. Lane 1: 100 bp ladder, Lanes 2 & 3: *Bcl*I digest of blue colonies pQE60-2 and pQE60-3 (566 bp & 239 bp), Lanes 4 & 5: *Bam*HI digest of blue colonies pQE60-2 and pQE60-3 (406 bp & 399 bp).

enzyme digest of the PCR (using A8 primers) of blue colonies pQE60-2 and pQE60-3 resulted in the appropriate band sizes of 566 bp & 239 bp (Figure 10, lanes 2 and 3). The *Bam*HI restriction enzyme digest of PCR (using A8 primers) of blue colonies pQE60-2 and pQE60-3 resulted in the appropriate band sizes of 406 bp & 399 bp (Figure 10, lanes 4 and 5). Unfortunately, pQE-60 post-digestion was not run on the either of the gels, which would have been beneficial to show that the digestion of the vector took place. It may also have been beneficial to gel-purify the vector following digestion and before insert ligation in order to remove residual nicked and supercoiled plasmid, which would have resulted in more efficient transformations (The QIAexpressionist, technical manual 5th edition, 2001). In addition, pQE-60 post-ligation should also have been run for confirmation of successful cloning into the vector. The DNA sequencing results of *ehl1* (01-1610) can be found in Appendix II. Cloned *ehl1*, colony pQE60-3, was found to be 98% identical to the GenBank sequence X70047 of the original isolate from Germany (C3888). From the DNA sequence, the Ehly1 protein was determined to be 97.4% identical to the original isolate, C3888.

Ehly1 was induced with IPTG or lipoprotein and were electrophoresed on SDS mini-gels, or on precast Ready Gel Tris-HCl Gels (Bio-Rad). Ehly1, pQE60-3, had a protein band at 30 kDa (Figure 11, Lane 2). This was in agreement with previous research, which had showed the Ehly1 protein to be 29,549.50 Da (Stroeher *et al*., 1993). After induction, the Ehly1 protein band shifted to 28 kDa (Figure 11, lane 3). The negative control, white colony pQE60-5, did not show any protein bands (Figure 11, lane 6). Inducing Ehly1 with lipoprotein (1 μl or 5 μl) had no significant affect on the protein (Figure 12).

An immunoblot was performed by semi-dry transfer of the SDS-PAGE to a PVDF transfer membrane and was unsuccessful since the Tetra-His and Penta-His HRP conjugate antibodies that were used did not bind to the 6xHis tag in the protein and no protein was seen on the western blot. Although a 6xHis protein ladder was used as a positive control for transfer and detection, the western blot experiment was completely unsuccessful since nothing was visualized on the blot after staining, including the 6xHis protein ladder positive control. Therefore, to confirm the induction of Ehly1, the SDS-PAGE was stained with In-Vision His-tag In-gel stain (Figure 13). The InVision His-tag In-gel stain was used for direct detection of the His-tagged fusion protein in the gel as it specifically stains His-tagged fusion proteins without the need for western blot and detection. It consists of a proprietary fluorescent dye conjugated to a Ni^{2+}: nitrilotriacetic acid (NTA) complex. The Ni^{2+} binds specifically to the oligohistidine domain of the His-tagged fusion protein allowing specific detection of His-tagged fusion proteins from a mixture of endogenous proteins (InVision His-tag In-gel Stain technical manual, version B, 2004). The fluorescent dye has an excitation of 300 – 560 nm and a maximum emission at 590 nm, allowing detection of the InVision His-tag Stain signal by using a UV transilluminator equipped with a camera (InVision His-tag In-gel Stain technical manual, version B, 2004). A 6xHis Protein ladder (Qiagen) was run as a marker (Figure 13, lane 2), and a BenchMark His-tagged Protein standard (Invitrogen) was used as a negative control as it would not stain (Figure 13, lane 1). The InVision His-tag In-gel stain confirmed the expression of Ehly1: the 30-kDa band appeared as in the SDS-PAGE (Figure 13, Lanes 3 and 4). In addition, Ehly1 induced with IPTG and lipoprotein also

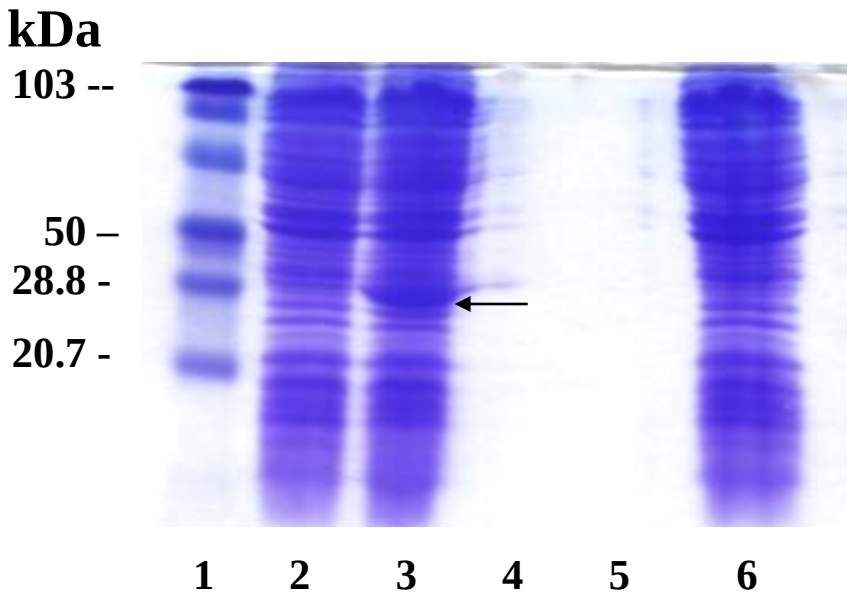

Figure 11: SDS-PAGE of Ehly1 Protein and Ehly1 Expression Induced with IPTG. Lane 1: Low Range Prestained SDS-PAGE standard (BioRad), Lane 2: Ehly1 blue colony pQE60-3 uninduced (30 kDa), Lane 3: Ehly1 blue colony pQE60-3 induced with IPTG (28 kDa), Lanes 4 and 5: blanks, Lane 6: negative control (white colony 5).

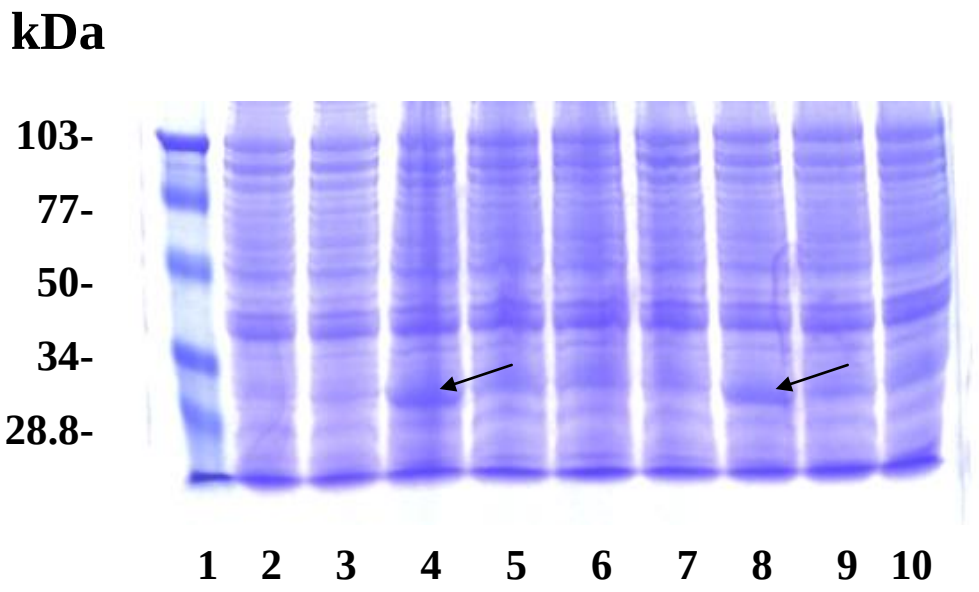

Figure 12: SDS-PAGE of Ehly1 Expression Induced with IPTG or Lipoprotein. Lane 1: Low Range Prestained SDS-PAGE standard (BioRad), Lane 2: negative control, white colony pQE60-5, Lane 3: Ehly1 blue colony pQE60-3A uninduced (30 kDa), Lane 4: Ehly1 blue colony pQE60-3A induced with IPTG (28 kDa), Lane 5: Ehly1 blue colony pQE60-3A induced with 1 μl of lipoprotein, Lane 6: Ehly1 blue colony pQE60-3A induced with 5 μl of lipoprotein, Lane 7: Ehly1 blue colony pQE60-3B uninduced (30 kDa), Lane 8: Ehly1 blue colony pQE60-3B induced with IPTG (28 kDa), Lane 9: Ehly1 blue colony pQE60-3B induced with 1 μl of lipoprotein, Lane 10: Ehly1 blue colony pQE60-3**A** induced with 5 μl of lipoprotein.

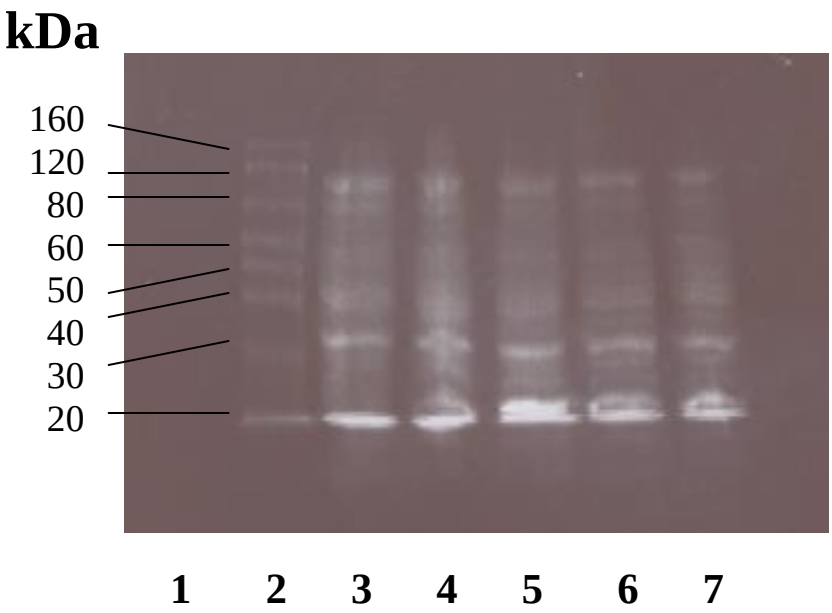

Figure 13: InVision His-tagged In-gel Stained SDS-PAGE of Ehly1. Lane 1: Low Range Prestained SDS-PAGE standard (BioRad), Lane 2: BenchMark His-tagged Protein standard (Invitrogen), Lane 3: Ehly1 blue colony pQE60-3, Lane 4: Duplicate Ehly1 blue colony pQE60-3, Lane 5: Ehly1 blue colony pQE60-3 induced with IPTG, Lane 6: Ehly1 blue colony pQE60-3 induced with 1 μl of lipoprotein, Lane 7: Ehly1 blue colony pQE60-3 induced with 5 μl of lipoprotein.

showed the same results, i.e. 28-kDa band, as it did in the SDS-PAGE (Figure 13, lanes 5, 6, and 7).

3.2.2 Coexpression of *ehl1* on Microarray

Ehly1 and Ehly1 induced with IPTG and Lipoprotein were run on the in-house *E. coli* Microarray chip containing 100 *E. coli* genes to look for coexpression of any genes with *ehl1*. The focus was limited to 21 of the 100 genes that were selected, as they were known *E. coli* virulence factor genes while the remaining genes were mostly housekeeping genes.

Briefly, RNA was extracted and the 3DNA Array 350Rp (Cy5) Kit was used for cDNA synthesis, purification of cDNA, and ligation of purified cDNA to 3DNA-specific capture sequence. The cDNA was concentrated and the 3DNA Array 350Rp (Cy5) Kit 2 step protocol was followed for successive hybridization of cDNA & 3DNA to microarray. The signal was detected on the Virtek chip reader using chipreader 3.0 software, and the signals were analyzed using Array Pro Analyzer software.

When comparing *ehl1* in pQE-60 (pQE60-3A) to the negative control (pQE60-5), the net intensity (signal intensity / background) was higher in all 21 virulence genes for pQE60-3A (Table 4). When comparing *ehl1* in pQE-60 (pQE60-3A) to the Argentina wild type stain 01-1610, the net intensity reading (signal intensity / background) was higher in all 21 virulence genes for pQE60-3A (Table 4). *ehl1* in pQE-60 (pQE60-3A) had an increase in net intensity for most genes when compared to the Germany wild type strain C3888, except for the following virulence genes: *ehl1*, alpha 2 intimin, *Esc* 2917,

and *iroN* (Table 4). *ehl1* in pQE-60 (pQE60-3A) had an increase in net intensity for most genes when compared to *E. coli* ATCC 25922 except for *papE* and *ehl1* (Table 4).

When comparing *ehl1* (pQE60-3A) to *ehl1* induced with IPTG (pQE60-3A-IPTG), the net intensity reading was higher once *ehl1* had been induced with IPTG for most genes except for *cdtIIIA*, *irp1p3*, and *iroN*. A decrease in net intensity reading was observed in these three genes (Table 4). When comparing *ehl1* (pQE60-3A) to *ehl1* induced with 1 μl of lipoprotein (pQE60-3A-1μl LP), there was an increase in net intensity reading for the following genes once *ehl1* was induced with 1 μl of lipoprotein: *espA*, *int*, *ehl1*, *irp1p3*, *hra1*, *papE*, and *iroN* (Table 4). A decrease in net intensity reading for the following genes was observed once *ehl1*was induced with 1 μl of lipoprotein: *bfpA*, *Cfaa*, *afaE3*, *fimH*, *invX*, *eaf*, *hlyA*, *rtx*, *alpha 2 intimin*, *Esc2917*, *Q* gene, *glyA*, and *cdtIIIA* (Table 4). When comparing *ehl1* (pQE60-3A) to *ehl1* induced with 5 μl of lipoprotein (pQE60-3A-5 μl LP), there was an increase in net intensity reading for the following genes once *ehl1* was induced with 5 μl of lipoprotein: *rtx*, *ehl1*, *alpha 2 intimin*, and *Esc2917* (Table 4). There was an decrease in net intensity reading for the following genes once *ehl1* was induced with 5 μl of lipoprotein: *espA*, *bfpA*, *cfaa*, *int*, *afaE3*, *fimH*, *invX*, *eaf*, *hlyA*, *Q* gene, *glyA*, *cdtIIIA*, *irp1p3*, *hra1*, *papE*, and *iroN* (Table 4).

When comparing gene function to net intensity reading for *ehl1* (pQE60-3A) versus *ehl1* induced with IPTG, a decrease in net intensity reading was observed for the following genes: repeats in toxin (*rtx*), intimin, *E. coli* cytolethal distending toxin genes (*cdtIIIA*), iron response protein (*irp1p3*), and EPEC siderphore receptor and urovirulence factor (*iroN*) (Table 5). When *ehl1* was induced with IPTG (pQE60-3A-IPTG) there was

an increase in net intensity reading for genes involved in pili production (*papE*), fimbriae production (*fimH*, *bfpA*, *cfaa*, *eaf*), adhesion and pore formation (*espA*, *afaE3*), genes involved in invasion (*invX*), integrase (*int*), genes of unknown function (*Ecs2917*), the gene for *E. coli* serine hydroxymethyltransferase (*glyA*), heat resistant agglutinin (*hra1*), and hemolysin genes (*ehl1*, *hlyA*) (Table 4). There was no change to a minimal increase in net intensity reading for the Lambda phage *Q* gene and EHEC Shiga toxin 1, *stx1*B (Table 5).

Table 4. Net Intensity Reading of *Ehl1* on 21 Genes of the In House *E. coli* Microarray.
(Net Intensity Reading = signal intensity / background)

GENE ID	pQE60-5	pQE60-3A	pQE60-3A-IPTG	pQE60-3A-1ul LP	pQE60-3A-5ul LP	01-1610	C3888	ATCC25922
espA	97.50911	1217.278	1761.073	708.7339	36.51686	298.8957	88.57752	643.475775
bfpA	1591.963	3115.731	4998.023	15.52907	76.26221	556.0793	102.5783	1308.10194
cfaa	687.1671	946.0853	1378.877	69.07965	42.30814	299.8186	237.4426	774.814147
int	62.25407	1451.889	1840.473	8964.944	127.4626	250.5295	33.00465	827.558333
afaE3	1495.938	2173.517	3123.717	0	204.489	327.7017	23.05562	1209.19845
fimH	919.3884	1650.911	2466.583	97.10136	205.537	456.4537	210.2339	1013.55911
invX	318.8589	7943.661	11330.89	981.8221	1201.762	1663.416	229.4717	3447.2
eaf	2351.179	3392.803	8487.151	71.91705	968.8101	1423.138	88.2905	3093.54593
hlyA	1840.127	2665.899	4314.479	79.15698	310.006	355.8167	49.65523	1314.93702
rtx	681.1101	3048.808	2205.671	8.396318	781.4578	383.0934	2566.158	1246.72868
→ *ehl1*	330.4672	539.9589	783.9603	832.574	840.8928	93.46047	676.3578	589.796512
alpha2 intimin	1398.785	5102.538	4956.574	4213.849	5343.134	1249.719	5197.972	3422.27791
Ecs2917	908.7769	2055.278	2610.609	15.87946	2830.054	617.5031	2970.197	1042.56996
Q gene	892.8826	2701.136	2977.703	718.1366	1633.528	672.3684	2905.042	1420.98333
glyA	1055.56	1513.056	2102.506	62.84109	94.71938	275.7686	101.0492	671.450388
cdtIIIA	10.3405	32.15795	0	0	0	13.67229	0	0
irp1p3	11.27791	57.49244	43.49419	466.0256	31.97752	0	22.04942	0
hra1	80.39012	91.42267	173.6316	173.7903	0	43.13702	25.06628	62.696705
papE	28.86008	48.11977	206.5029	106.7244	0	63.09709	0	137.600969
iroN	24.68178	83.96899	63.22287	1066.816	53.62287	20.47907	107.8242	16.121124
stx1B	294.5725	2547.514	2644.236	1324.335	742.2368	425.6618	1972.418	862.342054
stx2A-2	1280.961	1828.064	2552.874	17.40291	125.4207	366.5736	96.8343	1222.47132

pQE60-5 = Negative control; pQE60-3A = Ehl1; 01-1610 = *ehl1*; C3888 = *ehl1*;
ATCC 25922 = Standard *E. coli* Reference strain.

Table 5. Gene Function Compared to Net Intensity Reading of *Ehl1*. (Net Intensity = signal intensity / background)

GENE ID	Net Intensity Reading			Gene Function	References
	pQE60-5	pQE60-3A	pQE60-3A-IPTG		
espA	97.50911	1217.278	1761.073	EPEC Filament – pore formation, type III secretion, adhesins	Roe, *et al*. 2003. Infect Immun p 5900-5909
bfpA	1591.963	3115.731	4998.023	Structural unit of BFP, type IV fimbriae assoc. with microcolony formation and autoaggregation	Bustamante, *et al*. 1998. J Bact p. 3013-3016
cfaa	687.1671	946.0853	1378.877	Assembly of CFA/1 fimbriae = adhesion factor	Jordi, *et al*. 1993. J Bact p. 7976-7981
int	62.25407	1451.889	1840.473	Integrase = integrate, excise, or invert DNA segment	Collis, *et al*. 2002. Molec Micro p. 1415-1427
afaE3	1495.938	2173.517	3123.717	Structural adhesin gene that mediates specific binding to uroepithelial cells, HEp-2 & HeLa cells with diffuse adherence pattern	Le Bouguenec, *et al*. 1993. Infect Immun p. 5106-5114
fimH	919.3884	1650.911	2466.583	Adhesive subunit of type I fimbriae	Hommais, *et al*. 2003. Infect Immun p. 3619-3622
invX	318.8589	7943.661	11330.89	Invasion in EIEC	Hsia, *et al*. 1993. J Bact p. 4817-4823
eaf	2351.179	3392.803	8487.151	EPEC adherence factor plasmid – encodes genes for BFP; adherence to host cells & bacterial autoaggression	Abul-Milh, *et al*. 2001. Infect Immun p. 7356-7364
hlyA	1840.127	2665.899	4314.479	Plasmid encoded enterohemolysin in EHEC	Wang, *et al*. 2002. J Clin micro p. 3613-3619
rtx	681.1101	3048.808	2205.671	RTX family of toxins-hemolysin or leukotoxins, eg Ehx for EHEC-derived RTX toxin	Bauer, *et al*. 1996. Infect Immun p. 167-175
ehl1	330.4672	539.9589	783.9603	*E. coli* Enterohemolysin 1	Stroeher, *et al*. 1993. Gene p. 89-94
Alpha 2 intimin	1398.785	5102.538	4956.574	Intimin subtype - adhesin	Ramachandran, *el al*. 2003. J Clin Micro p. 5022-5032
Ecs2917	908.7769	2055.278	2610.609	Unknown function – possible virulence factor	
Q gene	892.8826	2701.136	2977.703	Lambda phage Q protein – turns on late phage genes, encodes antiterminator proteins	Yang, *el al*. 1989. J Mol Biol p. 453-460
glyA	1055.56	1513.056	2102.506	*E. coli* Serine Hydroxymethyltransferase	Iurescia, *el al*. 1996. Prot Express Purif p. 323-328
cdtIIIA	10.3405	32.15795	0	Encodes E. coli cytolethal distending toxin genes = cdtA, cdtB, cdtC. Cdt-III = genetic variant in E. coli	Clark, *el al*. 2002. J Clin Micro p. 2671-2674
irp1p3	11.27791	57.49244	43.49419	Iron Response Protein	Schubert, *et al*. 1998. Infect Immun p. 480-485
hra1	80.39012	91.42267	173.6316	E. coli Heat-Resistant Agglutinin 1 – mediates mannose-resistant hemagglutination.	Lutwyche, *et al*. 1994. Infect Immun p. 5020-5026
papE	28.86008	48.11977	206.5029	Gene involved in expression of pilus and adhesive phenotype in E. coli Pap Pili. .PapE = Production of pili with low binding capability.	Lindberg, *et al*. 1986. Proc Natl Acad Sci p. 1891-1895
iroN	24.68178	83.96899	63.22287	EPEC siderphore receptor and urovirulence factor.	Russo, *et al*. 2002. Infect Immun p. 7156-7160
stx1B	294.5725	2547.514	2644.236	Shiga toxin 1	Yokoyama, *et al*. 2000. Gene p. 127-139

4 DISCUSSION

4.1 Characterization of *E. coli* *ehl1*

The present study was established to characterize *E. coli* enterohemolysin 1, *ehl1*. There were two aims of this study: first to determine if *E. coli ehl1* has a synergistic effect with *E. coli* VT1 or VT2 cytotoxins, which may lead to HUS and other severe complications, and secondly, to determine if *ehl1* is coexpressed with other virulence factors and to determine if *E. coli ehl1* is induced by lipoprotein, which may lead to expression of *ehl1* and subsequent symptoms of diarrhea.

The first strategy involved cloning *ehl1* and it's upstream region into the promoter-less vector pRS551 and then transfecting into *E. coli* XL1-Blue cells. The XL-1 Blue cells containing *ehl1* were then grown alone or with VT1 or VT2 and were then tested in cell culture for their percent cytotoxicity. It was determined that *ehl1* alone has little to no cytotoxic effect on Vero cells. *Ehl1* does not have any synergistic effects with VT1 or VT2 on Vero cells. Cloned *ehl1* has no detectable cytotoxic effect on INT-407 and HEp-2 cells, and in Y-1 cells, *E. coli* isolate 01-1610 had an average cytotoxicity of 15.768%.

The second strategy involved cloning *ehl1* into the pQE-60 expression system and transfecting the plasmid DNA into XL1-Blue cells. The XL1-Blue cells containing e*hl1* were then induced by the addition of IPTG or lipoprotein. Expression of Ehly1 was confirmed through SDS-PAGE analysis by staining with In Vision His-tag In-gel stain, allowing for the specific staining of the His-tagged fusion protein, then viewing under a

UV transilluminator. After expression of Ehly1 was confirmed, the microarray chip was used to analyze differences in expression of Ehly1 and other virulence factors.

4.2 Challenges of *E. coli* Enterohemolysin 1 Research

Several problems were encountered during this study. Only minor problems occurred with the first part of the study. *Ehl1* and its upstream region were cloned into the promoterless vector pRS551 so that *ehl1* could potentially act under the regulation of its own promoter or regulators. Once it was confirmed through restriction enzyme digest and gel electrophoresis that *ehl1* and its upstream region were in the pRS551 vector, the clones were grown alone and in combination with either verotoxin 1 or verotoxin 2, then the supernatants were used to test monolayers of various cell types (Vero, HeLa, HEp-2, Y-1, and INT-407). The monolayers were observed for cell death and then stained, washed, and the absorbance was read at 620 nm using a plate reader. Overall, *ehl1* had no cytotoxic effect on of the cell monolayers except for the Y-1 cells in which isolate 01-1610 had an average cytotoxicity of 15.768%. In addition, *ehl1* had no synergistic effects with either verotoxin 1 or verotoxin 2.

While pRS551, had useful features such as a multiple cloning site with restriction sites, and antibiotic resistance selectable markers, it was originally chosen for its lack of promoter to see if the region upstream to *ehl1* would act as a promoter. Due to the lack of significant cytotoxicity produced by *ehl1* it is possible that the region upstream to *ehl1* that was cloned into pRS551 with *ehl1* had no promoter and Ehly1 was therefore not expressed. Thus, after the cytotoxicity experiments, it was decided that for the second part of the study a different vector would be used, specifically the expression vector pQE-

60. Thus, when examining the expression of Ehly1 and possible coexpression of other genes by microarray, the vector pQE-60 was used since it has the strong T5 promoter transcription-translation system regulated by the *lac* repressor protein.

In addition, the pQE-60 expression vector is ideal for expression studies since it results in the recombinant construct having the 6xHis-tag at the C-terminus on Ehly1. This expression system can be used in either *trans* or *cis* to *ehl1*. The pQE-60 expression vector uses *trans*-Repression when *E. coli* host strains containing the pREP4 plasmid, such as *E. coli* M15[pREP4], are used. However, all attempts at transformation into *E. coli* M15[pREP4] cells were unsuccessful possibly because the pREP4 repressor plasmid containing the $lacI^q$ gene may have been lost during the freeze-thaw process or cell propagation. To check if the pREP4 repressor plasmid was lost, the transformed M15 cells could have been plated on both, LB plates containing 100 μg/ml ampicillin and 25 μg/ml kanamycin, and on LB plates containing 100 μg/ml ampicillin only. If the M15 cells harbored the pREP4 plasmid, they would have grown on both types of LB plates. If the pREP4 repressor plasmid was lost, then the M15 cells would not have grown on the LB plates containing kanamycin. In addition, a negative control containing transformed cells with TE buffer was used. However, a positive control should also have been run to confirm the transformation. The recommended positive control was to transform competent cells with 1 ng of the pQE-40 control plasmid in 20 μl of TE (The QIAexpressionist, technical manual 5^{th} edition, 2001). Since the *E. coli* M15[pREP4] transformations were unsuccessful, *E. coli* XL1-Blue cells were used, which allows the pQE-60 expression vector to use *cis*-Repression because this strain has the $lacI^q$ mutation. Thus, expression relies upon the *E. coli* XL1-Blue cells making enough lac repressor to

block transcription but expression is regulated less tightly than in strains containing the pREP4 plasmid.

Cloning and expression were confirmed through PCR screening and restriction enzyme digest with *Bcl*I and *Bam*HI, and DNA sequencing. Cloned *ehl1*, colony pQE60-3, was found to be 98% identical to the GenBank sequence accession number X70047 of the original isolate from Germany (C3888). From the DNA sequence, the Ehly1 protein was determined to be 97.4% identical to the original isolate, C3888. Cloned *ehl1* had a highly similar identity with respect to its sequence and its protein to isolate C3888. The 2% difference between sequences and the 2.6% difference between the proteins occurred as mutations as the *E. coli* O18ac:H31 isolate made it way around the world from Germany to Argentina resulting in a new clone which is more virulent to neonates.

Analyzing the actual nucleotide sequence of *E. coli ehl1* from isolate 01-1610 (Appendix II) found that there were a total of fifteen mutations in the coding region. All of the mutations that occurred were base substitutions, either transitions (where a purine is substituted for a purine, or a pyrimidine is substituted for a pyrimidine) or transversions (where a purine is substituted with a pyrimidine or vice versa). Out of the fifteen base substitutions that occurred, nine of these mutations were synonymous mutations and therefore were not significant since they occurred in the third position of the codon and due to the degeneracy of the genetic code did not result in a change in the amino acid and therefore had no effect on the protein formed. However, six of the mutations were nonsynonymous mutations and did result in changes in the amino acid and thus would have affected the Ehly1 protein produced. At position 425 in the sequence, a transition mutation of Adenine (A) to Guanine (G) occurred resulting in a change of the amino acid

Serine to Glycine. At position 488 in the sequence, a transversion mutation of Cytosine (C) to G occurred resulting in a change of the amino acid Proline to Alanine. At position 589 in the sequence, a transversion mutation of G to Thymine (T) occurred resulting in a change of the amino acid Leucine to Valine. At position 687 in the sequence, a transition mutation of C to T occurred resulting in a change of the amino acid Alanine to Valine. At position 899 in the sequence, a transition mutation of A to G occurred resulting in a change of the amino acid Threonine to Alanine, and at positions 962 and 963 in the sequence, a transversion mutation of C to A and a transition mutation of G to A occurred respectively, resulting in the change of the amino acid Arginine to Asparagine. In addition, it is interesting to note that additional mutations occurred in the putative ribosome binding sequence (RBS). At position 344 in the putative RBS, a transition mutation of C to T occurred resulting in a change of the amino acid Arginine to a Stop codon. At positions 348 and 349 in the putative RBS, a transition mutation of C to T and a transversion mutation of G to C occurred respectively, resulting in a change of the amino acid Proline to Leucine, and at positions 353, 354, and 355 in the putative RBS, a transversion mutation of G to C, a transition mutation of C to T, and a transversion mutation of A to C occurred respectively, resulting in a change of the amino acid Alanine to Leucine. Since the putative RBS was altered by mutations that caused changes in the amino acids produced and also produced a stop codon in the RBS, it is possible that this would have resulted in the low levels of expression of Ehly1 that were seen in this study.

Protein purification of Ehly1 in pQE-60 was performed through both denaturing conditions and native conditions using the protocols supplied in The QIAexpressionist handbook (The QIAexpressionist, technical manual 5th edition, 2001). Qiagen Ni-NTA

Spin Columns (Qiagen) were used to purify the protein. All attempts at protein purification of Ehly1 were unsuccessful despite the fact that the recombinant protein containing *ehl1* with the 6xHis-tag fused at the C-terminus should have been bound to the special column in the kit and then elute out during the washes. Possibly the protein purifications were unsuccessful because the Ehly1 protein may have been expressed at levels that were too low resulting in using too much lysis buffer and subsequently an inadequate concentration factor of protein. Another possibility for the inability to purify the protein under native conditions was that the concentration of imiadazole in the buffer should have been reduced. The structure of histidine contains an imiadazole ring allowing it to bind to the nickel ions in the NTA group on the matrix but if the imiadazole concentration is too high it will prevent binding of the 6xHis-tagged protein (The QIAexpressionist, technical manual 5th edition, 2001). However, if this were the case the protein should have been purified and eluted out of the column under denaturing conditions for purification. It is also possible that the protein may not have been soluble thus the 6xHis tag may have been hidden by the tertiary structure of the Ehly1 protein. Purification under denaturing conditions should have solved both problems of imiadazole binding and protein solubility but this purification was still unsuccessful. This purification possibly failed since the procedure for purification under denaturing conditions was performed on a column. Another option would have been to perform a batch purification which involves binding the protein to the Ni-NTA resin in solution and then packing the protein-resin complex into the column for further washing and elution (The QIAexpressionist, technical manual 5th edition, 2001). This would allow more efficient binding of the 6xHis-tagged Ehly1 protein even if the protein were present at

very low concentrations or expressed at very low levels. Since the protein purifications that were attempted (both native and denaturing) were based on the protein containing the His tag, and the 6xHis tag binds to the Ni-NTA column as long as two histidine residues are available to interact with the nickel ion (The QIAexpressionist, technical manual 5th edition, 2001), it is also possible that the protein was out of frame, and there was no 6xHis tag translated. Thus, no protein would have been purified.

In addition to the set back with the protein purification, the immunoblot was also unsuccessful. The protocol for the immunoblot was followed from the QIAexpress Detection and Assay Handbook (Qiagen). The antibody used for the immunoblot was a general anti-His HRP conjugate solution and then the immunoblot was stained with HRP staining solution. Despite the fact that the recombinant protein should contain *ehl1* with the 6xHis-tag fused at the C-terminus which should bind to the anti-His HRP, the immunoblot was unsuccessful. This would make one think that the recombinant possibly did not contain the His tag. It is possible that there was no 6xHis tag translated due to the protein being out of frame, and thus no binding took place and no protein was seen on the western blot. However, it was later confirmed through staining the SDS-PAGE with In-Vision His-tag stain, to have the tag. This stain allowed for direct detection of the His-tagged fusion protein in the SDS-PAGE without the need for western blotting and detection. There was no color development on the immunoblot after the chemiluminescent detection. In addition, the 6xHis Protein Ladder was not visible leading to the conclusion that either the exposure/development time needed to be lengthened or possibly there was incomplete transfer during the western blot. The semi-dry transfer method can have an efficiency of transfer as low as 10% therefore transfer

time could have been lengthened, a second membrane could have been used to bind any proteins that may have passed through the first layer, or tank-blotting may have been more successful.

Ehly1 and Ehly1 induced with IPTG and Lipoprotein were run on the in-house *E. coli* Microarray chip, focusing on 21 of 100 *E. coli* virulence genes to look for coexpression of any genes with *ehl1*. Overall, induction of Ehly1 with IPTG had a greater affect on expression than it did to induced Ehly1 with lipoprotein. When using the microarray data to compare gene function to net intensity reading for *ehl1* versus *ehl1* induced with IPTG, a decrease in net intensity reading was observed for 5 of the 21 virulence genes studied, an increase in net intensity reading was observed for 13 of the 21 virulence genes studied, and no effect to a small increase in net intensity reading was observed in 3 of the 21 virulence genes studied.

The decrease in net intensity reading observed for 5 of the 21 virulence genes included the following genes: *rtx*, *cdtIIIA*, alpha 2 intimin, *irp1p3*, and *iroN*. The *rtx* and *cdt*IIIA genes both encode toxins. The *rtx* gene encodes hemolysins or leukotoxins from the Repeats in Toxin family that lyse red blood cells and other nucleated cells from many species (Bauer, *et al*. 1996). The *cdtIIIA* gene encodes *E. coli* cytolethal distending toxin gene that causes cellular effects that result in accumulation of F-actin assemblies that resemble stress fibers and progressive cell distension leading to eventual cell death (Clark, *et al*. 2002). The *alpha 2 intimin* gene is located on the locus of enterocyte effacement pathogenicity island and is an intimin subtype that acts as an adhesin (Ramachandran, *et al*. 2003). The last two virulence genes that showed a decrease in net intensity readings are *irp1p3* and *iroN*. The concentration of iron (Fe) is limited in sites

of extraintestinal infection due to the reduction of Fe availability from host factors. Thus iron acquisition is important for pathogens (Russo, *et al*. 2002). These two genes are both involved in iron acquisition. *Irp1p3* is an iron response protein and *iroN* is an EPEC siderphore receptor and urovirulence factor. These results show that both the *rtx* and *cdtIIIA* toxin genes and the *alpha 2 intimin* gene are not coexpressed with *ehl1*. In addition, the genes involved in iron uptake, *irp1p3* and *iroN*, are also not coexpressed with *ehl1* and therefore it is possible that iron uptake is not an important factor involved in *ehl1* regulation.

When *ehl1* was induced with IPTG there was an increase in net intensity reading for 13 of the 21 virulence genes which included: *espA, bfpA, cfaa, afaE3, fimH, eaf, glyA, hraI, papE, int, invX, HlyA*, and *ehl1*. Most of these virulence genes are involved in pili production, fimbriae production, adhesion, and pore formation. The *espA* gene product is the EspA filament that is produced on the bacterial surface. It allows formation of a pore on the host cell membrane when the additional secreted proteins EspB and EspD are present allowing for type III secretion (Roe, *et al*. 2003). *bfpA* encodes the structural unit of the bundle forming pilus (BFP). It is a type IV fimbriae associated with microcolony formation. *bfpA* is important since it is involved in the three stage model by which EPEC infection occurs involving the characteristic formation of the localized adherence pattern (Bustamante, et al. 1998). The *cfaa* virulence gene is a colonization factor antigen involved in assembly of CFA/I fimbriae in ETEC strains and acts as an adhesion factor. The *cfaa* gene itself is part of a 4 gene open reading frame that functions in fimbriae assembly (Jordi, *et al*. 1993). The *afa*E3 gene is a structural adhesin. The *afa* gene cluster encodes afimbrial adhesins (afa) and aid in colonization of the human mucosal

epithelium (Le bouguenec, *et al*. 1993). The *fimH* gene is part of the *fim* gene cluster encoding 9 genes that make the F1 pilus. It is one of the minor subunits of the F1 pilus, which mediates adhesion to D-mannose residues present in epithelial cells, macrophages, and other host cells (Vandemaele, *et al*. 2003). The *eaf* virulence gene encodes the EPEC adherence factor plasmid encoding genes for the bundle forming pilus that is involved in host adhesion and bacterial aggregation producing the localized adherence pattern typical of EPEC infection (Abul-Milh, *et al*. 2001). The *papE* gene is involved in expression of the Pap Pili and adhesive phenotype in *E. coli*. Where *papE* itself encodes production of pili with low binding capability (Lindberg, et al. 1986).

The remaining genes that had an increase in net intensity reading are involved in a variety of processes including: invasion, integration, the gene for *E. coli* serine hydroxymethyltransferase, heat resistant agglutinin, and hemolysin genes (*ehl1, hlyA).* The *int* gene is the integrase gene of Lambda phage and can integrate the phage genome into the bacterial chromosome or excise an integrated copy (Collis, *et al*. 2002). The *invX* gene is involved in invasion in EIEC. The *HlyA* gene encodes an enterohemolysin from EHEC where mature HlyA binds mammalian target cell membranes and causes lysis (Hertax, et al. 2003). It is the prototype of the RTX family of toxins. The *glyA* virulence gene is an *E. coli* serine hydroxymethyltransferase. This gene plays an important role in cell physiology since it catalyzes the conversion of 5,10-methylenetetrahydrofolate, serine, and glycine, and is the cells major source of one-carbon units which are used in the synthesis of purines, thymine and methionine and in the formation of aminoacylated initiator tRNA (Lorenz, *et al*. 1996). The *hraI* gene encodes *E. coli* heat-resistant agglutinin 1 and mediates mannose resistant

hemagglutination. It is strongly associated with the bacterial cells surface and strongly agglutinates human, pig, and dog erythrocytes (Lutwyche, *et al*. 1994).

Most of the 13 virulence genes that show an increase in net intensity reading are involved in involved in pili and fimbriae production, adhesion, and pore formation. These gene products are all involved in aiding the bacteria in entry into the host cell. Therefore, virulence genes that help bacterial cell entry into the host cell are coexpressed with *ehl1*. Possibly *ehl1* needs to use these coexpressed proteins to gain entry into the host cell before it can act as an important virulence factor.

There was no change to a minimal increase in net intensity reading for 3 of the 21 virulence genes studied which included: Esc2917, *Q* gene, and *stx1B*. The *Esc2917* gene is a potential virulence factor with unknown function. *Q* gene is from the Lambda phage and makes Q protein, which turns on late phage genes, and antiterminator proteins (Yang, *et al*. 1989). The *stx1B* gene is involved in *E. coli* verotoxin 1 production. It is interesting to note that the minimal increase in net intensity reading that occurred for the *stx1B* gene shows that verotoxin 1 is not coexpressed with *ehl1*, thus confirming the results from the cytotoxicity assay in the first part of this study which showed that there are no synergistic effects between *ehl1* and verotoxin 1.

4.3 Pros and Cons of Microarray

DNA Microarrays are becoming a more commonplace technology in many laboratories as a microbial research tool in areas such as: microbial physiology, pathogenesis, ecology, epidemiology, fermentation optimization, and pathway engineering (Ye, *et al*. 2001). An entire microbial genome can be represented in a single

array allowing for analysis of RNA abundance and DNA homology for thousands of genes in a single experiment and for genome wide analysis (Ye, *et al*. 2001). With current DNA microarray technology being used to study a variety of bacterial species including lab strains, environmental isolates, and clinical pathogens, it is important to look at the pros and cons of this rapidly emerging technology.

In general, microarrays are advantageous because one can rapidly examine an entire microbial genome on one chip, which may lead to significant scientific discoveries. Some disadvantages of microarrays are the costs, reproducibility of results, and quality control. The cost of the chip, or the cost of designing an in-house microarray, in addition to the supplies need to run a specimen, means that the ability to run a number of repeated observations is low. A few years ago, when microarray was a newly developing technology there was a lack of standards for data collection, analysis, and validation of data (Ye, *et al*. 2001). Currently, the "gold standard" assay for verification and confirmation of microarray gene expression levels is by quantitative real-time PCR (Qin *et al*., 2006). In addition, data collection and analysis is provided through the numerous software programs available to manage microarray data and analyze results.

When using the DNA microarray for this research project some of the advantages and disadvantages of microarray technology became apparent. The testing was rapid and a numerous amount of data was collected. Specifically, after one run on the chip, *ehl1* was examined for co-expression with 100 other virulence factors. However, the data was only examined for 21 of these virulence factor genes. In addition, it is difficult to isolate an intact mRNA population because mRNA is stable for only a short period of time. mRNA is not stable and can even be degraded while being translated with the half-life of

mRNA being about 3 minutes. In addition, specific genes can be induced or down-regulated during sample processing thus affecting the mRNA quality and quantity which is important to truly represent gene expression. When making conclusions on coexpression of genes with *ehl1*, it is important to remember that gene expression data may be a problem due to cross hybridizations between transcripts with high sequence homology. In addition, mRNA levels may not be proportional to protein levels because of different factors, such as variable efficiency of mRNA translation (Ye, *et al*. 2001).

While DNA microarray technology is still under new advances, it is possible that future projects using microarray technology may help find new disease mechanisms, biomarkers, aid in outbreak analysis, and potentially aid in the discovery of therapeutic remedies (Ye, *et al*. 2001). However, use of this technology is limited to laboratories that can afford the cost of running this type analysis.

4.4 Future Work

While it is still to be determined if *ehl1* is a significant virulence factor, the portion of the study focusing on microarray analysis and coexpression of virulence can still be looked at further. Firstly, it would be beneficial to see if the microarray data would be reproducible if run on the in-house microarray chip again. Secondly, it may be beneficial to focus on more of the 100 virulence genes on the microarray rather than just the 21 genes focused on in this study to see if any other virulence genes are coexpressed with *ehl1*. It may be beneficial to see if any housekeeping genes on the microarray were down regulated or not expressed at all thus possibly explaining why *ehl1* may not be acting as a

virulence factor. A bioinformatics specialist needs to review the data for statistical comparison of two or more trials for the data to be of any scientific value.

4.5 Conclusions

The following conclusions can be drawn from this study:

(1) *ehl1* has little to no cytotoxic effect on Vero cells, HEp-2 cells, HeLa cells, and INT-407 cells.

(2) *ehl1* and VT1 do not have a synergistic effect on the Vero cell assay.

(3) *ehl1* and VT2 do not have a synergistic effect on the Vero cell assay.

(4) In Y-1 cells, *E. coli* isolate 01-1610 (containing *ehl1* as its only virulence factor) has an average 15.8% cytotoxicity.

(5) When looking at co-expression of genes by microarray analysis once Ehly1 was induced with IPTG, it appears that there is a decrease in net intensity reading (gene expression) for the repeats in toxin gene, intimin, *E. coli* cytolethal distending toxin genes, iron response protein, and EPEC siderphore receptor and urovirulence factor. These results show that these genes are not coexpressed with *ehl1*. Since the genes involved in iron uptake, *irp1p3* and *iroN*, are not coexpressed with *ehl1*, it is possible that iron uptake is not an important factor involved in *ehl1* regulation.

(6) When looking at co-expression of genes by microarray analysis once Ehly1 was induced with IPTG, it appears that there is an increase in net intensity reading (gene expression) for genes involved in pili production, fimbriae production, adhesion and pore formation, genes involved in invasion, integrase, the gene for

E. coli serine hydroxymethyltransferase, heat resistant agglutinin, and hemolysin genes (*ehl1*, *hlyA)*. Most of the virulence genes that showed an increase in net intensity reading are involved in aiding the bacteria in entry into the host cell. Therefore, possibly *ehl1* needs to use these coexpressed proteins to gain entry into the host cell before it can act as an important virulence factor.

5 REFERENCES

Abul-Milh, M., Wu, Y., Lau, B., Lingwood, C. A., and Foster, D. B. (2001). Induction of Epithelial Cell Death Including Apoptosis by Enteropathogenic *Escherichia coli* Expressing Bundle-Forming Pili. *Infect Immun* **69**(12), 7356-7364.

Aittokallio, T., Kurki, M., Nevalainen, O., Nikula, T., West, A., and Lahesmaa, R. (2003). Computational strategies for analyzing data in gene expression microarray experiments. *J Bioinform Comput Biol* **1**(3), 541-586.

Argraves, G. L., Jani, S., Barth, J. L., and Argraves, W. S. (2005). ArrayQuest: a web resource for the analysis of DNA microarray data. *BMC Bioinformatics* **6**, 287.

Barry, M. (1992). Traveler's diarrhea: New perspectives. *Infect Agents Dis* **1**, 114-118.

Bauer, M. E., and Welch, R. A. (1996). Characterization of an RTX Toxin from Enterohemorrhagic *Escherichia coli* O157:H7. *Infect Immun* **64**(1), 167-175.

Benson, M., Olsson, M., Rudemo, M., Wennergren, G., and Cardell, L. O. (2004). Review. Pros and cons of microarray technology in allergy research. *Clin Exp Allergy* **34**, 1001-1006.

Beutin, L., Prada, J., Zimmermann, S., Stephan, R., Orskov, I., and Orskov, F. (1988). Enterohemolysin, a new Type of hemolysin produced by Some Strains of Enteropathogenic *E. coli* (EPEC). *Zbl Bakt Hyg* **267**, 576-588.

Beutin, L., Stroeher, U. H., and Manning, P. A. (1993). Isolation of enterohemolysin (Ehly2)-associated sequences encoded on temperate phages of *Escherichia coli*. *Gene* **132**, 95-99.

Behrman, R. E., Kliegman, R. M., and Jenson, H. B. (2003). Nelson Textbook of Pediatrics. 17th Edition. Saunders. 2407-2409.

Bettelheim, K. A. (1991). The genus *Escherichia*. In: Balows A, Truper HG, Dworkin M, Harder W, Schleifer K-H, eds. *The prokaryotes: A handbook on the biology of bacteria. Ecophysiology, isolation, identification, applications.* New York: Springer-Verlag, 1991; 2696-2736.

Bhakdi, S., Mackman, N., Nicaud, J. M., and Holland, I, B. (1986). *Escherichia coli* hemolysin may damage target cell membranes generating transmembrane pores. *Infect Immun* **52**(1), 63-69.

Bielaszewska, M., Fell, M., Greune, L., Prager, R., Fruth, A., Tschape, H., Schmidt, M. A., and Karch, H. (2004). Characterization of Cytolethal distending Toxin Genes and

Expression in Shiga Toxin-Producing *Escherichia coli* Strains of Non-O157 Serogroups. *Infect Immun* **72**(3), 1812-1816.

Boerlin, P., Chen, S., Colbourne, J. K., Johnson, R., De Grandis, S., and Gyles, C. (1998). Evolution of Enterohemorrhagic *Escherichia coli* Hemolysin Plasmids and the Locus for Enterocyte Effacement in Shiga Toxin-Producing *E. coli*. *Infect Immun* **66**(6), 2553-2561.

Bopp, C. A., Brenner, F. W., Wells, J. G., and Strockbine, N. A. *Escherichia, Shigella*, and *Salmonella*. In: *Manual of Clinical Microbiology*. Murray, P. R., Baron, E. J., Pfaller, M. A., Tenover, T. C., and Yolken, R. H. American Society for Microbiology. Washington, D.C. (1999). 7th Ed. pp. 459-465.

Burrowa, W., and Musteikis, G. M. (1966). Cholera infection and toxin in the rabbit ileal loop. *J Infect Dis* **116**, 183-190.

Bustamante, V. H., Calva, E., and Puente, J. L. (1998). Analysis of *cis*-Acting Elements Required for *bfpA* Expression in Enteropathogenic *Escherichia coli*. *J Bacteriol* **180**(11), 3013-3016.

Cavalieri S. J., and Snyder, I. S. (1982). Effect of *Escherichia coli* α-hemolysin on human peripheral leukocyte function in vitro. *Infect Immun* **37**, 966-974.

Chattopadhyay, K., Bhattacharyya, D., and Banerjee, K. K. (2002). *Vibrio cholerae* hemolysin. Implication of amphiphilicity and lipid-induced conformational change for its pore-forming activity. *Eur J Biochem* **269**, 4351-4358.

China, B., and Goffaux, F. (1999). Secretion of virulence factors by *Escherichia coli*. *Vet Res* **30**, 181-202.

Chinen, I., Rivas, M., Soriano, V., Miliwebsky, E., Galvez, G. F., Chillemi, G., Baschkier, A., Wang, G., Caldeira, R., Woodward, D. L., and Rodgers, F. (2002). *Escherichia coli ehl1* Gene-Positive Serotype O18ac:H31 Associated with an Outbreak of Diarrhea in a Neonatal Nursery in Neuquen City, Argentina. *J Clin Micro* **40**(4), 1225-1229.

Chirwa, N. T., and Herrington, M. B. (2003). CsgD, a regulator of curli and cellulose synthesis, also regulates serine Hydroxymethyltransferase synthesis in *Escherichia coli* K-12. *Microbiol* **149**, 525-535.

Clark, C. G., Johnson, S. T., Easy, R. H., Campbell, J. L., and Rodgers, F. G. (2002). PCR for Detection of *cdt-III* and the relative Frequencies of Cytolethal Distending Toxin Variant-Producing *Escherichia coli* Isolates from Humans and Cattle. J Clin Microbiol **40**(7), 2671-2674.

Cleary, J., Lai, L., Shaw, R. K., Straatman-Iwanowska, A., Donnenberg, M. S., Frankel, G., and Knutton, S. (2004). Enteropathogenic *Escherichia coli* (EPEC) adhesion to

intestinal epithelial cells: role of bundle-forming pili (BFP), EspA filaments and Intimin. *Microbiol* **150**, 527-538.

Cohen, M. B., and Giannella, R. A. (1991). Bacterial infections: Pathophysiology, clinical features, and treatment. In: Phillips SF, Pemberton JH, Shorter RG, eds. *The large intestine: Physiology, pathophysiology, and disease.* Mayo Foundation. New York: Raven Press, 1991; 395-428.

Coleman, W. H., Kaur, J., Iwert, M. E., Kasai, G. J., and Burrows, W. (1968). Cholera Toxins: Purification and Preliminary Characterization of Ileal Loop Reactive Type 2 Toxin. *J Bacteriol* **96**(4), 1137-1143.

Collis, C. M., Recchia, G. D., Kim, M., Stokes, H. W., and Hall, R. M. (2001). Efficiency of Recombination Reactions Catalyzed by Class 1 Integron Integrase IntI1. *J Bacteriol* **183**(8), 2535-2542.

Collis, C. M., Kim, M., Stokes, H. w., and Hall, R. M. (2002). Integron-encoded IntI integrases preferentially recognize the adjacent cognate *attI* site in recombination with a 59-be site. *Mol Microbiol* **46**(5), 1415-1427.

Cortajarena, A. L., Goni, F. M., and Ostolaza, H. (2003). A Receptor-binding Region in *Escherichia coli* α-Haemolysin. *J Biol Chem* **278**(21), 19159-19163.

Cozzone, A. J. (1998). Post-translational modification of proteins by reversible phosphorylation in prokaryotes. *Biochimie* **80**(1), 43-48.

Creasey, E. A., Friedberg, D., Shaw, R. K., Umanski, T., Knutton, S., Rosenshine, I., and Frankel, G. (2003). CesAB is an enteropathogenic *Escherichia coli* chaperone for the type-III translocator proteins EspA and EspB. *Microbiol* **149**, 3639-3647.

Dallas, P. B., Gottardo, N. G., Firth, m. J., Beesley, A. H., Hoffmann, K., Terry, P. A., Freitas, J. R., Boag, J. M., Cummings, A. J., and Kees, U. R. (2005). Gene expression levels assessed by oligonucleotide microarray analysis and quantitative real-time RT-PCR – how well do they correlate? *BMC Genomics* **6**(1), 59.

Dalton, C. B., Mintz, E. D., Tauxe, R. V., and Wells, J. G. (1995). *EnterotoxigenicE. coli* (ETEC) outbreaks in and around the United States, 1977-1994: a clinical and epidemiological profile, abstr. K-148, p.314. *In Program and Abstracts of the 35th Interscience Conference on Antimicrobial Agents and Chemotherapy.* American Society for Microbiology, Washington, D. C.

Ewing, W. H. (1986). Edwards and Ewing's Identification of Enterobacteriaceae, 4th edition. Elsevier Science Publishing Co., Inc., New York. 94-134.

Felmee, T., and Welch, R. A. (1988). Alterations of amino acid repeats in the *Escherichia coli* hemolysin affect cytotoxic activity and secretion. *Proc Natl Acad Sci* **85**, 5269-5273.

Figueiredo, P. M. S., Catani, C. F., and Yano, T. (2003). Serum high-density lipoprotein (HDL) inhibits in vitro enterohemolysin (EHly) activity produced by Enteropathogenic *Escherichia coli*. *FEMS Immunol Med Microbiol* **38**, 53-57.

Fischer, W., and Haas, R. (2004). The RecA Protein of *Helicobacter pylori* Requires a Posttranslational Modification for Full Activity. *J Bacteriol* **186**(3), 777-784.

Friedman, A. M., Fischmann, T. O., and Steitz, T. A. (1995). Crystal structure of lac repressor core tetramere and its implications for DNA looping. *Science* **268**, 1721-1727.

Fuchs, S., Muhldorfer, I., Donohue-Rolfe, A., Kerenyi, M., Emody, L., Alexiev, R., Nedkov, P., and Hacker, J. (1999). Influence of RecA on *in vivo* virulence and Shiga toxin 2 production in *Escherichia coli* pathogens. *Micro Path* 27, 13-23.

Garcia, M., Gounon, P., Courcoux, P., Labigne, A., and Le Bouguenec, C. (1996). The Afimbrial adhesive sheath encoded by the *afa-3* gene cluster of pathogenic *Escherichia coli* is composed of two adhesins. *Mol Microbiol* **19**(4), 683-693.

Gentschev, I., Mollenkopf, H. J., Sokolovic, Z., Ludwig, A., Tengel, C., Gross, R., Hess, J., Demuth, A., and Goebel, W. (1997). Synthesis and Secretion of Bacterial Antigens by Attenuated *Salmonella* via the *Escherichia coli* Hemolysin Secretion System. *Behring Inst Mitt* **95**, 57-66.

Gentschev, I., Dietrich, G., Mollenkopf, H. J., Sokolovic, Z., Hess, J., Kaufmann, S. H. E., and Goebel, W. (1997). The *Escherichia coli* Hemolysin Secretion Apparatus – a Versatile Antigen Delivery System in Attenuated *Salmonella*. *Behring Inst Mitt* **98**, 103-113.

Gentschev, I., Dietrich, G., and Goebel, W. (2002). The *E. coli* α-hemolysin secretion system and its use in vaccine development. *Trends Microbiol* **10**(1), 39-45.

Gentz, R., Langner, A., Chang, A. C., Cohen, S. N., and Bujard, H. (1981). Cloning and analysis of strong promoters is made possible by the downstream placement of a RNA termination signal. *Proc Natl Acad Sci USA* **78**(8), 4936-4940.

Ghosh, B., Grzadzielska, E., Bhattacharya, P., Peralta, E., DeVito, J., and Das, A. (1991). Specificity of Antitermination Mechanisms. Suppression of the Terminator Cluster T1-T2 of *Escherichia coli* Ribosomal RNA operon, *rrnB*, by Phage λ Antiterminators. *J Mol Biol* **222**, 59-66.

Gobel, W., Chakraborty, T., and Kraft, J. (1988). Bacterial Hemolysins as Virulence Factors. *Antonie van Leeuwenhoek* 54, 453-463.

Goni, F. M., and Ostolaza, H. (1998). *E. coli* α-hemolysin: a membrane-active protein toxin. *Braz J Med Biol Res* **31**, 1019-1034.

Griffin, P. M. (1995). *Escherichia coli* O157:H7 and other enterohemorrhagic *Escherichia coli*, p. 739-761. In M. J. Blaser, J. I. Ravdin, H. B. Greenberg, and R. L. Guerrant (ed), *Infections of the Gastrointestinal Tract*. Raven Press, New York, N. Y.
Grunstein, M., and Hogness, D. S. (1975). Colony hybridization: A method for the isolation of cloned DNAs that contain a specific gene. *Proc Natl Acad Sci* **72**:3961.

Gu, J., Stephenson, C. G., and Iadarola, M. J. (1994). Recombinant proteins attached to a Ni-NTA column: Use in affinity purification of antibodies. *BioTechniques* **17**, 257-262.

Gualerzi, C. O., and Pon, C. L. (1990). Initiation of mRNA Translation in Prokaryotes. *Biochem* **29**, 5881-5889.

Gyles, C., Johnson, R., Gao, A., Ziebell, K., Pierard, D., Aleksic, S., and Boerlin, P. (1998). Association of Enterohemorrhagic *Escherichia coli* Hemolysin with Serotypes of Shiga-Like-Toxin-Producing *Escherichia coli* of Human and Bovine origins. *Appl Environ Microbiol* **64**(11), 4134-4141.

Haile, D. J. (1999). Regulation of Genes of Iron Metabolism by the Iron-Response Proteins. *Am J Med Sci* **318**(4), 230-240.

Hamers, A. M., Pel, H. J., Willshaw, G. A., Kusters, J. G., van der Zeijst, B. A. M., and Gaastra, W. (1989). The nucleotide sequence of the first two genes of the CFA/I Fimbrial operon of human Enterotoxigenic *Escherichia coli*. *Microb Pathog* **6**, 297-309.

Herlax, V., and Bakas, L. (2003). Acyl chains are responsible for the irreversibility in the Escherichia coli α-hemolysin binding to membranes. *Chem Phys Lipids* **122**, 185-190.

Holland, I. B., Kenny, B., and Blight, M. (1990). Haemolysin secretion from *E. coli*. *Biochimie* **72**, 131-141.

Holland, I. B., Blight, M. A., and Kenny, B. (1990). The Mechanism of Secretion of Hemolysin and other Polypeptides from Gram-Negative Bacteria. *J Bioenerg Biomembr* **22**(3), 473-491.

Holst, O. (2000). Bacterial Toxins. Methods and Protocols. Methods in Molecular Biology, **145**, 41-63.

Hommais, F., Gouriou, S., Amorin, C., Bui, H., Rahimy, M. C., Picard, B., and Denamur, E. (2003). The FimH A27V Mutation Is Pathoadaptive for Urovirulence in *Escherichia coli* B2 Phylogenetic Group Isolates. *Infect Immun* **71**(6), 3619-3622.

Hsia, R., Small, P. L., and Bavoil, P, M. (1993). Characterization of Virulence Genes of Enteroinvasive *Escherichia coli* by Tn*phoA* Mutagenesis: Identification of *invX*, a Gene Required for Entry into HEp-2 Cells. *J Bacteriol* **175**(15), 4817-4823.

Huang, A., Friesen, J., and Brunton, J. L. (1987). Characterization of a Bacteriophage That Carries the Genes for Production of Shiga-Like Toxin 1 in *Escherichia coli*. *J Bacteriol* **169**, 4308-4312.

Hughes, C., Stanley, P., and Koronakis, V. (1992). *E. coli* Hemolysin Interactions with Prokaryotic and Eukaryotic Cell Membranes. *BioEssays* **14**(8), 519-525.

Hughes, C., Issartel, J. P., Hardie, K., Stanley, P., Koronakis, E., and Koronakis, V. (1992). Activation of *Escherichia coli* prohemolysin to the membrane-targetted toxin by HlyC-directed ACP-dependent fatty acylation. *FEMS Microbiol Immun* **105**, 37-44.

Iurescia, S., Condo, I., Angelaccio, S., Fratte, S. D., and Bossa, F. (1996). Site-Directed Mutagenesis Techniques in the Study of *Escherichia coli* Serine Hydroxymethyltransferase. *Protein Expr Purif* **7**, 323-328.

Jacob, F., and Monod, J. (1961). Genetic regulatory mechanisms in the synthesis of proteins. *J Mol Biol* **3**, 318-356.

James, B. W., and Keevil, C. W. (1999). Influence of oxygen availability on physiology, verocytotoxin expression and adherence of *Escherichia coli* O157. *J Appl Microbiol* **86**, 117-124.

Janda, J. M., and Abbott, S. L. The Enterobacteria. Weinberg, R. W. and Swan, K. Lippincott-Raven Publishers. Philidelphia,PA. 1998. pp. 13-60.

Janka, A., Bielaszewska, M., Dobrindt, U., Greune, L., Schmidt, M. A., and Karch, H. (2003). Cytolethal Distending Toxin Gene Cluster in Enterohemorrhagic *Escherichia coli* O157:H^- and O157:H7: Characterization and Evolutionary Considerations. *Infect Immun* **71**(6), 3634-3638.

Jordi, B. J. A. M., Willshaw, G. A., Van Der Zeijst, B. A. M., and Gaastra, W. (1992). The complete nucleotide sequence of region 1 of the CFA/I Fimbrial operon of human Enterotoxigenic *Escherichia coli*. *DNA Seq* **2**, 257-263.

Jordi, B. J. A. M., Op Den Camp, I. E. L., De Haan, L. A. M., Van Der Zeijst, B. A. M., and Gaastra, W. (1993). Differential Decay of RNA of the CFA/I Fimbrial operon and Control of Relative Gene Expression. *J Bacteriol* **175**(24), 7976-7981.

Jurgens, D., Ozel, M., and Takaisi-Kikuni, N. B. (2002). Production and Characterization of *Escherichia coli* Enterohemolysin and its Effects on the Structure of Erythrocyte Membranes. *Cell Biol Int* **26**(2), 175-186.

Koronakis, V., and Hughes, C. (1996). Synthesis, maturation and export of the *E. coli* hemolysin. *Med Microbiol Immunol* **185**, 65-71.

Lai, X. H., Wang, S. Y., and Uhlin, B. E. (1999). Expression of cytotoxicity by potential pathogens in the standard *Escherichia coli* collection of reference (ECOR) strains. *Microbiol* **145**, 3295-3303.

Lambert, P. F., and Reznikoff, W. S. (1985). Use of transcriptional Repressors to Stabilize Plasmid Copy Number of Transcriptional Fusion Vectors. *J Bacteriol* **162**(1), 441-444.

Law, D. (2000). Virulence factors of *Escherichia coli* O157 and other Shiga toxin-producing *E. coli*. *J Appl Microbiol* **88**, 729-745.

Le Bouguenec, C., Garcia, M. I., Ouin, V., Desperrier, J., Gounon, P., and Labigne, A. (1993). Characterization of Plasmid-Borne afa-3 Gene Clusters Encoding Afimbrial Adhesins Expressed by *Escherichia coli* Strains Associated with Intestinal or Urinary Tract Infections. *Infect Immun* **61**(12), 5106-5114.

Leitch, G. J., Iwert, M. E., and Burrows, W. (1966). Experimental cholera in the rabbit ligated ileal loop: toxin-induced water and ion movement. *J Infect Dis* **116**, 303-312.

Levine, M. M. (1987). *Escherichia coli* that cause diarrhea: Enterotoxigenic, enteropathogenic, Enteroinvasive, enterohemorrhagic, and enteroadherent. *J Infect Dis* **155**, 377-389.

Lewis, M., Chang, G., Horton, N. C., Kercher, M. A., Pace, H. C., Schumacher, M. A., Brennan, R. G., and Lu, P. (1996). Crystal structure of the lactose operon repressor and its complexes with DNA and inducer. *Science* **271**, 1247-1254.

Lin, W., Fullner, K., Clayton, R., Sexton, J., Rogers, M., Calia, K., Calderwood, S., Fraser, C., and Mekalanos, J. (1999). Identification of a *Vibrio cholerae* RTX toxin gene cluster that is tightly linked to the cholera toxin prophage. *Proc Natl Acad Sci USA* **96**, 1071-1076.

Lindberg, F., Lund, B., and Normark, S. (1986). Gene products specifying adhesion of uropathogenic *Escherichia coli* are minor components of pili. *Proc Natl Acad Sci* **83**, 1891-1895.

Liptakova, A., Siegfried, L., Podracka, B., Sabol, M., Sminalkova, H., Bogyiova, E., Rosocha, J., Kmetova, M., Kerestesova, H., and Kotulova, D. (2002). Detection of Shiga Toxins, Intimin and Enterohemolysin in *Escherichia coli* Strains Isolated from Children in Eastern Slovakia. *Folia Microbiol* **47**(2), 185-188.

Lorenz, E., and Stauffer, G. V. (1996). RNA polymerase, PurR and MetR interactions at the *glyA* promoter of *Escherichia coli*. *Microbiol* **142**, 1819-1824.

Lund, B., Lindberg, F., and Normark, S. (1988). Structure and Antigenic Properties of the Tip-Located P Pilus Proteins of uropathogenic *Escherichia coli*. *J Bacteriol* **170**(4), 1887-1894.

Ludwig, A., Benz, R., and Goebel, w. (1993). Oligomerization of *Escherichia coli* hemolysin (HlyA) is involved in pore formation. *Mol Gen Genet* **241**, 89-96.

Lutwyche, P., Rupps, R., Cavanagh, J., Warren, R. A. J., and Brooks, D. E. (1994). Cloning, Sequencing, and Viscometric Adhesion Analysis of Heat-Resistant Agglutinin 1, an Integral Membrane Hemagglutinin from *Escherichia coli* O9:H10:K99. *Infect Immun* **62**(11), 5020-5026.

Madrid, C., Nieto, J. M., Paytubi, S., Falconi, M., Gualerzi, C. O., and Juarez, A. (2002). Temperature- and H-NS-Dependent Regulation of a Plasmid-Encoded Virulence Operon Expressing *Escherichia coli* Hemolysin. *J Bacteriol* **184**(18), 5058-5066.

Mahajan, S., and Rodgers, F. G. (1990). Isolation, Characterization, and Host-Cell-Binding Properties of a Cytotoxin from *Campylobacter jejuni*. *J Clin Microbiol* **28**(6), 1314-1320.

Makino, S., Watarai, M., Tabuchi, H., Shirahata, T., Furuoka, H., and Takeda, Y. (2001). Genetically modified Shiga toxin 2e (Stx2e) producing *Escherichia coli* is a vaccine candidate for porcine edema disease. *Microb Pathog* **31**(1), 1-8.

Mann, M., and Jensen, O. N. (2003). Proteomic analysis of post-translational modifications. *Nat Biotechnol* **21**(3), 255-261.

Marques, L. R. M., Abe, C. M., Griffin, P. M., and Gomes, T. A. (1995). Association between Alpha-Hemolysin production and HeLa Cell-Detaching Activity in fecal isolates of *Escherichia coli*. *J Clin Microbiol* **33**(10), 2707-2709.

Martinez-Laguna, Y., Calva, E., and Puente, J. L. (1999). Autoactivation and environmental regulation of *bfpT* expression, the gene coding for the transcriptional activator of *bfpA* in enteropathogenic *Escherichia coli*. *Mol Microbiol* **33**(1), 153-168.

Maurer, M., Molidor, R., Sturn, A., Hartier, J., Hackl, H., Stocker, G., Prokesch, A., Schneideler, M., and Trajanoski, A. (2005). MARS: microarray analysis, retreval, and storage system. *BMC Bioinformatics* **6**, 101.

Mercurio, A., and Manning, P. A. (1985). Cellular localization and export of the soluble haemolysin of *Vibrio cholerae* El Tor. *Mol Gen Genet* **200**, 472-475.

Messier, N., and Roy, P. H. (2001). Integron Integrases Possess a Unique Additional Domain Necessary for Activity. *J Bacteriol* **183**(22), 6699-6706.

Moore, A. E., Sabachewsky, L., and Toolan, H. W. (1955). Culture Characteristics of Four Permanent lines of Human Cancer Cells. *Cancer Res.* 15: 598-602.

Moxley, R. A., Berberoy, E. M., Francis D. H., Xing, J., Moayeri, M., Welch, R. A., Baker, D. R., and Barletta, R. G. (1998). Pathogenicity of an Enterotoxigenic *Escherichia coli* hemolysin (hlyA) mutant in Gnotobiotic Piglets. *Infect and Immun* **66**, 5031-5035.

Mourino, M., Munoa, F., Balsalobre, C., Diaz, P., Madrid, C., and Juarez, A. (1994). Environmental regulation of α-haemolysin expression in Escherichia coli. *Microb Pathog* **16**, 249-259.

Muhldorfer, I., Hacker, J., Keusch, G. T., Acheson, D. W., Tscharpe, H., Kane, A. V., Ritter, A., Olschlager, T., and Donohue-Rolfe, A. (1995). Regulation of the Shiga-Like Toxin II Operon in *Escherichia coli*. *Infect Immun* **64**, 495-502.

Muniesa, M., and Jofre, J. (1998). Abundance in Sewage of Bacteriophage That Infect *Escherichia coli* O157:H7 and That Carry the Shiga Toxin 2 Gene. *Appl Environ Microbiol* **64**, 2443-2448.

Nataro, J. P., and Kaper, J. B. (1998). Diarrheagenic *Escherichia coli*. *Clin Microbiol Rev* **11**, 142-201.

Neely, M. N., and Friedman, D. I. (1998). Arrangement and functional identification of genes in the regulatory region of lambdoid phage H-19B, a carrier of a Shiga-like toxin. *Gene* **223**, 105-113.

Neely, M. N., and Friedman, D. I. (1998). Functional and genetic analysis of regulatory regions of coliphage H-19B: location of shiga-like toxin and lysis genes suggest a role for phage functions in toxin release. *Molec Microbiol* **28**(6), 1255-1267.

Negre, V. L., Bonacorsi, S., Schubert, S., Bidet, P., Nassif, X, and Bingen, E. (2004). The Siderophore Receptor IroN, but Not the High-Pathogenicity Island or the Hemin Receptor ChuA, Contributes to the Bacteremic Step of *Escherichia coli* Neonatal Meningitis. *Infect Immun* **72**(2), 1216-1220.

Oh, M. K., and Liao, J. C. (2000). DNA Microarray Detection of Metabolic Responses to protein overproduction in *Escherichia coli*. *Metab Eng* **2**, 201-209.

Okhuysen P. C. X., and Ericsson, C. D. (1992). Traveler's diarrhea: Prevention and treatment. *Med Clin North Am* **76**, 1357-1373.

Oscarsson, J., Westermark, M., Beutin. L., and Uhlin, B. E. (2001). The bacteriophage-associated Ehly1 and Ehly2 determinants from *Escherichia coli* O26:H$^-$ strains do not encode enterohemolysins per se but cause release of the ClyA cytolysin. *Int J Med Microbiol* **291**, 625-631.

Pan, T., Chen, L., and Su, Y. (2002). Identification of *Escherichia coli* O157:H7 by Multiplex PCR with Primers Specific to the HlyA, EaeA, Stx1, Stx2, FliC and Rfb Genes. *J Formos Med Assoc* **101**(9), 661-664.

Paton, J. C., and Paton, A. W. (1998). Pathogenesis and Diagnosis of Shiga Toxin-Producing *Escherichia coli* Infections. *Clin Microbiol Rev* **11**(3), 450-479.

Plunkett III, G., Rose, D. J., Durfee, T. J., and Blattner, F. R. (1999). Sequence of Shiga Toxin 2 Phage 933W from *Escherichia coli* O157:H7: Shiga Toxin as a Phage Late-Gene Product. *J Bacteriol* **181**, 1767-1778.

Puente, J. L., Bieber, D., Ramer, S. W., Murray, W., and Schoolnik, G. K. (1996). The bundle-forming pili of enteropathogenic *Escherichia coli*: transcriptional regulation by environmental signals. *Mol Microbiol* **20**(1), 87-100.

Purves, W. K., Sadava, D., Orians, G. H., and Heller, C. (2004). Life: The Science of Biology, 7th Ed. Sinauer Associates, W. H. Freeman & Co., and Sumanas, Inc.

Qin, L. X., Beyer, R. P., Hudson, F. N., Linford, N. J., Morris, D. E., and Kerr, K. F. (2006). Evaluation of methods for oligonucleotide array data via quantitative real-time PCR. *BMC Bioinformatics* **7**, 23.

Quackenbush, J. (2001). Computational analysis of microarray data. *Nat Rev Genet* **2**(6), 418-427.

Ramachandran, V., Brett, K., Hornitzky, M. A., Dowton, M., Bettelheim, K. A., Walker, M. J., and Djordjevid, S. P. (2003). Distribution of Intimin Subtypes among *Escherichia coli* Isolates from Ruminant and Human sources. *J Clin Microbiol* **41**(11), 5022-5032.

Riley, L. W., Reims, R. S., and Helgerson, S. D., McGee, H. B., Wells, J. G., Davis, B. R., Hebert, R. J., Olcott, E. S., Johnson, L. M., Hargrett, N. T., Blake, P. A., and Cohen, M. L. (1983). Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. *N Engl J Med* **308**, 681-685.

Roe, A. J., Yull, H., Naylor, S. W., Woodward, M. J., Smith, D. G. E., and Gally, D. L. (2003). Heterogeneous Surface Expression of EspA Translocon Filaments by *Escherichia coli* O157:H7 Is Controlled at the Posttranscriptional Level. *Infect Immun* **71**(10), 5900-5909.

Rommens, J., MacKnight, D., Pomeroy-Cloney, L., and Jay, E. (1983). Gene expression: chemical synthesis and molecular cloning of a bacteriophage T5 (T5P25) early promoter. *Nucl Acid Res* **11**(17), 5921.

Russo, T. A., McFadden, C. D., Carlino-MacDonald, U. B., Beanan, J. M., Barnard, T. J., and Johnson, J. R. (2002). IroN Functions as a Siderophore Receptor and Is a Urovirulence Factor in an Extraintestinal Pathogenic Isolate of *Escherichia coli*. *Infect Immun* **70**(12), 7156-7160.

Sambrook. J., Fritsch, E., and Maniatis, T. In *Molecular cloning: a laboratory manual.* Ford, N., Nolan, C., and Ferguson. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. 1989. 2nd Ed. pp 1.3-1.110.

Sandhu, K. S., Clarke, R. C., and Gyles, C. L. (1997). Hemolysin phenotypes and genotypes of *EAE*A-positive and *EAE*A-negative bovine verotoxigenic *Escherichia coli*. *Mech Path Enteric Dis* **49**, 295-302.

Savkovic, S. D., Villanueva, J., Turner, J. R., Matkowskyj, K. A., and Hecht, G. (2005). Mouse Model of Enteropathogenic *Escherichia coli* Infection. *Infect Immun* **73**(2), 1161-1170.

Schindel, C., Ziter, A., Schulte, B., Gerhards, A., Stanley, P., Hughes, C., Koronakis, V., Bhakdi, S., and Palmer, M. (2001). Interaction of *Escherichia coli* hemolysin with biological membranes. A study using cysteine scanning mutagenesis. *Eur J Biochem* **268**(3), 800-808.

Schmidt, H., Karch, H., and Beutin, L. (1994). The large-sized plasmids of enterohemorrhagic *Escherichia coli* O157 strains encode hemolysins which are presumably members of the *E. coli* α-hemolysin family. *FEMS Microbiol Lett* **117**, 189-196.

Schmidt, H., Beutin, L., and Karch, H. (1995). Molecular Analysis of the Plasmid-Encoded Hemolysin of *Escherichia coli* O157:H7 strain EDL 933. *Infect Immun* **63**(3), 1055-1061.

Schmidt, H., and Karch, H. (1996). Enterohemolytic Phenotypes and Genotypes of Shiga Toxin-Producing *Escherichia coli* O111 Strains from Patients with Diarrhea and Hemolytic-Uremic Syndrome. *J Clin Microbiol* **34**(10), 2364-2367.

Schmidt, H., Scheef, J., Janetzki-Mittmann, C., Datz, M., and Karch, H. (1997). An *ileX* tRNA gene is located close to the Shiga toxin II operon in enterohemorrhagic *Escherichia coli* O157 and non-O157 strains. *FEMS Microbiol Lett* 149, 39-44.

Schubert, S., Rakin, A., Karch, H., Carniel, E., and Heesemann, J. (1998). Prevalence of the "High-Pathogenicity Island" of *Yersinia* Species among *Escherichia coli* Strains That Are Pathogenic to Humans. *Infect Immun* **66**(2), 480-485.

Sha, J., Lu, M., and Chopra, A. K. (2001). Regulation of the Cytotoxic Enterotoxin Gene in *Aeromonas hydrophila*: Characterization of an Iron Uptake Regulator. *Infect. Immun* **69**(10), 6370-6381.

Shine, J., and Dalgarno, L. (1974). The 3'-terminal sequence of Escherichia coli 16S ribosomal RNA: Complementarity to nonsense triplets and ribosome binding sites. *Proc Nat Acad Sci* **71**, 1342-1346.

Simons, R. W., Houman, F., and Kleckner, N. (1987). Improved single and multicopy *lac*-based cloning vectors for protein and operon fusions. *Gene* **53**, 85-96.

Small, P. L. C., and Falkow, S. (1988). Identification of Regions on a 230-Kilobase Plasmid from Enteroinvasive *Escherichia coli* That Are Required for Entry into HEp-2 Cells. *Infect Immun* **56**(1), 225-229.

Smith, H. W., Green, P., and Parsell, Z. (1983). Vero Cell Toxins in *Escherichia coli* and Related Bacteria: Transfer by Phage and Conjugation and Toxic Action in Laboratory Animals, Chickens and Pigs. *J Gen Microbiol* **129**, 3121-3137.

Snyder, L., and Champness, W. (1997). Molecular Genetics of Bacteria. ASM Press. Washington, D. C.

Sokurenko, E. V., Feldgarden, M., Trintchina, E., Weissman, S. J., Avagyan, S., Chattopadhyay, S., Johnson, J. R., and Dykhuizen, D. E. (2004). Selection Footprint in the FimH Adhesin Shows Pathoadaptive Niche Differentiation in *Escherichia coli*. *Mol Biol Evol* **21**(7), 1373-1385.

Srinivasan, U., Foxman, B., and Marrs, C. F. (2003). Identification of a Gene Encoding Heat-Resistant Agglutinin in *Escherichia coli* as a Putative Virulence Factor in Urinary Tract Infection. *J Clin Microbiol* **41**(1), 285-289.

Strachan, Tom., and Read, Andrew P. (1996). Human Molecular Genetics. BIOS Scientific Publishers Limited.

Stroeher, U. H., Bode, L., Beutin, L., and Manning, P. A. (1993). Characterization and sequence of a 33-kDa enterohemolysin (Ehly1)-associated protein in *Escherichia coli*. *Gene* **132**, 89-94.

Sung, L. M., Jackson, M. P., O'Brien, A. D., and Holms, R. K. (1990). Transcription of the Shiga-Like Toxin Type II and Shiga-Like Toxin Type II Variant Operons of *Escherichia coli*. *J Bacteriol* **172**, 6386-6395.

Taichman, N. S., Simpson, D. L., Sakurada, S., Cranfield, M., DiRienzo, J., and Slots, J. (1987). Comparative studies on the biology of *Actinobacillus actinomycetemcomitans* leukotoxins in primates. *Oral Microbiol Immunol* 2, 97-104.

Todd, E. C. D. (1989). Costs of acute bacterial foodborne disease in Canada and the United States. *Int J Food Microbiol* **9**, 313-326.

Ullmann, A., Jacob, F., and Monod, J. (1967). Characterization by *in vivo* complementation of a peptide corresponding to an operator-proximal segment of the β-galactosidaseeee structural gene of *Escherichia coli*. *J Mol Biol* **24**: 339.

Vandemaele, F., Vandekerchove, D., Vereecken, M., Derijcke, J., Dho-Moulin, M., and Goddeeris, B. M. (2003). Sequence analysis demonstrates the conservation of *fimH* and variability of *fimA* throughout Avian Pathogenic *Escherichia coli* (APEC). *Vet Res* **34**, 153-163.

van der Goot, G. (2001). Pore-Forming Toxins. *Curr Top Microbiol Immunol* **257**, 86-111.

Venkateswaran, K., Murakoshi, A., and Satake, M. (1996). Comparison of commercially available kits with standard methods for the detection of coliforms and *Escherichia coli* in foods. *Appl Environ Microbiol* **62**, 2236-2243.

von Gabain, A., and Bujard, H. (1977). Interaction of *E. coli* RNA polymerase with promoters of coliphage T5: the rates of complex formation and decay and their correlation with in vitro and in vivo transcriptional activity. *Mol Gen Genet* **157**(3), 301-311.

von Gabain, A., and Bujard, H. (1979). Interaction of *Escherichia coli* RNA polymerase with promoters of several coliphage and plasmid DNAs. *Proc Natl Acad Sci USA* **76**(1), 189-193.

Wandersman, C., and Delepelaire, P. (1990). TolC, an *Escherichia coli* outer membrane protein required for hemolysin secretion. *Proc Natl Acad Sci* **87**, 4776-4780.

Wang, G., Clark, C. G., and Rodgers, F. G. (2002). Detection in *Escherichia coli* of the Genes Encoding the Major Virulence Factors, the Genes Defining the O157:H7 Serotype, and Components of the Type 2 Shiga Toxin Family by Multiplex PCR. *J Clin Microbiol* **40**(10), 3613-3619.

Wang, G., Clark, C. G., Liu, C., Pucknell, C., Munro, C. K., Kruk, T. M. A. C., Caldeira, R., Woodward, D. L., and Rodgers, F. G. (2003). Detection and Characterization of the Hemolysin Genes in *Aeromonas hydrophila* and *Aeromonas sobria* by Multiplex PCR. *J Clin Microbiol 41*(3), 1048-1054.

Watson, J. D., Gilman, M., Witkowski, J., and Zoller M. (1992). Recombinant DNA. 2nd Ed. Distributed by W. H. Freeman and Company. New York, New York.

Weiler, L. H., Tiggs, M., Ebel, F., Schaferkordt, S., Djafari, S., Schlapp, T., Baljer, G., and Chakraborty, T. (1996). The enterohemolysin phenotype of bovine Shiga-like toxin-producing *Escherichia coli* (SLTEC) is encoded by the EHEC-hemolysin gene. *Vet Micro* 52, 153-164

Welch, R. A. (1991). Pore-forming cytolysins of gram-negative bacteria. *Mol Micriobiol* **5**, 521-528.

Willshaw, G. A., Smith, H. R., McConnell, M. M., and Rowe, B. (1985). Expression of Cloned Plasmid Regions Encoding Colonization Factor Antigen I (CFA/I) in *Escherichia coli*. *Plasmid* **13**, 8-16.

Willshaw, G. A., Smith, H. R., Scotland, S. M., Field, A. M., and Rowe, B. (1987). Heterogeneity of *Escherichia coli* Phages Encoding Vero Cytotoxins: Comparison of Cloned Sequences Determining VT1 and VT2 and Development of Specific Gene Probes. *J Gen Microbiol* **133**, 1309-1317.

Wolfe, M. S. (1990). Acute diarrhea associated with travel. *Am J Med* **88**(Suppl 6A), 34S-37S.

Xia, X., McClelland, M., and Wang, Y. (2005). WebArray: an online platform for microarray data analysis. *BMC Bioinformatics* **6**, 306.

Yamagami, S., Motoki, M., Kimura, T., Izumi, H., Takeda, T., Katsuura, Y., and Matsumoto, Y. (2001). Efficacy of postinfection treatment with anti-Shiga toxin (Stx humanized monoclonal antibody TMA-15 in mice lethally challenged with Stx-producing *Escherichia coli*. *J Infect Dis* **184**(6), 738-742.

Yang, X, Goliger, J. A., and Roberts, J. W. (1989). Specificity and Mechanism of Antitermination by Q Proteins of Bacteriophages λ and 82. *J Mol Biol* **210**, 453-460.

Ye, Rick W., Wang, Tao., Bedzyk, Laura., and Croker, Kevin M. (2001). Applications of DNA microarrays in microbial systems. *J Microbiol Meth* **47**, 257-272.

Yokoyama, K., Makino, K., Kubota, Y., Wantanabe, M., Kimura, S., Yutsuno, C. H., Kurokawa, K., Ishii, K., Hattori, M., Tatsuno I., Abe, H., Yoh, M., Iida, T., Ohnishi, M., Hayashi, T., Yasunaga, T., Honda, T., Sasakawa, C., and Shinagawa, H. (2000). Complete nucleotide sequence of the prophage VT1-Sakai carrying the Shiga toxin 1 genes of the enterohemorrhagic Escherichia coli O157:H7 strain derived from the Sakai outbreak. *Gene* **258(1-2)**, 127-139.

Zamora, J., Reinhardt, G., Polette, M., and Macias, P. (1997). Hemolysins and Verotoxin (VT) in Enteric *Escherichia coli* Isolated from pigs. *Rev Lat Amer Microbiol* **39**, 159-165.

Zhang, W. L., Kohler, B., Oswald, E., Beutin, L., Karch, H., Morabito, S., Caprioli, A., Suerbaum, S., and Schmidt, H. (2002). Genetic Diversity of Intimin Genes of Attaching and Effacing *Escherichia coli* Strains. *J Clin Microbiol* **40**(12), 4486-4492.

APPENDIX I

F = forward primer, R = reverse primer, _____ = coding sequence
Restriction enzyme sites in capital letters

A) Primers used for cloning *E. coli ehl1* into pRS551 and pQE-60

Primer Name	Primer Sequence (5' to 3')	Nucleotide Position	Mastermix	Plasmid
ehl1prlesf1	GAGCTCGAGCTCatgaatcaactggtgcg *Sac*I *Sac*I	356 - 372	A1	pRS551
ehl1prlesr1	AAGCCTAAGCCTttacatagatgtttcgtttaattc *Hind*III *Hind*III	1136 - 1159	A1	pRS551
ehl1prlesf2	GAGCTCGAGCTCatgaatcaactggtgcgtttcg *Sac*I *Sac*I	356 - 377	A2	pRS551
ehl1prlesr2	AAGCCTAAGCCTttacatagatgtttcgt *Hind*III *Hind*III	1143 - 1159	A2	pRS551
ehl1promoterf1	gccgggcacgctctgttc	157 - 174	A3	pRS551
ehl1promoterr1	gtgtggcgccggtggtggttac	1156 - 1177	A3	pRS551
ehl1promoterf2	ccgccgggatctgaatac	174 - 191	A4	pRS551
ehl1promoterr2	gtggcgccggtggtggttacata	1154 - 1175	A4	pRS551
ehl1promoterf3	CTGCAGCTGCAGgccgggcacgctctgttc *Pst*I *Pst*I	157 - 174	A5	pRS551
ehl1promoterr3	AAGCCTAAGCCTgtgtggcgccggtggtggttac *Hind*III *Hind*III	1156 - 1177	A5	pRS551
ehl1promoterf4	CTGCAGCTGCAGccgccgggatctgaatac *Pst*I *Pst*I	174 - 191	A6	pRS551

APPENDIX I cont'd

A) Primers used for cloning *E. coli ehl1* into pRS551 and pQE-60 (cont'd)

Primer Name	Primer Sequence (5' to 3')	Nucleotide Position	Mastermix	Plasmid
ehl1promoterr4	AAGCCTAAGCCTgtggcgccggtggtggttacata *Hind*III *Hind*III	1154 - 1175	A6	pRS551
ehl1prlesf7	TCGAGatgaatcaactggtgcg *Sac*I (portion)	356 - 372	A7	pCAL-n
ehl1prlesr7	AGCTAATTAGttacatagatgtttcgtttaattc *Hind*III – stop codons (portion)	1136 - 1159	A7	pCAL-n
ehl1prlesf8	cgaCCATGGggcatgaatcaactggtgcg *Nco*I	344 - 372	A8	pQE-60
ehl1prlesr8	AGATCTtaggaaattcccctgcgcgct *Bgl*II	1280 - 1300	A8	pQE-60

APPENDIX I cont'd

B) Primers used for sequencing *E. coli ehl1* after cloning into pQE-60

Primer Name	Primer Sequence (5' to 3')	Nucleotide Position	Method (Mastermix)
Ehly1Fa	atagccgtgatccagaagaaatg	972 - 994	Did not use
Ehly1Ra	gtggcgccggtggtggttaca	1155 - 1175	Did not use
Ehly1Fb (GPF1)	ctcctgatgtgcttctcggtgttt	467 - 490	PCR confirmation (GP mastermix)
Ehly1Rb (GPR1)	aggccatcagcatttcttct	648 - 667	PCR confirmation (GP mastermix)
pQE-F2	cggataacaatttcacacag	pQE Vector	Sequencing (mm-QE)
pQE-R1	gttctgaggtcattactgg	pQE Vector	Sequencing (mm-QE)
LGP1-1	cgccatacctgatggtaagttc	355 - 376	Sequencing
LGP1-2	ggagagttacgcactccggc	379 - 398	Sequencing
LGP1-3	gccacgggcaaagggactgtg	132 - 152	Sequencing
LGP1-4	gctggaagaggatacgaccgc	208 - 228	Sequencing
LGP2-1	gagttgcgcgccacggcaaaggac	509 - 532	Sequencing
LGP2-2	gcctcattacagagcgag	530 - 547	Sequencing
LGP2-3	gtatctacaaagcaaattaag	799 - 819	Sequencing
LGP2-4	gaaccaacgtcaggccgc	842 - 859	Sequencing
LGP2-5	gcgatgcagaagaaatgctgatgg	641 - 665	Sequencing
LGP2-6	ggccttttaccagcgcagcg	663 - 681	Sequencing
LGP2-7	gaataacagcactgtctc	684 - 701	Sequencing
LGP2-8	ctgtctccgaactggataagg	695 - 715	Sequencing

APPENDIX II

A) Nucleotide sequence of *Escherichia coli* Enterohemolysin 1 (isolate 01-1610) coding region DNA. Start (ATG) and stop (TAA) codons are in red. Deviations of base pairs from GenBank Sequence Accession Number X70047 are in blue.

CATGACTCGAGCTCATGAATCAACTGGTGCGTTTCGCGGAGTTGATGTCACA
AAGCAAAGCGACTGTACCGAAACATCTTGAAGGCAAACCTGCCGATTGCCTG
GCGGTGACCATGCAGGCGGCACAGTGGGGAATGAACCCTTTCGCCGTGGCGC
AGAAAACGCATGTGGTAAACGGAACGTTAGGCTACGAAGCACAGTTGGTAA
ACGCGGTCGTATCCTCTTCCAGCCTGCTAGCGACACGCCTTAATTATCGCTGG
AGCGGTGACTGGTCGAATGTTAACGGCAAAACAGATAAATCACCGAATCTGA
CGGTAACTGTGTCAGCAGTTCTTAAAGGAGAAGTAGAACCACGTGAGCTTAC
CATCAGTATGGCGCAAGCCGGAGTGCGTAACTCTCCATTGTGGGAACAGGAT
CCGCGCCAGCAACTTGCCTATCTTTGCACGAAACGATGGGCTCGCCTGCACG
CTCCTGATGTACTTCTTGGTGTTTACACCCCTGACGAATTACAGGAAACGGCA
CCGCGCGTTGAGCGAGACATTACTCCGCAAACAACTGCTGCTGCGGGAATGA
ACAGTCTGATCAACGCTAAACCAGTGAAAAAGCCTGATGAGCAAACGAATA
AAGCGGATAGCCGTGATCCAGAAGAAATGCTGATGGCCTTTACCAGCGCAGC
GATGAATTACAGCACTGTCTCCGAACTGGATAAGGCTTACAAATACATTGCA
CAAAAACTTTCAGATGATGACGAACTGCTGGCAAAAGCCACCGACGTTTACA
GCGTTCGTCGGGAAGAATTAAACGAAACATCTATGTAASS*ACCACCGGCGCC
ACACGCGCCGGACTGCAACCAAGAGAGGTATTTATGAAAGGTGCATTAGGTA
AGAAGGAACTCCTGGCGGTGGTGCCACTGTCATGGAGCACTATCGACCGTAT
GGAGCGCGCAGGGGAATTTCCTA

*S represents C or G. S is inserted into sequence data when it is undetermined whether the nucleotide is C or G.

APPENDIX II

B) *E. coli ehl1* sequence (GenBank sequence accession number X70047). Coding sequence is from 356 bp – 1159 bp.

1 gaattccgct accacgtgca ggacgcaatg taccgcgaag gacgaatgag ggttactggt

61 cagccgcatg gttttttctt tcttgccgtg agcgaaagca ttgattgtgg tcggtatccg

121 gtacgcgtgt tcgagctgga tgcgcaggat gtcgatgccg ggcacgctct gttccgccgg

181 gatctgaata cctatcacga atgccgcatc aacgatgagt ggggcggagt ggaaattatt

241 aaacgccctg actgggcacg taaacaggat atgtacgtat gagcaatgat atcgcaatca

301 catcacaacc aggtgcaact gtaggtactg ctgcggcaat cttcgaccgg agggcatgaa

361 tcaactggtg cgtttcgcgg agttgatgtc acaaagcaaa gcgactgtac cgaaacatct

421 tgaaagcaaa cctgccgatt gtctggcggt gaccatgcag gcggcacagt ggggaatgaa

481 ccctttgccc gtggcgcaga aaacgcatgt ggtaaacgga acgttaggct acgaagcaca

541 gttggtaaac gcggtcgtat cctcttccag cctgctggcg acacgcctga attatcgctg

601 gagcggtgac tggtcgaatg ttaacggcaa aacagataaa tcaccgaatc tgacggtaac

661 tgtgtcagca gttcttaaag gagaagcaga accccgtgaa cttaccatca gtatggcgca

721 agccggagtg cgtaactctc cattgtggga acaggatccg cgccagcagc ttgcctatct

781 ttgcacgaaa cgatgggctc gcctgcacgc tcctgatgtg cttctcggtg tttacacccc

841 tgacgaatta caggaaacgg caccgcgcgt tgagcgagac attactccgc aaacaactac

901 tgctgcggga atgaacagtc tgatcaacgc taaaccagtg aaaaagcctg atgagcaaac

961 tcgtaaagcg gatagccgtg atccagaaga aatgctgatg gcctttacca gcgcagcgat

1021 gaattacagc actgtctccg aactggataa ggcttacaaa tacattgcac aaaaactttc

1081 agatgatgac gaactgctgg caaaagccac cgacgtttac agcgttcgtc gggaagaatt

1141 aaacgaaaca tctatgtaac caccaccggc gccacacgcg ccggactgca accaagagag

1201 gtatttatga aaggtgcatt aggtaagaag gaactcctgg cggtggtgcc actgtcatgg

APPENDIX II cont'd

B) *E. coli ehl1* sequence (GenBank sequence accession number X70047) (cont'd).

1261 agcactatcg accgtatgga gcgcgcaggg gaatttccta aacgctggta tatcactgac

1321 aaacctgcgc atggaaccgt gacgaagttg agcggttggc ttgatgaacg tcaggcagca

1381 agccaggcag agttccaggg taaaaaacct cctgttcagc aacgtgtata tcgtcccgtg

1441 agcaacgctg catgagtgcg ctgctaaggc actggagcaa atggtcagga tggtacttat

1501 tcctggcctc tgtttctgca tggctttatc tgctggcatt aattttcaga gagggttgga

1561 ttaagtgaga aagttaagcc gacttgaaaa atatcacatg aacaaggttt caatgcgcag

1621 tccttcaaag gttgttgccg ttactcctgc ggcgatagag atcgaaaaac gcgcgattga

1681 aagagagaaa aaagggcagt tccgcattgc cgcccacctt tggcttcagt gtatggatgt

1741 tgcttctggt gatgttgagc gtgcaaggat cgcggttcgt agggatcaat gtatcacaaa

1801 aggtaaacgg ccttcgccgt ggcgactata gcggtatagg atgttgcggg gtggtttatg

1861 actaagaaat acacgctaat ctatgcagat cccccatggg tataccggga caaagccgca

1921 gatggtaatc gtggtgccgg ttttaaatat ccagttatga gtgtgctgga tatctgccgc

1981 cttcctgtgt gggatttagc cgatgaaaac tgcctactgg ccatgtggtg ggtgccaana

2041 caaccactcg aagcactaaa agttgttgaa gcctggggat tccgtctgat gaccatgaag

2101 ggcttcacgt ggataaaatg tggtagtcga cctgcagcca agct

APPENDIX III

This appendix has been written to address the scientific flaws within the presented research. The thesis as written above contained four major concept errors, which affected the scientific research and data obtained from the experiments. The four main concepts that need to be addressed are: screening systems for cloning, transcriptional and translational vectors, induction of the pQE-60 vector, and ribosomal binding site mutations. This appendix will identify the flawed experiments, explain the problems within the experiments and their resulting affect on the data, and clarify the topics through explaining how the research should have been properly carried out.

In the cloning portion of the presented research, two vectors (pRS551 and pQE-60) were used. Blue and white color screening of colonies was used to screen transformants from both of the vectors for the recombinant protein. This was the first major scientific error within this research, as both of the vectors used do not use color screening for identification of colonies containing the recombinant protein.

Identification of bacterial colonies that contain recombinant plasmids is usually done through one of five common methods: insertional inactivation, restriction analysis of small-scale preparations of plasmid DNA, screening by hybridization, α-complementation, or PCR-DNA sequencing (Sambrook *et al*., 1989; Snyder and Champness, 1997). Insertional inactivation is typically used with vectors that have at least two antibiotic resistance genes and sites for restriction enzymes to cleave the vector (Sambrook *et al*., 1989). The insert DNA and the plasmid DNA is cut with a restriction enzyme that has a site within one of the antibiotic resistance genes, the insert DNA and

plasmid are ligated together, and transformants are selected for resistance to the second antibiotic (Sambrook *et al*., 1989). Bacterial colonies that contain recombinant plasmids can also be screened by restriction analysis. The transformants are grown in small-scale cultures and plasmid DNA is isolated and screened through digestion with appropriate restriction enzymes and gel electrophoresis (Sambrook *et al*., 1989). Screening colonies through hybridization involves lysis of bacterial colonies onto a nitrocellulose filter and noncovalent attachment of the released DNA into the filters (Sambrook *et al*., 1989). The DNA is then hybridized to an appropriate nucleic acid probe such as a ^{32}P-labeled probe (Grunstein and Hogness, 1975). Bacterial colonies that contain recombinant plasmids can also be screened by α-complementation. When screening by α-complementation, the vector that is used would have DNA containing the first portion of the beta-galactosidase gene, l*acZ,* and a polycloning site that results in the addition of a few amino acids to the amino-terminus of beta-galactosidase (Jacob and Monod, 1961; Sambrook *et al*., 1989). Host cells are used that have the carboxy-terminus fragment of beta-galactosidase. Both the host fragments and the plasmid fragments do not have an active beta-galactosidase enzyme but they combine together to form the active beta-galactosidase enzyme (Jacob and Monod, 1961; Ullmann *et al*., 1967). When screening by PCR-DNA sequencing, PCR is performed using primer to amplify the gene of interest and then either Maxam-Gilbert sequencing or Sanger dideoxy sequencing sequences the DNA. Sanger dideoxy sequencing is the more commonly used of the two methods, and uses 4 separate polymerizing reactions with each reaction containing one of the dideoxynucleoside triphosphates (Snyder and Champness, 1997). When a dideoxynucleoside triphosphate is incorporated into the growing DNA chain, termination of the chain takes place. Each of

the four reaction mixtures will have a different set of shortened DNA chains that can then be used to determine the sequence of the template DNA (Snyder and Champness, 1997).

Blue/white colony selection is based on the Lac operon and is used on plasmids containing the LacZ gene. The LacZ gene contains an internal multiple cloning site (MCS) that is cut using a restriction enzyme and the gene of interest is inserted. An intact LacZ gene encodes the enzyme beta-galactosidase that metabolizes galactose into lactose and glucose (Purves *et al*., 2004). A LacZ gene containing an insert is not functional and disrupts the activity of beta-galactosidase. To perform color screening, bacteria are plated on an enriched agar plate containing 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside, X-Gal, and isopropyl b-D-thiogalactoside, IPTG (Sambrook *et al*., 1989). X-Gal, a substrate for beta-galactosidase, acts as a color indicator, and IPTG induces the Lac operon to produce beta-galactosidase. The X-gal is metabolized by beta-galactosidase and produces a bright blue color in the colonies that contain a functional LacZ gene. Therefore, blue colonies do not contain the inserted gene of interest. White colonies contain the gene of interest and are white because the interrupted LacZ gene no longer produces the beta-galactosidase enzyme and X-gal will not be metabolized. Therefore, when selecting colonies, white color colonies, which would contain the inserted gene of interest, would be selected (Sambrook *et al*., 1989).

In the process of screening transformants from pRS551, blue white color screening was used. This was a major error for two reasons. First, the pRS551 MCS is an operon fusion to the LacZ gene (Simons *et al*, 1987). This means that the MCS is directly in front of the LacZ gene. Thus, the LacZ gene would not be cleaved by the insertion of the *ehl1* gene, and color selection would not be able to be used for selection.

The recombinant phage should have an intact kanamycin resistance gene (kan) and would be selected for by plating on agar plates containing ampicillin and kanamycin (Simons *et al*, 1987). Secondly, *ehl1* was not cloned into the MCS but into the kan gene in the flanking region (Figure 4). Therefore, there was no operon fusion to LacZ. In addition, colonies now needed to be selected for by plating on agar plates containing ampicillin only. It was noticed that the *ehl1* gene was inserted into the kan gene and not the MCS, therefore the experiment was adjusted to correct for this error by plating the transformed cells on plates containing ampicillin only. However, blue white color selection for screening colonies was still performed.

A possible reason for the flawed application of blue white color screening when using the pRS551 vector was due to confusion on how to properly use this plasmid. pRS551 and the insert of interest can be transferred back into a multicopy plasmid, pRS308, which was specifically designed to examine mutations within the fusion insert (Simons *et al*., 1987). The recombinants from this transfer can be identified through color screening. These protein fusion vectors have very low levels of the beta-galactosidase enzyme and allow for quantitation of transcription and translation levels from weakly expressed genes (Simons *et al.*, 1987). It also allows for identification of clones containing the DNA of interest since virtually any increase in expression produces a detectable enhancement of the blue color, while both single and multicopy vectors are either light blue or white (Simons *et al*., 1987).

Even though color selection was incorrectly used for selecting colonies from pRS551 cloning, the transformations were plated on the correct type of screening plates (LB + Amp) and both blue and white colonies were selected as positive and negative

controls. The misunderstanding of the pRS551 plasmid resulted in the use of color screening and choosing blue colonies as *ehl1* positive colonies and white colonies as *ehl1* negative colonies. The blue and white color would not aid in identification of recombinant colonies. However, the colonies were screened through PCR restriction enzyme digest to confirm whether or not they contained the insert. Errors for cloning *ehl1*into pRS551 incorrectly were accounted for by the restriction enzyme digest screen and colonies containing the insert were selected. However, an additional error was made during the cytotoxicity assay. Preparation of the culture supernatants containing the recombinant colonies should have been adjusted to account for inserting *ehl1* into the kan gene of pRS551. This was overlooked and cultures were grown in the presence of ampicillin and kanamycin when they should have been grown in the presence of ampicillin only. Although, when grown in combination with either VT1 or VT2 no antibiotic was added. Thus, one cannot draw any conclusions from the cytotoxicity data as to whether or not *ehl1* is cytotoxic other than when grown in combination with VT1 or VT2, which did not result in any change in cytotoxicity.

Transcriptional fusion vectors contain a gene, the expression of which is under the control of a cloned promoter, and whose gene product is assayable (Lambert and Reznikoff, 1985). *ehl1* and its upstream region which may have possibly contained a promoter were cloned into the transcriptional fusion vector pRS551 which contained the LacZ gene with b-galactosidase as the gene product. If recombinant pRS551 was cloned into pRS308, beta-galactosidase activity could have been used to detect gene expression. In a translational fusion vector, it is important that the insert is cloned in frame with the ATG start codon and the correct coding reading frame must be maintained through to the

stop codon. In the translational fusion vector pQE-60, which also has a C-terminus 6xHis tag, the insert must be cloned in frame with both the ATG start codon and the 3' 6xHis coding sequence (The QIAexpressionist, technical manual 5th edition, 2001).

In cloning *ehl1* into pQE-60, two major errors occurred. First, in the process of screening for colonies from cloning with pQE-60, blue white color screening was used. The application of this type of colony selection when using this vector was entirely flawed. pQE-60 is a translational fusion vector and has a C-terminus 6xHis tag, therefore the insert must be cloned in frame with both the ATG start codon and the 3' 6xHis coding sequence (The QIAexpressionist, technical manual 5th edition, 2001). Color selection is irrelevant, and if cloned in the correct reading frame, the colonies grown from the transformation would contain the insert. When using pQE-60, transformants can be screened for correct insertion of the coding fragment by direct screening of bacterial colonies for the expressed protein by colony blot, by restriction analysis of the pQE-60 plasmid DNA, sequencing for correct insertion of the cloning junctions, or by preparing small scale expression cultures (The QIAexpressionist, technical manual 5th edition, 2001).

When using the pQE-60 translational fusion vector, the primers must be designed to insure the insert is in frame and the correct coding reading frame must be maintained through to the C-terminus 6xHis tag. An undetected frame-shift mutation or translational stop codon would lead to premature termination and would result in peptides without the 6xHis tag (The QIAexpressionist, technical manual 5th edition, 2001). The second major error to occur in the presented research was that the forward primer, ehl1prlesf8, for cloning into the pQE-60 MCS, was designed incorrectly. In this case, the primer was

designed to incorporate the *Nco*I restriction site upstream to the ATG start codon of *ehl1*, resulting in the entire insert being out of frame. It was overlooked that the *Nco*I recognition sequence, 5'-CCATGG-3', contains an ATG start codon, which resulted in *ehl1* being out of frame and a stop codon at position 421 bp in the *ehl1* sequence (GenBank Accession number X70047, Appendix II). Therefore, no Ehly1 protein was produced. In addition, the C-terminus 6xHis tag was not translated.

The effects of this cloning error are seen throughout the rest of the research and can help to explain why the protein purifications and western blot were unsuccessful. The protein purifications that were attempted (both native and denaturing) were based on the protein containing the His tag. The 6xHis tag binds to the Ni-NTA column as long as two histidine residues are available to interact with the nickel ion (Gu *et al*., 1994). In this case, there was only a small-truncated Ehly1 protein, which was out of frame, and there was no 6xHis tag translated. Thus, no protein was purified. Unfortunately, only the final protein elution of the purifications was tested on SDS-PAGE and although no protein was purified, it was not realized that there was no complete Ehly1 protein and no C-terminus 6xHis tag. It was incorrectly assumed that Ehly1 could not be purified but was still present. During the protein purifications, the cell lysate, flow-through, and washes, as well as the final elution containing the protein should have been examined on SDS-PAGE as controls and to aid in trouble shooting the purifications. This would have shown that the Ehly1 protein did not bind to the Ni-NTA resin, meaning that the 6xHis tag was not present. In addition, the ligation junctions could have been sequenced to check if the reading frame was correct and to see if there was a premature stop codon. In the western blot, the Tetra-His and Penta-His HRP conjugate antibodies that were used

should bind to the 6xHis tag in the protein. Since there was no 6xHis tag translated due to the protein being out of frame, no binding took place and no protein was seen on the western blot. There was no negative control run during the western blot. A suitable negative control would have been a cell lysate or extract of material that is similar to the sample but lacking the 6xHis protein (QIAexpress Detection and Assay Handbook, technical manual 3rd edition, 2002). Although a 6xHis protein ladder was used as a positive control for transfer and detection, the western blot experiment was completely unsuccessful since nothing was visualized on the blot after staining, including the 6xHis protein ladder positive control. Since the 6xHis protein ladder was not visible, the blot procedure should have been reviewed to determine the following: whether development time was suboptimal, if incomplete transfer of the protein took place, if the washing conditions were inappropriate, if the HRP enzyme was inactive, if the membrane was shaken during development, and if the blocking buffer reagent was not prepared correctly (QIAexpress Detection and Assay Handbook, technical manual 3rd edition, 2002). During color development with the HRP staining solution, the incubation took place on a rocker, which would have caused the color precipitate to disperse, resulting in no visualization of the 6xHis ladder positive control. However, this was overlooked at the time of the western blot procedure.

The unsuccessful protein purifications and western blot should have lead to questions being raised as to whether or not a complete Ehly1 protein with the C-terminus His tag was expressed. These problems were overlooked and thus the second major error of this research, the improperly designed forward primer resulting in an out of frame Ehly1 protein with no C-terminus His tag, occurred. Unfortunately, the protein

purification and western blots were not examined carefully and this error was not detected until after all the microarray experiments were performed. Thus, no conclusions from the microarray data as to the expression of Ehly1 and coexpression with other virulence factors would be correct.

The third major concept error within the presented research occurred during the induction of the pQE-60 vector. Either IPTG or lipoprotein were used to induce expression of Ehly1 in pQE-60. The use of lipoprotein to induce pQE-60 was scientifically flawed, as lipoprotein cannot induce the pQE-60 vector.

The pQE-60 plasmid is based on the T5 promoter transcription-translation system, where high levels of the *lac* repressor protein are necessary to regulate and repress the high transcription rate of the T5 promoter. An advantage to using the bacteriophage T5 early promoters is its ability to exceed other promoters in the rate the formation of a complex with RNA polymerase (von Gabain and Bujard, 1977; von Gabain and Bujard, 1979), even though the T5 promoter does not contain a complete –35 region consensus sequence (Rommens *et al*., 1983). A disadvantage in using the bacteriophage T5 promoter is that a strong termination signal for transcription is needed (Gentz et al., 1981). In the pQE-60 plasmid, the T5 promoter is recognized by *E. coli* RNA polymerase and the vector has two *lac* operator sequences to increase the binding of *lac* repressor to ensure the strong T5 promoter is repressed (The QIAexpressionist, technical manual 5th edition, 2001).

The pQE-60 expression system uses *E. coli* host strains that use the *lac* repressor gene either in *trans* or *cis* to the gene to be expressed. pQE-60 uses *trans*-Repression when *E. coli* host strains containing the pREP4 plasmid, such as *E. coli* M15[pREP4], are

used. A gene is considered to have a *trans*-acting function when the gene product can act on DNAs in the cell other than the DNA from which it was produced (Snyder and Champness, 1997). In this case, *lac* repressor is produced from the *lac*I gene on the pREP4 plasmid, which is in the same cell as the recombinant protein, but not on the same DNA. *lac* repressor protein is produced within the cell and acts to repress the recombinant protein, thus it is considered to act through *trans*-Repression. In the *trans* system, the host strains contain the low-copy plasmid pREP4 and constitutively expresses the *lac* repressor protein encoded by the *lac* I gene (The QIAexpressionist, technical manual 5th edition, 2001). The host cells contain numerous copies of pREP4 and thus numerous copies of the *lac* repressor protein, which ensures that the *lac* repressor protein will bind to the operator sequence and tightly regulate recombinant protein expression (The QIAexpressionist, technical manual 5th edition, 2001). pQE-60 uses *cis*-Repression when *E. coli* strains that have the *lac*I^{q} mutation, such as *E. coli* XL1-Blue, are used. A gene is considered to be *cis*-acting when it affects the DNA molecule in which it occurs and not other DNA molecules in the same cell (Snyder and Champness, 1997). In this case, *lac* repressor is produced from the *lac*I^{q} gene in recombinant pQE-60. The *lac*I^{q} gene is on the same DNA as the recombinant protein. The *lac* repressor protein acts to repress the recombinant protein on the same DNA molecule and is thus considered to act through *cis*-Repression. The *E. coli* XL1-Blue strain makes enough *lac* repressor from the *lac*I^{q} gene to block transcription but expression is regulated less tightly than in strains containing the pREP4 plasmid. This is because in *E. coli* XL1-Blue, there is only one *lac*I^{q} gene to produce the *lac* repressor, while in strains containing the pREP4 plasmid, such as *E. coli* M15, there are many copies of pREP4 and therefore many copies of the

*lac*I gene to make *lac* repressor. Thus, strains with the pREP4 plasmid would have more *lac* repressor protein present to block transcription.

To induce the recombinant protein, IPTG is added and binds to the *lac* repressor protein and inactivates it. The *lac* repressor protein is inactivated because the binding of IPTG to the *lac* repressor protein changes the shape of the *lac* repressor protein so it can no longer bind to the DNA. The *lac* repressor protein is a tetramere with four identical components (Lewis *et al*., 1996). It has three domains: the NH_2 terminal domain that is involved in binding to the DNA, the coreprotein domain also known as the inducer binding domain, and the COOH-terminal domain (Lewis *et al*., 1996). IPTG binds to the *lac* repressor through the coreprotein domain by four hydrogen bonds in the hydroxyl group of IPTG (at positions: Asn246, with O2 of IPTG, Arg197 with O3 and O4, and Asp149 with O6) and by van der walls contact with the Trp220 amino acid with an isopropyl group of IPTG (Lewis *et al.*, 1996). In addition, a hydrophobic sugar pocket in the core protein is also produced upon IPTG binding. Once the *lac* repressor is inactivated, the host cell's RNA polymerase can transcribe the sequences downstream from the promoter; transcripts are produced and then translated into the recombinant protein (Jacob and Monod, 1961; The QIAexpressionist, technical manual 5th edition, 2001).

During this portion of the experiments, either IPTG or lipoprotein were used to induce the recombinant protein in pQE-60. In the case of induction with IPTG, IPTG inactivates the *lac* repressor protein by binding to it, the repressor is inactivated and transcription takes place (Jacob and Monod, 1961; Lewis *et al*., 1996). In the case of induction with lipoprotein, lipoprotein would not inactivate the *lac* repressor protein and

transcription would still remain blocked since the lipoprotein does not have the same structure as the IPTG coreprotein domain that binds to the *lac* repressor (Lewis *et al.*, 1996). Therefore, it was entirely flawed to try to induce the pQE-60 vector with lipoprotein.

Lipoprotein was incorrectly used to try to induce *ehl1* in pQE-60 while to trying to investigate whether the presence of lipoprotein would have an effect on Ehly1 expression since previous studies have shown that lipoprotein may inhibit enterohemolysin activity (Figueiredo *et al*., 2003). Since neonates have minimal levels of lipoprotein compared to adults (Behrman *et al*., 2003), the effect of lipoprotein on Ehly1 may have been a possible explanation as to why only some of the neonates and not the hospital support staff were affected in the Argentinean outbreak.

The effect of lipoprotein on Ehly1 was not examined in the pQE-60 vector since lipoprotein does not induce the T5 promoter in pQE-60. It would have been more accurate to investigate the effects of lipoprotein on Ehly1 expression during cloning with pRS551. pRS551 is a promoterless vector and *ehl1* and its upstream region, which may have a potential promoter region, could have been examined to see if lipoprotein would have an effect on the potential promoter (Figueiredo *et al*., 2003). While it was originally thought that lipoprotein might induce *ehl1* (and thus was used to induce the pQE-60 vector) it would have been more accurate to examine the effects of lipoprotein as a repressor, since neonates have minimal lipoprotein levels and are affected by Ehly1, and adults, with higher levels of lipoprotein than neonates, are not affected by Ehly1 (Figueiredo *et al*., 2003). In addition, lipoproteins, such as cholesterol, have been found to inhibit enterohemolysin activity (Figueiredo *et al*., 2003). Inducing the pRS551 vector

with lipoprotein would examine if lipoprotein negatively regulated *ehl1* and its presence would prevent transcription of *ehl1* by binding to an operator site and turning off the promoter. Unfortunately, the concept of induction of vectors was not completely understood at the time of the experimentation, thus leading to the induction of pQE-60 with lipoprotein, and not trying to test the effects of lipoprotein on *ehl1* and its upstream region in the promoterless vector pRS551.

In the presented research, induction of pQE-60 with lipoprotein had shown changes in net intensity readings for some genes (Table 4) as discussed in the results and discussion section. This could possibly be explained by errors such as: experimental protocol errors, detection of background signal, error in use of the software, or error in detecting signal in the chip reader (Ye *et al*., 2001). The experiment was performed only one time and thus, no statistics were applied to the data. In addition, it was overlooked how similar the data for net intensity readings were from induced Ehly1 and uninduced Ehly1. If the experiments had been repeated or statistics applied, there would have been no statistically significant change in net intensity readings since both inductions did not work. Inducing pQE-60 with lipoprotein was irrelevant since it does not bind to the *lac* repressor and thus transcription from the T5 promoter would not take place (Lewis *et al*., 1996), and inducing with IPTG would have shown no change since only a truncated Ehly1 protein would have been induced. Therefore, any data obtained from the induction of recombinant protein from pQE-60, including the microarray experiment, was flawed, as lipoprotein cannot induce recombinant Ehly1 in pQE-60 since it cannot bind to the *lac* repressor as it does not have the same structure as IPTG coredomain or lactose (Lewis *et al*., 1996).

Although, the recombinant Ehyl1 protein in pQE-60 was accurately induced by IPTG, the data was still affected by the previous error of having the incorrectly designed primer resulting in the recombinant protein being out of frame, which resulted in the truncated Ehly1 protein with no C-terminus His tag. Unfortunately, the lack of understanding of induction and how to induced pQE-60 was not completely understood until submitting the presented research. This is evident, in Figure 13, where an attempt at trying to confirm induction of the recombinant protein took place. It was originally claimed that recombinant Ehly1 in pQE-60 was induced by both IPTG and lipoprotein. However, one can clearly see that the protein does not appear to be changed from the uninduced protein. In addition, had this been noted at the time of experimentation, it would have been beneficial to confirm the plasmid through sequencing, which again would have shown a truncated Ehly1 protein with no C-terminus 6xHis tag.

The fourth and final major error to occur in the presented research did not occur during the experimentation portion of the research but in the discussion. This concept error took place while describing the mutations that occurred in *ehl1* sequenced from *E. coli* strain 01-1610. It was incorrectly stated that the mutation in the putative ribosome binding site, RBS, would cause a change of the amino acid Arginine to a stop codon. This conclusion was scientifically flawed since the RBS does not encode amino acids, and any mutation in the RBS would prevent the ribosome from binding to the mRNA and translation of mRNA would not take place.

The relationship between DNA, RNA, and protein is known as the central dogma of molecular biology (Watson *et al*., 1992). Single stranded DNA undergoes the process of transcription to produce complementary RNA, and the newly synthesized RNA serves

as the template for the order of amino acids within a growing protein through the process of translation (Watson *et al*., 1992).

In bacteria, specific nucleotide segments of DNA, known as promoters, are recognized by RNA polymerase to initiate transcription of DNA into RNA (Watson *et al*., 1992). The RNA polymerase recognizes a specific T or C in the promoter region known as the start point of transcription (Snyder and Champness, 1997). RNA polymerase recognizes a promoter by the different type of sigma factor that is attached to it (Snyder and Champness, 1997). In addition, a promoter sequences has two important consensus sequences: the –10 sequence which is a short AT-rich region about 10 bp upstream to the transcription start site, and the –35 sequence which is about 35 bp upstream to the transcription start site (Snyder and Champness, 1997). To begin transcription, RNA polymerase must bind both of these consensus sequences within the promoter (Snyder and Champness, 1997). After transcription initiation, a polymerization reaction takes place, which involves RNA polymerase moving 3' to 5' along the DNA to polymerize RNA in the 5' to 3' direction (Snyder and Champness, 1997). The process is complete when an antiparallel complementary copy known as a transcript of RNA has been made. Transcription continues until RNA polymerase reaches a termination site in the DNA. Termination is either Rho dependent where termination occurs due to the presence of transcription termination factor Rho, or Rho independent in which termination occurs due to the formation of a hairpin loop in the RNA (Snyder and Champness, 1997).

In bacteria, both the start codon, AUG, and the RBS are necessary for translation initiation (Shine and Delgarno, 1974). The mRNA sequence that contains the initiation codon and the RBS is known as the translational initiation region, TIR (Snyder and

Champness, 1997). The start codon may not be the first sequence in the mRNA. In addition, the RNA copy of the promoter may be a distance from the TIR and the start codon, in a region called the leader sequence or 5' untranslated region (Snyder and Champness, 1997). The RBS contains a purine rich region known as the Shine-Delgarno sequence, which is about 10 nucleotides upstream from the AUG start codon (Gualerzi and Pon, 1990). The 3' end of the 16S rRNA of 30S subunit of the ribosome is complementary to the Shine-Delgarno sequence and binds to it. This helps the 30S subunit of the ribosome bind to the mRNA and to properly align the ribosome relative to the start codon. After the ribosome is properly aligned relative to the start codon, an fmet-tRNA (coding for formylmethionine and starting the process of translation) base pairs to the mRNA codon AUG, and the large ribosomal subunit joins the complex ending the process of initiation (Gualerzi and Pon, 1990). After initiation, the elongation process occurs where elongation of the polypeptide chain takes place by addition of amino acids to the carboxy end of the peptide chain, and the growing protein exits the large subunit of the ribosome. The ribosome continues to translate the codons on the mRNA until it reaches one of three stop codons (UAA, UAG, and UAG). This causes the termination process of translation since the stop codons are not recognized by any tRNAs. Proteins called release factors recognize the stop codons and release the newly synthesized protein from the ribosome (Snyder and Champness, 1997).

It was entirely flawed to state that a mutation in the RBS would result in a change in the amino acid Arginine to a stop codon, since the RBS does not encode amino acids or stop codons. However, the RBS plays a major role in the initiation of protein translation. A mutation in the RBS would prevent translation initiation from taking place

and no protein would be translated. While this error did not affect any of the experiments or any data acquired from the experiments, it was important to address this major concept error as it was discussed incorrectly in the discussion portion of the presented research.

This appendix addressed the four main concept errors that occurred within the presented research, namely: screening systems for cloning, transcriptional and translational vectors, induction of the pQE-60 vector, and ribosomal binding site mutations. It was necessary to address these errors by identifying the flawed experiments and the impact on the data analyzed. In addition, it was important to clarify these topics through explaining how the research should have been properly carried out to ensure that those whom read the presented research could learn from these errors.