SHIGA TOXIN-PRODUCING BACTERIOPHAGE IN *ESCHERICHIA COLI* O157:H7

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ABSTRACT

Shiga toxin-producing *E. coli* (STEC) including *E. coli* O157:H7 are potential food and water borne zoonotic bacterial pathogens capable of causing outbreaks of severe illness in humans. The virulence of *E. coli* O157:H7 strains may be related to the type of Stx produced and several Stx2 variants have been identified which appear to differ in their ability to cause disease. Two lineages exist within O157 strains where lineage I is associated mainly with human and bovine isolates and lineage II is associated mainly with bovine isolates. The goal of this study was to identify and characterize a lineage II EC970520 Stx2c phage and determine if variations in the phage compared to Stx2 phage found within the lineage I strain, EDL933, can result in differences in virulence observed between the lineages. This study suggests: 1) that the lineage II strain EC970520 contains a highly heterogeneous Stx2c variant phage; 2) that location of integration of the phage within the genome of a bacterium may be important for host selection; 3) that EC970520 Stx2c phage genes are lineage II specific but only a subset of EDL933 phage genes are lineage I specific; 4) that differences in the stability of phages within bacteria contribute to the evolution of new pathogens; 5) that variation in phage genes can be used to detect different strains of *E. coli* O157:H7 and other STEC; and 6)that the type of phage may result in phenotypic differences between lineages and occurrence of human disease. Results of this study indicate that lineage II strains may be less virulent than lineage I strains due to specific genetic differences and the ability to release phage which is important to the evolution of new pathogenic strains.

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LIST OF ABBREVIATIONS

A/E	Attaching and Effacing
bp	base pair
CGH	Comparative Genomic Hybridization
CT	Cholera toxin
DAEC	Diffuse-adhering *Escherichia coli*
DNA	deoxyribonucleic acid
ds	double stranded
EAEC	Enteroaggregative *Escherichia coli*
EHEC	Enterohemorrhagic *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
ELISA	Enzyme-linked immunosorbent assay
EPEC	Enteropathogenic *Escherichia coli*
ETEC	Enterotoxigenic *Escherichia coli*
g	gram
h	hour
HC	haemorrhagic colitis
HUS	hemolytic uremic syndrome
kb	kilobase
KM	kanamycin
l	liter
LB	Luria-Beratani
LEE	Locus of enterocyte effacement

LPSA	Lineage-specific polymorphism assay
LI	lineage I
LII	lineage II
m	milli
M	Molar
min	minute
MLE	Multi-locus enzyme analysis
MLVA	Multi-locus variable tandem repeat analysis
n	nano
NCBI	National center for biotechnology information
NM	Non-Motile
OBGS	Octamer-based genome scanning
OD_{600}	Optical Density at 600nm
ORF	Open reading frame
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field gel electrophoresis
PT	Phage type
RAPD	Random amplification of polymorphic DNA
s	second
SD	standard deviation
SP	seropathotype
STEC	Shiga toxin producing *Escherichia coli*
Stx	Shiga toxin

TCP	Toxin co-regulated pili
TE	Tris/EDTA
TMAC	Tetramethyl ammonium chloride
U	Unit
UV	Ultra violet
VBNC	viable but non-culturable
VT	verocytotoxin
VTEC	verocytotoxin-producing *Escherichia coli*
w/v	weight per volume
µ	micro
ºC	degree celsius

BACTERIA REFERRED TO IN THIS STUDY

Citrobacter freundii

Enterobacter cloacae

Escherichia coli

Escherichia coli O157:H7

Group A *Streptococcus*

Salmonella

Shigella dysenteriae type I

Shigella flexneri

Staphylococcus aureus

Vibrio cholera

CHAPTER ONE

1. Shiga toxin-producing bacteriophage in *Escherichia coli* O157:H7

1.1. Introduction

Bacteriophage or "phage" are viruses capable of infecting bacterial cells. There are ~4500 different dsDNA phage described that are capable of infecting a variety of bacteria. Comparison of the genomes from these phage shows that a majority of them have a common ancestry. It seems that phage are capable of undergoing a constant exchange of genetic elements from a large shared pool and may create recombinant phage variants (Hendrix *et al.*, 1999).

Phage are capable of existing in two states: lysogenic and lytic. During lysogeny, bacteriophage DNA is inserted into the bacterial chromosome and in this inactive state the phage DNA is referred to as a prophage. Upon induction or spontaneous replication of the phage or bacterial virus, it will enter a lytic cycle resulting in production of new phage progeny using the host's ribosomes resulting in death of the host, and spread of the phage to new bacterial hosts. During packaging of the phage DNA into phage heads prior to release, DNA fragments of the host genome are sometimes packaged instead of the phage DNA resulting in phage-mediated gene transfer (Brussow *et al.*, 2004). Therefore, phage serve as vectors for dissemination of genes by horizontal transfer which can contribute to the evolution of new strains, as well as encode structural components associated with bacterial pathogenesis. The study of these recombinant phage in bacteria will aid in understanding the role phage play in the evolution of new pathogenic bacterial species.

Several emerging pathogenic bacterial strains have recently acquired virulence factors which are encoded by phage (Saunders *et al*., 2001). These virulence factors, such as bacterial toxins are associated with human illness (Miao and Miller, 1999). These phage-encoded virulence factors contribute to the fitness of the bacterial host and contribute to its pathogenic capabilities (Brussow *et al.*, 2004).

Typing of bacterial strains has allowed us to trace the sources of infection. This source information is invaluable in preventing the spread of infections in epidemics. Characterization of these phage in bacteria is important as it will aid in the identification of new pathogens as well as specific clones of well known pathogens responsible for epidemics. By studying phage, we will learn more about their evolution, and how these viruses contribute to the virulence of bacteria and allow us to develop methods to identify these markers of virulence in well known and newly emerging pathogens.

1.2. Roles of phage in bacterial evolution

Phage have played an important role in the emergence of pathogenic bacteria. Some bacteria may contain specific toxin-encoding phage and/or have multiple fitness factor-encoding prophage that coexist in the same bacterial host. This is referred to as polylysogeny. The configuration and gene content of these phages may account for variability in virulence and the emergence of strains responsible for increased disease severity and the number of hosts infected (Brussow *et al*., 2004). It is thought that most pathogenic bacterial species have emerged by acquisition of virulence factors by phage. A select few of these include *Vibrio cholerae*, *Staphylococcus aureus, Group* A *Streptococcus* (GAS), *Salmonella* and *Escherichia coli* O157:H7. All possess one or

more phage and appear to display varying levels of pathogenesis depending on their phage content (Table 1.2.1.).

1.2.1. Bacteriophage in *V. cholerae*

V. cholerae causes severe secretory diarrhea and is responsible for epidemic cholera. In *V. cholerae*, the toxins essential for infection of the bacteria into the host are encoded on phage or phage-like elements. Acquisition of these phage virulence genes appears to have been recent; *i.e.*, phage structure is still relatively intact and, GC content and codon usage is unique and different from that seen in the host chromosomal DNA (Sanchez and Holmgren, 2005). *V. cholerae* requires two main regulated factors for virulence; *i.e.*, cholerae toxin (CT) and a toxin-coregulated pilus (TCP) (Faraque and Mekalanos, 2003). CT is composed of a single toxic A subunit (CTA) which triggers rapid chloride and water efflux into the intestinal lumen causing watery diarrhea and a circular homopentamer B subunit (CTB) responsible for toxin binding to intestinal cells (Sanchez and Holmgren, 2005). The CT genes (*ctxAB)* are found on the phage CTXΦ which is capable of transferring genes between toxigenic and non-toxigenic strains of bacteria (Waldor and Mekalanos, 1996). CTXΦ uses TCP pili which promote bacterial interactions that allow *V. cholerae* to establish colonies on the intestinal epithelia. Interestingly, the TCP gene cluster is flanked by *att*-like attachment sequences carrying integrase and transposase genes which may have been initially involved in horizontal transfer of phage genes (Karaolis and Kaper, 1999). The ability of TCP gene products to

Table 1.2.1. Summary of important phage related toxins and co-virulence factors associated with select representative bacterial pathogens; *i.e., E. coli, V. cholerae, S. aureus*, *Group* A *strepotococci* (*GAS)* and *Salmonella*.
Virulent strains of *E. coli* and *V. cholerae* have specific toxins whereas *S. aureus*, *GAS* and *Salmonella* contain several toxins and co-virulence factors associated with virulence.

Phage-encoded proteins	***E. coli* O157:H7**	***V. cholerae***	***S. Aureus***	***GAS***	***Salmonella***
Toxins	Shiga toxin (Stx)	Cholera toxin (CT)	enterotoxin A	exotoxins SpeA,K,C,L, M	SopE
			cytotoxin leukocidins (lukS/lukF)	superantigen SSA	
			exfoliative toxin A (ETA)		
			enterotoxin P		
Co-virulence factors	Sp1-18, multigenic regions	Pili (TCP)	Satellite and helper phages for tst, enterotoxins B,C	DNAse Spa1	GIFSY-1
	SpLE1-6			phospholipase Sla	GIFSY-2
					Fels-1
					Fels-2

participate in DNA excision and transfer to other bacteria suggests that this element is phage-related although the evidence for this is disputed (Faraque and Mekalanos, 2005).

1.2.2. Bacteriophage in *S. aureus*

S. aureus causes a wide range of diseases in humans from food poisoning and skin infections to invasive diseases (Brussow, 2004). More than 250 staphylococcal phage have been described in the literature and many of the virulence factors associated with *S. Aureus* are coded for by a family of phage rather than one particular phage (Pantucek *et al.*, 2004). The enterotoxin A (food poisoning), exfoliative toxin A (scalded skin syndrome) and Panton-Valentine leukocidin (PVL) which encodes the cytotoxin leukocidins, lukS and lukF which assemble into pore-forming transmembrane complexes that can lyse human leukocytes are all carried on phage (Lindsay and Holden, 2004). Some strains are also known to carry prophage that encode enterotoxin P, a superantigen involved in the symptoms of food poisoning, and staphylokinases, which are suspected of being involved in proteolytic destruction of host tissues (Canchaya *et al.*, 2003). Phage-mediated transfer of pathogenicity islands by satellite and helper phage may also be responsible for the spread of the superantigen family of toxins which cause disease conditions such as toxic shock syndrome and encode entertoxins B and C (Lindsay and Holden, 2004; Brussow *et al.*, 2004).

1.2.3. Bacteriophage in *Group* A *Streptococcus* (GAS)

GAS are responsible for many different diseases but are frequently associated with invasive infections such as toxic shock syndrome, necrotizing fasciitis and may

result in death (Banks *et al.,* 2002). Genome sequencing of the *Group* A *Streptococcus* (GAS) pathogen serotypes M1, M3 and M18 has revealed that phage or phage-like elements account for the majority of variation in gene content seen in these bacteria and may play a vital role in the evolution and diversification of GAS strains; e.g., integrated bacteriophage make up as much as 12% of the genome in some pathogenic serotypes (Beres *et al.,* 2002). In M1 serotypes Spa1, which is from a family of DNAses, and the pyrogenic exotoxin SpeC are encoded adjacent to one another on the same prophage. Other prophage-encoded exotoxins, SpeL and SpeM found in M18, have been known to be lethal in rabbits (Brussow *et al.*, 2004). The highly virulent M3 serotype is associated with several phage-encoded genes that contribute to pathogenesis such as exotoxins SpeK, SpeA, the superantigen SSA, and phospholipase, Sla. These proteins found in M13 are not encoded in contemporary M1 strains of the bacteria and may have been lost from the genome by phage-mediated recombination which has resulted in excision of these genes (Beres *et al.*, 2002).

1.2.4. Bacteriophage in *Salmonella*

Salmonella, which encompass a variety of serovars, are bacterial pathogens responsible for bacterial gastroenteritis and may result in death. *Salmonella* strains have different combinations of phage which enhance the pathogen fitness; e.g., GIFSY-1 and GIFSY-2 are fully functional lambdoid prophages which are competent to undergo excision and viral assembly. These phage encode several factors thought to contribute to systemic infections such as superoxide dismutases which protect bacteria from oxygen radicals produced by host macrophages (Brussow *et al.*, 2004). Besides GIFSY, two

other P2-like prophage have been identified (Fels-1 and Fels-2) which contain the genes for putative fitness factors such as another superoxide dismutase and a neuraminidase whose role in virulence is not clearly defined (Brussow *et al.*, 2004). *Salmonella* host interactions are largely mediated by type III secretion systems which enable bacterial effector proteins to translocate into eukaryotic cells (Bakshi *et al.*, 2000). The SopE protein belongs to a class of toxins that are involved in type III-secreted Rho-GTPase function and is encoded by SopEΦ, another P2-like bacteriophage. Bacterial isolates found to carry SopEΦ are known to belong to epidemic strains (Hopkins and Threlfall, 2004). Spontaneous phage induction in *Salmonella* results in phage-mediated killing of sensitive bacteria and lysogenic conversion of survivors; this contributes to the diversity and evolution of these pathogenic strains (Bossi *et al.*, 2003).

1.2.5. Bacteriophage in *E. coli* O157:H7

E. coli O157:H7 is responsible for severe diarrhea which may result in hemorrhagic colitis, hemolytic uremic syndrome and death. Comparative genomic analysis of *E. coli* O157:H7 strains (EDL-933 and Sakai) to *E. coli* K-12 MG1655, a non-pathogenic laboratory strain of these bacteria, determined several regions in *E. coli* O157:H7 which possessed an atypical base composition suggesting that horizontal gene transfers had occurred relatively recently from a donor species of foreign origin (Perna *et al.,* 2001; Hayashi et al., 2001). Eighteen multigenic regions in EDL-933 were found to be related to bacteriophage (Perna *et al.*, 2001). In Sakai, 18 prophage (Sp1-18) and 6 prophage-like elements (SpLE 1-6) were present in the chromosome suggesting that bacteriophage have played a key role in generating diversity in *E. coli* (Ohnishi *et al.*,

2002). In particular, considerable diversity of the main virulence factors, the Shiga toxins (Stx, also known as Verocytotoxins or VTs) which are encoded by phage, exists among different isolates of the bacteria (Ohnishi *et al.*, 2002). These Stx phage are thought to contribute greatly to the diversity and pathogenicity found within the serotype and other *E. coli* strains and will be the focus of this review.

1.3. Stx bacteriophage

Shiga toxins (Stx) are a family of bacterial heterodimeric protein toxins that are involved in human and animal disease and have multiple damaging effects on eukaryotic cells. These toxins are produced in *Shigella dysenteriae* type I, *Citrobacter freundii*, *Enterobacter cloacae*, *Shigella flexneri* and *E. coli* (Herold *et al.*, 2004). In *E. coli*, strains that are capable of producing Stx are termed Shiga toxin-producing *E. coli* (STEC).

Stx's bind the glycolipid receptor globotriaosylceramide (Gb_3) in eukaryotic cells and function as rRNA-N-glycosidases to inhibit protein biosynthesis and in some cases, induce apoptosis (Lingwood, 1993). Stx's are composed of a single A subunit which possess N-glycosidase activity and can catalyze the release of an adenine from 28S rRNA preventing it from interacting with elongation factors EF1 and EF2, needed for protein synthesis. Five identical B subunits facilitate binding of Stx to the Gb_3 receptor found in eukaryotic membranes (Takeda *et al.*, 1993; Fraser *et al.*, 2004). Some STEC such as *E. coli* O157:H7 adhere to the enterocytes of the colon and cause effacement of the microvilli and diarrhea. If sufficient Stx is produced, damage to the blood vessels in the colon results in bloody diarrhea and release of toxins into the bloodstream of the host

(Gyles, 2007). This can lead to systemic illness such as hemolytic uremic syndrome (HUS) which is caused by thrombotic microvascular lesions in the renal glomeruli, the gastrointestinal tract and other organs, such as the brain, pancreas and lungs (Karmali, 2004).

There are two major types of antigenically distinct Stx termed Stx1 and Stx2. In *E. coli*, Stx1 is identical to the Stx found in *Sh. dysenteriae* and Stx2 has 55% identity to Stx1 at the amino acid level (Gamage *et al.*, 2004; Strauch *et al.,* 2001; Kozlov *et al.*, 1988). A STEC strain can produce either Stx1 or Stx2 or both toxins. The Stx's found in *E. coli* O157:H7 and other STEC strains are encoded by lysogenic bacteriophage (Yokoyama, *et al.*, 2000; Plunkett, *et al.*, 1999; Makino *et al.*, 1999). The complete genome sequences from at least seven Stx-converting phage in *E. coli* O157:H7 are available in GenBank. Sequence analysis has shown that *Stx* genes from these phage are uniformly flanked by phage sequences indicating a bacteriophage origin (Schmidt *et al.,* 2001). These phage are variable in both structure and gene composition (Herold *et al.*, 2004). Stx2- encoding phage include Φ933W which was isolated from EDL-933, prophage VT2-Sakai and Stx2Φ-I which were isolated from O157-Sakai strains, and ΦP297 which was obtained from an STEC strain isolated from a patient in Belgium with HUS (Plunkett *et al.*, 1999; Makino *et al.,* 1999; Sato *et al.*, 2003; De Greve *et al.*, 2002). A study of eight *E. coli* O157:H7 strains by Ohnishi *et al.* (2002) shows that only one of the Stx2 phage characterized was identical to that of O157-Sakai and that these phage share limited homology to each other and exhibit high structural and positional diversity indicating that various types of Stx phage exist within *E. coli* O157:H7 populations.

Studies of Stx2 have revealed the presence of a family of closely related Stx2 variants including Stx2, Stx2c, Stx2d, Stx2e and Stx2f, all of which appear to differ in their ability to cause severe disease (Recktenwald *et al.,* 2002; Pierard *et al.,* 1998; Strauch *et al.*, 2004; Schmidt *et al.*, 2000; Friedrich *et al.*, 2002). Several of these Stx variants appear to be widely distributed among STEC serotypes (e.g. Stx2) whereas others seem to be much more serotype restricted; e.g., Stx2e is only produced by *E. coli* serotypes associated with edema disease in pigs. These variants have been shown to have different physiochemical properties, stability, receptor affinity and LD50 in mice (Law, 2000; Karmali, 2004; Gyles, 2007; Melton-Celsa *et al.,* 2002). Therefore, virulence of *E. coli* O157 strains may be related to the type of Stx produced. Severe human disease associated with HUS has been linked to bacterial strains possessing Stx2 and Stx2c. STEC strains possessing Stx2d and Stx2e are more likely to be isolated from patients with an uncomplicated infection suggesting that Stx2d and Stx2e may be less toxic for humans than Stx2 and Stx2c (Friedrich *et al.*, 2002).

In contrast to the Stx2 family, Stx1 genes appear to be more homogeneous, with only one variant, Stx1c, isolated thus far, which is usually associated with asymptomatic or mild disease (Zhang *et al.*, 2002).

Despite the heterogeneity of Stx2 phage, most of these phage analyzed to date possess a typical lambdoid morphology with dsDNA and, regular or elongated hexagonal heads and non-contractile flexible tails (Herold *et al.*, 2004; Strauch *et al.*, 2004). The typical lambda genome structure which most Stx2 phage possess is seen in Figure 1.3.1. In Stx-containing phage, s*tx1* and s*tx2* both appear to be inserted downstream of the promoter, $p_{R'}$ and are regulated by the Q antiterminator protein (Herold *et al.*, 2004;

Plunkett, *et al.,* 1999; Yokoyama *et al*., 2000). Final assembly of Stx-phage and lytic release from a host cell are controlled by the *Q* gene which is situated near the start of the "Late Lytic Expression" region of the prophage genome (Fig. 1.3.1.). The Q protein acts by antiterminating rho-dependent and rho-independent terminators and stabilizing polymerases initiating from the p_R promoter which will ultimately allow transcription through the entire late gene operon (Calendar, 2006). Since late phage genes are only expressed during the lytic cycle, induction of the lytic cycle may trigger production of Stx in phage-infected cells.

Induction of phage excision and release can occur after exposure of lysogenized bacteria to DNA damaging agents such as UV light, mitomycin C, neutrophil activation products such as hydrogen peroxide or antibiotics which interfere with DNA synthesis; e.g., Stx production has been shown to increase after induction of phage-containing bacteria with ciprofloxacin (Malone *et al*., 2007; Muhldorfer *et al.,* 1996; Wagner *et al.*, 2001; Zhang *et al.*, 2000; Walterspiel *et al.,* 1992). Ciprofloxacin is a quinolone antibiotic which is known to induce the SOS response in bacterial cells by trapping DNA gyrase on DNA and blocking movement of the replication fork (Drlica and Zhao, 1997). In the SOS pathway of *E. coli*, an activated RecA protein promotes autoproteolysis of the DNA repair repressor, LexA, as well as the phage repressor, cI, resulting in derepression of late phage lytic genes (Goerke *et al.*, 2006; Smith and Walker, 1998) (Figure 1.3.1.). Treatment of *Stx* containing bacteria with ciprofloxacin resulted in enhanced Stx production as well as Stx phage release (Herold *et al.*, 2004; Schmidt *et al.*, 2001; Goerke *et al.*, 2006). Children and adults infected with *E. coli* O157:H7 show increased risk of HUS and death when they are prescribed antibiotics at the early stages of infection, since

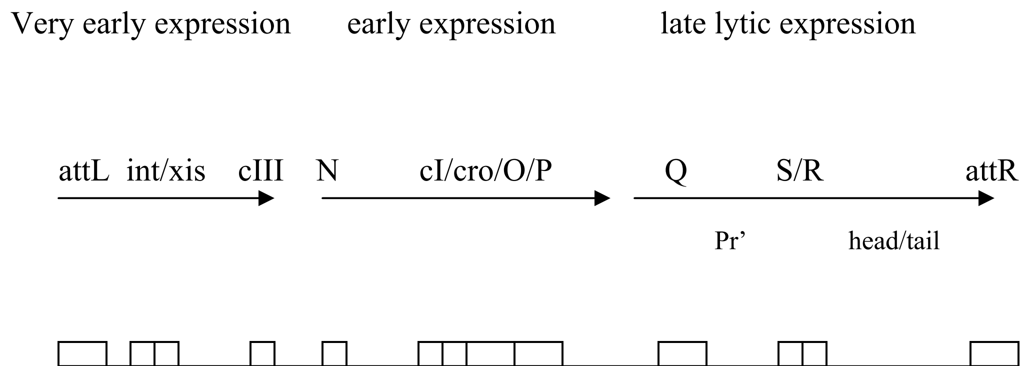


Figure 1.3.1. Structure of typical bacteriophage λ prophage.
Left and right attenuation sites (AttL/R) flank prophage regions and are used in site specific recombination. (Int) and (xis) are for integration and excision of the prophage. The (cIII) and (N) are recombination genes. The (cI) and (cro) are early expression regulators. During the SOS response, the (cI) is cleaved by RecA resulting in derepression of late lytic gene expression. (O) and (P) are replication genes. The (Q) gene is an antiterminator which initiates transcription from the promoter Pr' and late gene expression. The (S) and (R) are lysis proteins which lie upstream of the main head and tail genes (Calendar, 2006).

phage induction may lead to an increase in the amount of Stx toxin released (Herold *et al.*, 2004). Not all STEC strains produce the same amount of phage after induction and the diversity found in phage may be related to variations in virulence. It has been suggested that greater levels of phage release could result in more toxin production which in turn could produce more damage to the host although more evidence is needed to support this (Muniesa *et al.*, 2003).

1.4. Clinical disease associated with STEC and *E. coli* O157:H7

There are over 200 serotypes of STEC but *E. coli* O157:H7 is the most common serotype isolated in North America. Only a limited number of STEC serotypes are associated with clinical disease in humans and illness associated with other pathogenic STEC tends to be less common, sporadic in nature, and less severe than that associated with *E. coli* O157:H7 (Boyce *et al.,* 1995; Johnson *et al.,* 1996; Besser *et al.,* 1999; Brooks *et al.,* 2005). *E. coli* O157:H7 (and strains of a few other STEC serotypes) are responsible for most outbreaks of hemorrhagic colitis (HC) which can result in hemolytic uremic syndrome (HUS) or death (Karmali *et al.*, 1983; Obrig *et al.,* 1998; Ochoa and Cleary, 2003). Illness occurs after a 3 - 4 day incubation period during which the bacteria colonize the bowel resulting in non-bloody diarrhea and abdominal cramps. Infected individuals may develop HC which is characterized by severe abdominal cramps, watery and bloody diarrhea with little or no fever (Wells *et al.*, 1983). In 5-10% of the children who are infected, HUS develops and leads to acute renal failure, thrombocytopenia and microangiohemolytic anemia and, may even result in death (Besser *et al.,* 1999, Boerlin *et al.*, 1999). Severe neurological impairment of the central nervous system resulting in

seizures, coma, stroke and/or cerebral edema occurs in 20-30% of patients with HUS (Pickering *et al.*, 1994; Siegler, 1994). Many individuals with *E. coli* O157:H7 infections develop intestinal symptoms but historically, only 6% of patients in U.S. outbreaks, mainly infants and young children, develop life threatening HC or HUS while 1% of these cases result in death (Besser *et al.*, 1999). However, comparison of the incidence of hospitalization and death caused by *E. coli* O157:H7 infections from 1982 to 2002 with the incidence of these parameters during the past five years suggests that new, more virulent strains of *E. coli* O157:H7 are emerging as a serious threat to human health (Manning *et al*., 2008).

1.5. Serotypes/pathogroups/seropathotypes

E. coli strains have been classified by several methods over the last century. When referring to the serotype of *E. coli* strains, the O refers to the somatic antigen and H to the flagellar antigen. There are 174 O antigens and 53 H flagellar antigens and several non-motile (NM) strains (Gyles, 2007). *E. coli* can be commensal organisms but some strains are pathogenic, capable of causing disease including diarrhea. Diarrheagenic *E. coli* are categorized into groups based on their virulence properties, clinical symptoms and host range (Table 1.5.1.). These categories or pathogroups include enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), diffuse-adhering *E. coli* (DAEC) and enterohemorrhagic *E. coli* (EHEC) (Orskov and Orskov, 1984; Meng *et al.,* 2001). EHEC strains including *E. coli* O157:H7 produce Stx, attaching and effacing (A/E) lesions and have a characteristic large plasmid (Plunkett *et al.*, 1999). However, not all

Table 1.5.1. Pathogroups of *E. coli*.
Categories are based on virulence properties and clinical symptoms (Parry and Palmer, 2002).

Pathogroup	Abbreviation	Virulence property	Clinical symptoms
Enteropathogenic	EPEC	Bundle forming pili and a type III secretion encoded enterocyte attaching and effacing activity	acute diarrhea in children
Enterotoxigenic	ETEC	heat stable or heat labile toxins	acute watery diarrhea in infants, calves, lambs and piglets
Enteroinvasive	EIEC	cell invasion	acute dysentery
Enteroaggregative	EAEC	aggregative adherence	Persistent diarrhea
Diffuse-adhering	DAEC	fimbrial adhesion	Persistent diarrhea
Enterohemorrhagic	EHEC	A type III secretion encoded enterocyte attaching and effacing activity as in EPEC, shiga toxin production	bloody diarrhea, HC, HUS

E. coli that produce Stx's fit the strict definition of EHEC, therefore these organisms have also been termed Shiga toxin-producing *E. coli* (STEC) or verocytotoxin-producing *E. coli* (VTEC). STEC first were recognized after the description of a cytotoxin that was produced by *E. coli* strains associated diarrhea in infants and swine. This toxin was referred to as vero toxins because it killed Vero cells (Konowalchuk *et al.*, 1977). O'Brien *et al.* (1983) also described an *E. coli* toxin that had many characteristics in common with that produced by *Sh. dysenteria* type 1 and termed the cytotoxin a "Shiga-like" toxin. It was subsequently discovered that these toxins were the same and since then both terms have been used.

A classification system of STEC groups has also been developed based on reported occurrence of certain serotypes in human disease outbreaks and HUS (Karmali *et al*., 2003). Within this model STEC strains were grouped into five seropathotypes A to E. Seropathotype A (SPA) contains strains of STEC O157:H7 and O157:NM that are considered to be highly virulent. These bacteria are associated with a high frequency of disease in humans and are commonly isolated from outbreak situations. Seropathotype B (SPB) contains bacterial serotypes that are similar to O157 but, are only moderately virulent to humans and are not commonly isolated under outbreak conditions. Both SPA and SPB have been associated with HUS and HC. Seropathotype C (SPC) is associated with sporadic incidence of HUS and HC, but only rarely infects humans and is not typically isolated under outbreak situations. Seropathotypes D (SPD) and E (SPE) are associated with diarrhea and include animal strains normally not implicated in human disease. This system was designed to identify those STEC isolated from food or the environment that pose a significant risk of disease in humans.

1.6. Pathogenesis of STEC and *E. coli* O157:H7

Virulence factors contribute to the ability of a microorganism to cause disease. The main virulence factors associated with STEC are the Stx's which target the intestinal epithelial barrier, enter the bloodstream and damage vascular cells of the colon, the kidneys and the central nervous system (Brussow *et al.*, 2004). No single factor is responsible for the virulence of STEC and several virulence factors are thought to contribute to the pathogenesis of STEC, in particular, *E. coli* O157:H7 (Table 1.6.1.). Both EPEC and STEC strains including *E. coli* O157:H7 are able to colonize the intestine by type III secretion system-mediated injection of a receptor, the translocated intimin receptor (Tir), into epithelial cells in the intestine and attach to Tir using an adhesin called intimin which is encoded by the *eae* gene (Chen and Frankel, 2005). These virulence genes are located in a 45 kb pathogenicity island termed the locus of enterocyte effacement (LEE) (Mcdaniel *et al.,* 1995). In addition to encoding proteins which mediate adhesion, LEE encodes proteins (e.g., EspF, EspG, EspH, EspZ) which alter the structure of the cell and its microvilli and change the cell's physiology (Gyles, 2007). Areas of bacterial attachment are characterized by effacement of microvilli and the accumulation of cytoskeletal actin under the bacteria, destroying the microvilli and allowing delivery of toxins to the cell surface (Gyles, 2007; Kaper *et al.*, 1998).

When *E. coli* O157:H7 and other STEC serotypes are considered, Boerlin *et al.* (1999) reported that the combination of *eae* and *Stx*2 genes has been more frequently isolated from *E. coli* serotypes associated with human disease. Similarly, a 60 MDA plasmid in EHEC is also more common in STEC associated with severe clinical disease in humans. This has led some researchers to conclude that factors encoded by this large

Table 1.6.1. Putative virulence factors in *E. coli* O157:H7.
STEC acid tolerance systems not listed here.

Virulence factor	Gene	Location	Size (~)	Putative function
Shiga toxins	stx1, stx2	Stx prophage	60kb (variable)	secreted toxins which target endothelial cells in the intestine
Intimin	eae	LEE island	45kb	A/E lesion
translocated intimin receptor	tir	LEE island	45kb	adherence
effector proteins	EspF, EspG, EspH, EspZ	LEE island	45kb	adherence
type III secretion system	several genes	LEE island	45kb	translocation of proteins
enterohemolysin	ehxA	plasmid	60 MDA	May cause mucosal Hemorrhage
serine protease	espP	plasmid	60 MDA	May cause mucosal Hemorrhage
catalase peroxidase	katP	plasmid	60 MDA	interacts with hemolysin

plasmid such as an enterohemolysin (*ehx*A) and, a serine protease (*esp*P) may contribute to disease associated with EHEC (Schmidt *et al.*, 1995). The catalase peroxidase encoded by *kat*P also found on the same plasmid is present in all enteric *E. coli* pathotypes and, may act together with the hemolysin in *E. coli* O157:H7 (Brunder *et al.*, 1996). However, the association of these factors with more virulent STEC strains does not necessarily demonstrate cause and effect. These genes could simply be markers of specific pathogenic lineages or clades and may not play a direct role in illness associated with these pathogens.

E. coli O157:H7 has three identified acid resistance systems; *i.e*., an acid-induced oxidative system, an acid-induced arginine-dependent system, and a glutamate-dependent system. These systems allow the bacteria to adapt to and survive in highly acidic environments (Meng and Doyle, 1996). This acid tolerance is important to STEC pathogen virulence as it allows bacterial survival in mildly acidic foods and, protects it in the stomach, allowing for a low infectious dose of the pathogen to colonize the intestinal tract (Leyer *et al.*, 1995).

1.7. Epidemiology

1.7.1. Incidences of STEC and *E. coli* O157:H7

In Canada, the National Laboratory of Enteric Pathogens (NLEP), Laboratory Center for Disease Control and Health Canada have been collecting information on STEC incidence since the 1970's; they have defined an outbreak as two or more linked cases (Spika *et. al*, 1996). In 1993-1995, *E. coli* O157:H7 was the most commonly isolated STEC identified by the NLEP, with 93% isolates confirmed, followed by serotypes 055

(1.1%), 0125 (0.8%), 026 (0.6%), 0126 (0.6%), 0128 (0.6%), 018 (0.5%) and other STEC strains (2.8%). In Canada, the incidence of *E. coli* O157:H7 in the population from 1990 to 1999 ranged from 4.1 to 7.1 / 100,000 with an average of 1407 cases reported annually (Alberta Health and Wellness, February, 2008). However, levels of incidence can vary geographically, with those in southern Alberta reaching levels as high as 5.6 – 10.9 / 100,000. However, it should be taken into consideration that frequency of serotypes in these reports are likely biased toward O157 due to routine use of *E. coli* O157:H7 selective isolation methods in clinical laboratories. It is clear that there is need to develop strategies to isolate and accurately identify strains with other STEC serotypes.

1.7.2. Outbreaks of *E. coli* O157:H7

E. coli O157:H7 was first investigated after multiple outbreaks occurred in Oregon and Michigan in 1982, after consumption of contaminated hamburgers (Wells *et al.*, 1983; Riley, *et. al.*, 1983). The *E. coli* O157:H7 strain EDL-933 was isolated from a Michigan ground beef outbreak and later the entire genome sequence of this pathogen was determined (Perna *et al.*, 2001). In Japan, several large outbreaks of *E. coli* O157:H7 occurred between May and December, 1996. One of the largest outbreaks occurred in Sakai City, Osaka, Japan in July 1996 where 6000 individuals exhibited gastrointestinal illness, and 1000 people were hospitalized. Among the hospitalized there were 100 cases of HUS and 3 deaths. Many of the victims were school children and the source of the infection was believed to have been white radish sprouts served at a school cafeteria (Michino *et al.*, 1999; Watanabe, 1996). The Sakai strain of *E. coli* O157:H7 isolated

during this outbreak, like EDL933 has been sequenced and is now commonly used as a reference strain (Hayashi *et al.*, 2001; Yokoyama *et al.*, 2000; Makino *et al.*, 1999).

1.7.3. Sources of disease

STEC strains are found mainly in the gastrointestinal tracts of cattle, sheep and goats but have also been found in swine, cats and dogs and can be shed and transmitted to humans via contaminated food, direct contact with animals or person to person spread in human populations (Koch *et al.*, 2001; Beutin *et al.*, 1995; Smith *et al.*, 1996). The most common form of contamination in North America of STEC is in ground beef, but outbreaks have also been linked to improper cooking of meats such as roast beef, salami and venison. Most contamination of meats occurs during slaughter and meat processing (Griffin, 1996). Rural areas in Canada are at particular risk especially in areas where intensive animal agriculture occurs in close proximity to human populations and direct contact of humans with cattle populations or exposure to contaminated surface and groundwater is of concern (Gannon *et al*., 2004; Hyland *et al*., 2003; Spika *et al.*, 1996). STEC can also be spread to vegetables, fruits and other plants through irrigation water or soil contaminated with fecal material (Meng and Doyle, 1996; Besser *et al.*, 1999). In Canada 70.9% of cases originated within a family setting (Spika *et al.*, 1996). However, outbreaks also were found to occur elsewhere; *i.e*., within the community (9.2%), in nursing homes (7.6%), restaurants and at catered events (4.4%), in other institutions (2.5%), day car centers (2.2%), and at other places (3.2%). These data suggest that illness may be attributable to common sources of infection such as foods or water, that

some illness may be associated with food which is not properly cleaned or cooked and that secondary person to person spread also plays a role in transmission of the organism.

1.8. Evolution of STEC and *E. coli* O157:H7

1.8.1. Origin of Stx

The Stx genes found in STEC and other enteropathogenic bacteria are similar immunologically and biochemically to those of pathogen *Sh. dysenteriae* type 1 (Kozlov *et al*., 1988). The Stx in *Sh. dysenteria* are considered to be chromosomally encoded rather than encoded by a lambdoid bacteriophage as is seen in most STEC strains (Herold *et al.*, 2004; Paton *et al.*, 1993). Genetic studies suggest that *E. coli* O157:H7 may have evolved from an EPEC that acquired the Stx gene by phage-mediated gene transfer or conversion in which a fully functional mobile phage infected a Stx-containing *Shigella* strain and horizontally transferred the *Stx* gene to an EPEC strain (Besser *et al.*, 1999). Evidence for continuous evolution of bacteria such as *Shigella* and *E. coli* O157 through phage infection and horizontal gene transfer has been reported and it has been suggested that lysogenic phages are responsible for picking up chromosomal genes such as *Stx* and transferring them horizontally to other non-pathogenic bacteria (Greco *et al.,* 2004). The *Stx* genes are incorporated into the genomes of these bacteria through lysogenic conversion and are responsible for converting them into more pathogenic forms. The Stx phage have been known to convert strains of *E. coli* that do not produce Stx's to Stx-producers; e.g., multiple phage infections have been shown to lead to the coexistence of three *Stx* genes in the same bacteria (Furst *et al.,* 2000). Mobile phage containing *Stx*

genes may be major vehicles for their dissemination and, could lead to emergence of new STEC strains.

1.8.2. Evolutionary groups

Investigations into *E. coli* O157:H7 evolutionary relationships based on multi-locus enzyme analysis and sequence typing have identified four clonal groups of *E. coli* in which Stx-producing strains are concentrated; *i.e*., EHEC1, EHEC2, STEC1 and STEC2 (Whittam, 1996). In addition to *E. coli* O157:H7, the EHEC1 grouping also contained non-motile *E. coli* as well as *E. coli* 055:H7. Whittam *et al*. (1996) found that both *E. coli* 055:H7 and *E. coli* O157:H7 were able to produce A/E lesions in human tissue culture cells. These strains were genetically similar and may be closely related (Wick *et al*., 2005; Whittam *et al.*, 1993). Furthermore, *E. coli* O157:H7 is not closely related to other clones of the O157 serotype, suggesting that *E. coli* O157:H7 is not recently derived from other strains of the O157 serogroup (Whittam *et al.*, 1988). Rather, Wick *et al*. (2005) have shown that many of the virulence genes found in *E. coli* O157:H7 are already present in O55:H7. They suggest that a >100 kb region around the O-antigen gene cluster was transferred in its entirety from a nonpathogenic *E. coli* O157 strain to an O55:H7-like progenitor to create the pathogen *E. coli* O157:H7. A stepwise process of evolution of the pathogen *E. coli* O157:H7 has been proposed (Whittam *et al.*, 1993; Wick *et al.*, 2005); 1) evolution of an ancestral *E. coli* which contained chromosomal gene sequences that produced A/E lesions (*i.e*., EPEC); 2) acquisition of virulence factors such as *stx* and plasmid-encoded hemolysins by horizontal transfer into an *E. coli* 055:H7-like progenitor which already possesses A/E lesion forming capabilities. The

modern human pathogen *E. coli* O157:H7 is unique among other *E. coli* serotypes in that it does not ferment sorbitol or produce β-glucuronidase. However, some *E. coli* O157:H7 (NM) strains have been described that ferment sorbitol and have β-glucuronidase activity. It is felt that these features and loss of motility in these *E. coli* O157 strains are characteristic of intermediate types that likely diverged from an ancestral form of the now dominant pathogenic clone of *E. coli* O157:H7 at various points along its evolutionary pathway (Kim *et al.*, 2001).

1.8.3. Comparative genomics

Comparative genomic analysis of *E. coli* K-12 MG1655, a non-pathogenic laboratory strain of these bacteria with two known isolates of pathogenic *E. coli* O157:H7, (Sakai and EDL-933) revealed that each of these strains possesses a common 4.1Mb backbone of highly conserved chromosomal DNA (> 98 % nucleotide identity in a comparison of orthologous genes), suggesting that these bacteria all share a common ancestor (Perna *et al.*, 2001; Hayashi, 2001). Most differences in gene content can be attributed to horizontal transfer of large islands of DNA which are interspersed within the chromosomal DNA and, are referred to as "K-islands" in *E. coli* K-12 and either "S-loops" or "O-islands" depending on whether they were identified in pathogenic isolates of either *E. coli* O157 Sakai or EDL-933 respectively. Strain-specific sequences totaled 0.5 MB for *E. coli* K12 (K-loops) and 1.4 MB for O157 Sakai (S-loops) in which the S-loops consisted mainly of virulence related genes (Ohnishi *et al.*, 2002). Similarly, comparative analysis of *E. coli* O157 strain EDL-933 with *E. coli* K12 revealed strain-specific sequences totaling 0.53 mB for K12 (K-Islands) and 1.34 mB for EDL-933 (O-

Islands) (Perna *et al.*, 2001). These O-Islands include nine large regions (>15 kb), also termed pathogenicity islands (Table 1.8.1.). Smaller O-Islands that were found are suspected to be involved in fimbrial biosynthesis, iron uptake and utilization gene clusters, and coding regions for suspect non-fimbrial adhesions (Perna *et al.*, 2001).

Use of genome-based oligonucleotide microarrays has allowed comparison of the sequenced reference strains of *E. coli* O157 (Sakai and EDL-933) with other *E. coli* O157 strains. These studies have shown that there is a high level of genome diversity among different *E. coli* O157 strains (Ohnishi *et al.*, 2002; Ogura *et al*., 2006; Ogura *et al*., 2007); e.g., Ogura et al. (2006) identified more than 400 "variably absent or present" genes in different isolates of *E. coli* O157, many of which were virulence related. Zhang *et al*. (2007) extended these observations to identify 1,751 "variably absent or present" open reading frames among 31 different *E. coli* O157 strains belonging to different phage types (PT) and bacterial variants which were identified using the lineage-specific polymorphism assay (LSPA) (Kim *et al*., 1999). This approach resulted in identification of 15 lineage I strains in which 11 different genomic regions were present in most isolates and, 12 lineage II strains in which these genomic regions were mostly absent. Most "variably absent or present" regions of the genome were associated with S-loops and O-islands from *E. coli* O157 Sakai and EDL933. In other studies Steele *et al*. (2007) were able to identify 12 conserved regions of genomic difference that were present in most lineage I bacteria and, absent in most lineage II bacteria. Eleven of these conserved regions were associated with S-loops from *E. coli* O157 Sakai and the corresponding O-islands from *E. coli* O157 EDL933. One conserved region was located on the pO157 virulence plasmid from *E. coli* O157.

Table 1.8.1. Strain specific O-Islands >15 kb present in EDL-933 (Perna *et. al*, 2001).

O-Island >15kb	**Putative virulence factors**
OI#7	Macrophage toxin, ClpB-like toxin
OI#28	RTX-toxin-like exoprotein and transport system
OI#43, OI#48	2 urease gene clusters
OI#47	Adhesion and polyketide or fatty-acid biosystem system
OI#115	Type III secretion system
OI#122	2 toxins, PagC-like virulence factor
OI#138	Fatty acid biosynthesis system
OI#148	Locus of enterocyte effacement (LEE)

1.8.4. Lineages

Octamer based genome scanning (OBGS) is a high resolution genotyping method which has been used to identify genome variation in populations of *E. coli* O157:H7. It is based on the use of strand-biased octamers to amplify regions of the bacterial genome. Through this analysis, two major lineages of *E. coli* O157:H7 have been identified. An examination of the data from strains of these lineages showed that they were non-randomly distributed among host source. Lineage I strains were isolated from both humans and cattle; however, lineage II strains were commonly isolated from cattle but were rarely isolated from humans. These data suggest that lineage I may be more virulent for humans or may be more efficiently transmitted to humans from bovine sources than lineage II strains (Kim *et al.*, 1999). The lineage-specific polymorphism assays (LPSA) using six lineage-specific markers based on the OBGS were introduced to simplify strain lineage typing. Based on LSPA-6 typing 91% of the strains are represented by five genotypes, suggesting that these subtypes are widespread (Yang *et al.*, 2004). Both OBGS and LPSA-6 data suggest that divergence of lineages was probably an ancestral event that preceded geographic dissemination (Kim *et al.,* 2001). It is likely that phage-mediated events contributed to the divergence of these *E. coli* lineages (Kim *et al.*, 1999).

1.9. Typing of STEC and *E. coli* O157:H7

1.9.1. Detection/ Identification of STEC and *E. coli* O157:H7

The majority of O157 strains are unable to ferment sorbitol rapidly, lack β-glucuronidase production and are unable to grow at temperatures >45˚C (Besser *et al.*,

1999; Doyle and Schoeni, 1984). The most widely used isolation methods for *E. coli* O157:H7 are culture based detection methods such as MacConkey-sorbitol medium (SMAC) and SMAC with tellurite and cefixime (CT-SMAC) for increased selectivity followed by slide agglutination tests and immunomagnetic separation with O157 specific antibodies attached to paramagnetic beads (Strockbine *et al.,* 1996). Isolates for *E. coli* O157 isolates can be screened on 4-methylumbelliferyl-β-D-glucuronidase (MUG) in which a fluorescent product is detectable under UV light and by using PCR which is also valuable for identifying non-O157 STEC isolates (Strockbine *et al.*, 1996). Cytotoxic assays and Enzyme-linked immunosorbent assays (ELISA) have also been developed to confirm STEC strains (Lopez *et al.*, 1996).

1.9.2. Typing Methods

The enormous diversity found between STEC and within *E. coli* O157:H7 has lead to the need to develop methods to identify and characterize these pathogens. Subtyping or fingerprinting of pathogens is important for identification of pathogen isolates, tracing sources of infections and identifying outbreaks for the prevention of disease. Subtyping is a standardized method in which there is a consensus criteria of reading and interpreting results, whereas fingerprinting involves comparison of isolates obtained by a single lab and is not always reproducible in all labs.

Several fingerprinting methods such as plasmid profile analysis, antimicrobial susceptibility testing, and ribotyping have been evaluated as typing methods for *E. coli* O157:H7 but, are not considered discriminating enough to generate epidemiological meaningful data (Grif *et al.*, 1998; Strockbine *et al.*, 1996). Phage typing (PT), is a

standardized typing method for which 88 PT's have been described. In this method lysis patterns for a panel of lytic phages are used in subtyping *E. coli* O157:H7 strains. Unfortunately, many epidemiologically unrelated strains may belong to the same PT. Therefore, this typing method is best used as a first-line subtyping method especially if there is a rare phage-type (Khakhria *et al.*, 1990; Barrett *et al.*, 1994). Multi-locus enzyme analysis (MLE), Random Amplification of Polymorphic DNA (RAPD) and Multi-Locus variable-number tandem repeat analysis (MLVA) also provide evidence for characterization of isolates but, like PT, varying degrees of sensitivity have been noted among methods (Grif *et al.*, 1998). PFGE is currently the "gold standard" in *E. coli* O157:H7 typing since it allows discrimination between O157:H7 isolates from different geographic regions in specific outbreaks. Barett *et al.* (1994) found that 25 out of 74 *E. coli* O157:H7 isolates in a Washington outbreak and, 10 out of 27 isolates from other states associated with the same outbreak had the same PT, yet only one isolate had the same PFGE pattern indicating that PFGE may be more sensitive but, should still be used in conjunction with other molecular subtyping methods for reliable typing. PFGE is also not consistent between laboratories and requires complex computer-dependent analysis. Genotyping microarrays have advantages over PFGE and ribotyping because they can discriminate between specific genes of interest and have a higher resolution than MLE (Borucki *et al.*, 2004). Microarray-based comparative genomic hybridizations has been developed to identify genomic differences within *E. coli* O157 lineages and support phylogeny evidence of other methods such as PT and LSPA (Zhang *et al.*, 2007). However, experimental procedures and analysis of strain data from planar microarrays is time-consuming, expensive and laborious, so new methodologies need to be developed.

Multiplex PCR has been widely used to provide fast, accurate results for identifying STEC strains, as well as variants of *E. coli* O157:H7 (Nakao *et al.,* 2002; Bastian *et al.*, 1998). Gannon *et al*. (1997) developed a multiplex PCR which targets the genes coding for Stx (*stx1* and *stx2*), intimin (*eae*) and flagellar (*flic*C) proteins of *E. coli* O157:H7; this method can be used to supplement other molecular fingerprinting methods and is a valuable tool for use in epidemiological investigations and clinical diagnosis.

1.9.3. Suspension arrays for detection and identification of strains

Nucleic acid suspension microarrays used with the Luminex (Luminex, TX) and Bioplex (BioRad, CA) microbead technologies can be used for rapid detection of multiple genes, allowing up to 100 different reactions in a single well. These systems use nucleic acid or protein-coupled microbeads, each with its own fluorescent spectral address in conjunction with a reporter dye which are activated by lasers to produce a signal which is detected by a high-speed digital photomultiplier. Several assays for nucleic acids have been developed with this technology. Most detect DNA by direct DNA to DNA hybridization to bead-coupled probes; e.g., single base chain extension, allele specific primer extension and ligation end-labeling (Dunbar and Jacobson, 2007; Dunbar, 2006). Direct DNA hybridization, often using several DNA oligonucleotides in the same multiplex reaction mixture, can be used simultaneously to detect multiple genes amplified in an unknown sample. This approach can be used for, genotyping as well as for virus and bacterial gene detection. Colinas *et al.* (2000) developed a Luminex-based

multiplex genotyping scheme that uses allele-specific beads to distinguish between mutant and wild-type β-globin genes from newborn, blood spot DNA. Multiplex assays for viruses such as HIV, Hepatitis C and Herpes Simplex virus also have been developed for detection of multiple infections in susceptible patients (Smith *et al.*, 1998). Asymmetric PCR is another useful way to identify multiple infections by pathogens and has been expanded for genotyping strains of animal pestivirus (Deregt *et al.*, 2006). Spiro *et al.* (2000) used multiplex hybridization of 16S/23S intergenic spacer region DNA to develop probes to detect and quantify microorganisms found in ground water at a nuclear waste site. Serovar-specific probes for the food-borne pathogen, *Listeria monocytogenes* were developed using oligonucleotide dendrimers to detect up to 98% of known strains and classify four serogroups (Borucki *et al.,* 2005). *L. monocytogenes* along with three common bacterial pathogens, *E. coli, C. jejuni* and *Salmonella* were detected simultaneously using organism-specific 23S ribosomal RNA gene (rrl) coupled probes (Dunbar *et al.*, 2003). These studies suggest that the suspension array technology can be useful in detection, typing, and genotypic analysis of *E. coli* O157:H7.

1.10. Thesis Goals and Objectives

Stx2 production is thought to be one of the most important virulence attributes of *E. coli* O157:H7. Characterization of the Stx2 phage found in *E. coli* O157:H7 strains will not only be important for detecting genetic variations within this serotype but will also help us to identify an important phenotypic trait associated with virulent *E. coli* O157:H7 strains.

This study will help us to detect and identify factors that may be contributing to the evolution and virulence of *E. coli* O157 and other STEC strains. The main thesis goals and specific objectives are:

I) Contribute to efforts of the laboratory team in identification and characterization of a Stx2c-encoding bacteriophage from an *E. coli* O157:H7 lineage II strain

1) Help clone and determine the nucleotide sequence of a Stx2c phage
2) Help annotate the genes in the Stx2c phage and determine the integration site of the phage in the chromosome
3) Determine if regions of this Stx2c phage are lineage-specific
4) Determine if the integration sites for Stx2c and Stx2 are lineage-specific

II) Detection of Stx2-encoding bacteriophage in lineage I and II strains

1) Develop methods for specific detection of genes found within *E. coli* O157:H7 and other STEC
2) Assess the variation in lineage-specific genes found within *E. coli* O157:H7 containing Stx2 and Stx2c in different isolates of the bacteria
3) Assess the variation in lineage-specific genes found within seropathotypes of STEC which contain Stx2 and Stx2c

III) Examine phenotypic differences between Stx2-encoding bacteriophage from *E. coli* O157:H7 lineage I and II strains.

1) Determine if Stx2 phage is released in a lineage-specific manner after exposure to specific chemical and physical inducers
2) Determine if relative toxin release following induction by strains is lineage-specific

CHAPTER TWO

2. Characterization of a Shiga toxin 2c-encoding bacteriophage from an *E. coli* O157:H7 lineage II strain

2.1. Introduction

Escherichia coli (*E. coli*) O157:H7 is a food- and waterborne bacterial pathogen responsible for outbreaks and sporadic cases of hemorrhagic colitis (HC). The disease is most severe in children and 5-10% of infections in children with HC develop the sometimes fatal hemolytic uremic syndrome (HUS) (Besser *et al.,* 1999; Monnens *et al.*, 1998, Karmali *et al.,* 1983; Obrig *et al.,* 1998; Ochoa and Cleary, 2003).

The single most important virulence factor of this pathogen is thought to be the production of Shiga toxins (Stxs) (Law, 2000; Karmali, 2004; Gyles, 2007). These toxins are bipartite protein molecules composed of one large A subunit (32 kDa) and a pentamer of smaller B-subunits (7.7 kDa). The A subunit contains the enzymatically active portion of the toxin which inhibits protein synthesis in eukaryotic cells by N-glycosidase cleavage of an adenine residue of the 28 S ribosomal RNA subunit. The five B-subunits of Stx 1 and Stx 2 bind to glycolipid receptor globotriaosylceramide (Gb3) on cells and trigger internalization of the toxin molecule. Stx produced by the organism in the colon is taken up and released into the bloodstream of the host. It is thought that the toxin binds to receptor-bearing endothelial cells lining the human microvasculature resulting in cell death, release of cytokines, and formation of microthrombi. This leads to anoxic damage to organs such as the brain and kidney and complications such as HUS.

While *E. coli* O157:H7 is the most common serotype of Stx-producing *E. coli* (STEC) in North America (Boyce *et al.*, 1995; Johnson *et al.*, 1996; Besser *et al.*, 1999;

Brooks *et al.*, 2005), these toxins are also produced by over 200 other serotypes of *E. coli*. However, only a limited number of other STEC serotypes are associated with clinical disease in humans and illness associated with these other pathogenic STEC serotypes tends to be less common, sporadic in nature and less severe than that associated with *E. coli* O157:H7. Further, many other STEC serotypes have never been associated with human disease. The apparent serotype-specific differences in virulence among STEC has lead to the classification of these organisms into different seropathotypes (SP) (Karmali *et al*., 2003).

Two major antigenic types of Stx are produced by *E. coli* O157:H7; Stx1 and Stx2, and individual strains of this serotype produce either Stx1 or Stx2 or both toxins. In addition, several variants of Stx2 (Stx2c, Stx2d, Stx2e, and Stx2f) have been identified as being produced by *E. coli* O157:H7 and other STEC. Several of these Stx variants appear to be widely distributed among STEC serotypes (e.g., Stx2) whereas others seem to be much more serotype-restricted; e.g., Stx2e is only produced by *E. coli* serotypes associated with edema disease in pigs. These toxin variants have been shown to have different physiochemical properties, stability, receptor affinity and LD50 in mice (Law, 2000; Karmali, 2004; Gyles, 2007; Melton-Celsa *et al*., 2002). It is therefore not surprising that *E. coli* which produce different toxin variants also differ in their ability to cause disease (Strauch *et al.*, 2004; Pierard *et al.*, 1998; Recktenwald *et al.*, 2002; Schmidt *et al.*, 2000; Freidrich *et al.*, 2002). However, the virulence of the *E. coli* strains which produce these toxins may not only be influenced by the type of Stx but also the quantity of the toxin produced (Lejeune *et al.,* 2004).

In *E. coli* O157:H7 and several other STEC, Stx1 and Stx2 have been shown to be encoded by bacteriophage integrated into the host genome. The genomes of at least seven Stx-converting phage from various STEC serotypes have been sequenced and characterization of these phage shows that they are heterogeneous in structure and gene composition (Herold *et al.,* 2004). Stx-containing phage integrate into at least four different sites in STEC genomes. These include the genes *wrbA*, *yehV*, *sbcB* and *yecE* (Ohnishi *et al.,* 2002). In *E. coli* O157:H7 EDL933, the Stx1 bacteriophage is integrated into the *yehV* gene and the Stx2 phage is integrated into *wrbA* (Plunkett *et al.,* 1999).

However, it is clear that the Stx phage, the toxin variants they produce and the phage integration sites not only differ among STEC serotypes but also among *E. coli* O157:H7 strains themselves (Besser *et al.,* 2007; Ohnishi *et al.,* 2002; Serra-Moreno *et al.,* 2007; Shaikh and Tarr, 2003). Lejeune *et al.* (2004) reported that a collection of *E. coli* O157:H7 strains isolated from cattle produced less Stx2 than strains of the pathogen isolated from human infections and that these bovine strains lacked the phage-encoded Q anti-terminator gene present in the human pathogen *E. coli* O157:H7 strain EDL933. Besser *et al.* (2007) have also reported that bovine strains were significantly more likely to produce Stx2c than strains of human origin.

Kim *et al.* (1999) reported that *E. coli* O157 strains could be divided into two lineages using octamer-based genome scanning (OBGS). Lineage I strains were the common genotype and were equally likely to be isolated from humans with clinical disease and cattle, however, lineage II strains were commonly isolated from the faeces of cattle but rarely isolated from clinical disease in human disease. Recently, Ziebell *et al.* (2008) have made a "phenotypic link" which could explain these observations. They

reported that Stx2 type and levels of Stx production are associated with lineage and phage types (PT) specific to different *E. coli* O157:H7 strains. These workers found that *E. coli* O157:H7 lineage II strains were significantly more likely to produce the Stx2c variant while lineage I strains commonly carry Stx2. This difference in toxin type and levels of toxin production could explain differences in apparent virulence of lineage I and II strains as inferred from their biased host distribution. While the Stx2 phage sequence from lineage I strains EDL 933 and Sakai has been determined little is known about the phage associated with Stx2c variant production in lineage II strains.

In this study, the nucleotide sequence and the integration sites for one lineage II Stx2c phage was determined. The nucleotide sequence was annotated and compared to that of other Stx2 phage that have been sequenced. The distribution of lineage II Stx2c and lineage I Stx2 phage in a collection of *E. coli* O157:H7 strains was also determined using PCR screening. Specific objectives of this study were to:

1) clone and determine the nucleotide sequence of Stx2c prophage from the *E. coli* O157:H7 lineage II strain EC970520.
2) annotate the genes in the new Stx2c phage sequences and determine the integration site of the Stx2c phage into the chromosome of strain EC970520
3) determine if regions of the Stx2c phage genome from strain EC970520 are lineage II-specific
4) determine if regions of this Stx2 phage genome from EDL933 are lineage I-specific
5) determine if the integration sites for Stx2 and Stx2c phage are lineage-specific

2.2. Material and Methods

2.2.1. Bacterial strains

In this study, 30 lineage-specific strains of *E. coli* O157:H7 which were isolated from different sources and contained several different phage types (PT) were used (Kim *et al.*, 1999; Khakhria *et al.,* 1990) (Table 2.2.1.). These strains previously used by Zhang *et al.*, 2007 were determined to possess different lineage-specific polymorphism assay (LPSA) types by microarray-based comparative genomic hybridization (CGH). LI/II strains were treated as subgroups often within lineage I strains but were identified as LI/II strains. Studies involving integration of the bacteriophage into host bacteria were done on an extended group of *E. coli* O157:H7 strains consisting of 112 isolates (Table 2.2.2.).

2.2.2. Screening an EC970520 cosmid genomic library for Stx2

A cosmid genomic library for EC970520 was prepared to allow isolation or 'fishing' of conserved Stx2 prophage DNA. Genomic DNA was digested and ligated into Supercos I according to the manufacturer's instructions (Stratagene, CA) and stored in glycerol stocks at -80 ° C (personal communication, Zhang). Bacteria were resuscitated by placing about $3*10^8$ CFU in LB broth in a 37 º C shaker for exactly 2 hours. Serial dilutions of cells containing the cosmid were prepared, then plated on kanamycin (KM) agar plates (50 µg/ml) and incubated at 37 ºC overnight; the Supercos I cosmid contains an neo^r gene which confers kanamycin resistance and bacteria containing this construct can be selected by growth on KM. Fifty individual colonies were picked and transferred

Table 2.2.1. *E. coli* O157:H7 lineage-specific strains used in this study. Strains were previously used and characterized for LPSA type by Zhang *et al*. (2007) by analysis of microarray-based comparative genomics hybridizations (CGH).

Strain	Serotype	Phage type	Source	LSPA type	Lineage
LRH16	O157:H7	14	Human	IIIIII	I
LRH27	O157:H7	14	Human	IIIIII	I
LRH6	O157:H7	14	Human	IIIIII	I
TS97	O157:H7	14	Bovine	IIIIII	I
F1299	O157:H7	14	Bovine	IIIIII	I
F5	O157:H7	14	Bovine	IIIIII	I
EDL933	O157:H7	21	Human	IIIIII	I
63154	O157:H7	31	Human	IIIIII	I
58212	O157:H7	31	Human	IIIIII	I
F1095	O157:H7	31	Bovine	IIIIII	I
Sakai	O157:H7	32	Human	IIIIII	I
H4420	O157:H7	87	Bovine	IIIIII	I
E2328	O157:H7	87	Bovine	IIIIII	I
F1082	O157:H7	87	Bovine	IIIIII	I
59243	O157:H7	2	Human	2IIIIII	I/II
71074	O157:H7	2	Human	2IIIIII	I/II
EC20030338	O157:H7	2	Human	2IIIIII	I/II
Zap0046	O157:H7	2	Human	2IIIIII	I/II
LRH13	O157:H7	23	Human	222222	II
R1797	O157:H7	23	Human	222222	II
LS68	O157:H7	23	Bovine	222222	II
F12	O157:H7	23	Bovine	222222	II
F1081	O157:H7	23	Bovine	222222	II
FRIK 920	O157:H7	23	Bovine	222222	II
FRIK 1999	O157:H7	23	Bovine	222222	II
FRIK 1985	O157:H7	45	Bovine	22222I	II
FRIK 1990	O157:H7	54	Bovine	2222II	II
FRIK 2001	O157:H7	54	Bovine	222222	II
EC970520	O157:H7	67	Bovine	222222	II
12491	O157:H7	74	Human	2222I2	II

Table 2.2.2. Extended list of *E. coli* O157:H7 strains used for the integration studies. Some strains from Table 2.2.1 are included in this list.

STRAIN	SOURCE	PHAGE TYPE	LINEAGE
F1150	Bovine	1	I
TS104	Bovine	1	I
EC20020294	Human	1	I
R1388	Human	1	I
E1572	Human	2	I
107718	Human	2	I
111570	Human	2	I
R910	Human	14	I
F832	Bovine	14	I
F1042	Bovine	14	I
F1135	Bovine	14	I
S2476	Human	14	I
45550	Human	14	I
72143	Human	14	I
65576	Human	14	I
33336	Human	14	I
AA7821	Water	14	I
TS52	Bovine	14	I
LS7	Bovine	14	I
LS225	Bovine	14	I
EC20020290	Human	14	I
TS97	Bovine	14	I
LRH6	Human	14	I
LRH16	Human	14	I
LRH27	Human	14	I
97701	Human	14	I
F5	Bovine	14	I
F1299	Bovine	14	I
E1112	Human	14	I
EC20020292	Human	21	I
EC20020293	Human	21	I
EC20030103	Human	21	I
R776	Human	31	I
R1277	Human	31	I
LRH17	Human	31	I
63154	Human	31	I
58212	Human	31	I
F1095	Bovine	31	I
F1104	Bovine	32	I
93178	Human	32	I
Sakai	Human	32	I
R904	Human	33	I
EC20020291	Human	34	I
120290	Human	45	I
EC19980119	Bovine	87	I
74909	Human	87	I
LN7264	Water	87	I
F1082	Bovine	87	I
E2328	Bovine	87	I
F801	Bovine	87	I
F1099	Bovine	87	I

STRAIN continued	SOURCE	PHAGE TYPE	LINEAGE
94023	bovine	87	I
F1083	bovine	87	I
F1084	bovine	87	I
F1085	bovine	87	I
F1090	bovine	87	I
F1097	bovine	87	I
F1106	bovine	87	I
EDL933	human	21	I
71074	human	2	I/II
59243	human	2	I/II
EC20020462	bovine	15	I,II
EC20030338	bovine	15	I,II
EC20030282	bovine	15	II
EC20030348	bovine	15	II
EC20030351	bovine	15	II
EC19920026	bovine	23	II
EC19920171	bovine	23	II
EC19920283	human	23	II
EC19930086	human	23	II
EC19930200	bovine	23	II
F1081	bovine	23	II
EC20030186	bovine	23	II
EC20030216	bovine	23	II
EC20030226	bovine	23	II
EC20030275	bovine	23	II
EC20030277	bovine	23	II
EC20030278	bovine	23	II
EC20030289	bovine	23	II
EC20030340	bovine	23	II
EC20030495	human	23	II
LS68	bovine	23	II
FRIK 920	bovine	23	II
FRIK 1999	bovine	23	II
LRH13	human	23	II
R1797	human	23	II
EC20000623	bovine	23	II
EC19920283	human	23	II
EC19930086	human	23	II
R1797	human	23	II
LRH13	human	23	II
EC20030528	human	23	II
EC20030547	human	23	II
EC20030292	bovine	45	II
FRIK 1985	bovine	45	II
R834	human	54	II
EC20030154	bovine	54	II
FRIK 1990	bovine	54	II
FRIK 2001	bovine	54	II
EC19920005	bovine	67	II
EC19920192	bovine	67	II
EC19970520	bovine	67	II
EC20030317	bovine	67	II
12491	human	74	II
EC20030193	bovine	74	II

STRAIN continued	SOURCE	PHAGE TYPE	LINEAGE
EC20030223	bovine	74	II
EC20030281	bovine	74	II
EC20030422	human	74	II
EC20011139	bovine	82	II
AA579-2	unknown	87	II
EC20030496	human	-	II
EC20030497	human	-	II

to two separate KM plates and then incubated at 37 ºC over night. Colonies from one of the duplicate plates were prepared by adding 5 ml of sterile milli Q water to the surface and were screened using a l μl aliquot of the cosmid mixture for the presence of Stx2 by PCR using VT2 conserved primers (Table 2.2.3.). Individual cosmid clones were subsequently isolated by individual colony picking of Stx2 positive plates. Regions upstream (personal communication, Zhang) and downstream of the Stx2 were screened by PCR analysis and cosmid clone isolation.

2.2.3. Screening of Stx2 bacteriophage genes by PCR

Forward and reverse primers either were identified within the literature (Gannon *et al.,* 1997) or were designed from a previous sequence (933W accession no. AF125520) or a partial sequence as each cosmid was sequenced (EC970520) (Table 2.2.3) (personal communication, Zhang). PCR's were performed using genomic DNA (100 ng), a 1 μl aliquot of cosmid mixture as previously described or a picked colony template. PCR's producing amplified products <2 kb in size were performed in a 25 μl reaction volume which contained 200 μM of each deoxynucleoside triphosphate, 10 pmol of each primer, 1X PCR buffer (Qiagen) and 1U DNA Taq polymerase (Qiagen). PCR's were conducted using the following parameters: 3 min denaturation at 94 ºC, followed by 25 cycles of 45 s at 94 ºC, 30 s at 55 ºC and 90 s at 72 ºC with a final 10 min 72 ºC extension step. PCR products were visualized under UV light on a 1.0% (w/v) agarose gel stained with ethidium bromide (0.5 μg/ml). PCR's producing amplified products >2 kb were performed as long template PCR's which also used genomic DNA (100 ng), a 1 μl aliquot of cosmid mixture or picked colony templates. However, each 25 μl reaction

volume contained 200 μM of each deoxynucleoside triphosphate, 10 pmol of each primer, 1X Advantage 2 PCR buffer (BD) and 1X Advantage 2 Taq polymerase (BD).

Table 2.2.3. Nucleotide sequences and locations of primers used for fishing Stx2 phage from a cosmid genomic DNA library, PCR screening for lineage-specific strains and identification of potential integration sites.

Primers	Nucleotide sequence (5'-3')	Location	Predicted amplicon size (~bp)	Source
P7	ATCACCTTCCGCCGCAATGG	Upstream stx2c	n/a	EC970520
pyz2	TCCAGTACCTATTACGCCTTG	Upstream stx2c	n/a	EC970520
pyz6	GCTCTTCTGTTGCCAGTCTTA	Upstream stx2c	n/a	EC970520
pyz7	AGAAGGTGGAGAACTCCATG	Upstream stx2c	n/a	EC970520
R104-13	CGCCACATAGACAAAGCCCT	Downstream stx2c	6500	EC970520
R104-RC	TGTGCCTTGATCTCTTCCAGTG	Downstream stx2c		
VT2-F	CCATGACAACGGACAGCAGTT	Stx2 conserved gene	2000	Gannon *et al.* (1997)
VT2-R	CCTGTCAACTGAGCACTTTG	Stx2 conserved gene		
R104-23r	TGCAGAAGGCGGTGAATGA	Downstream stx2c	10,000	EC970520
302-T3-9r	TCGTTGGTCCGTCTGGCTCA	Downstream stx2c		
pyz1	CCTAAGACTGGCAACAGAAG	Downstream stx2c	9000	EC970520
rstx1	GTGGTATAACTGCTGTCCGTTGTC	stx2c region		
stx2-f	TATATCAGTGCCCGGTGTGA	stx2 in EDL933	753	933W (accession no. AF125520)
stx2-r	TTCTTTCCCGTCAACCTTCA	stx2 in EDL933		
stx2c-f	TATATCAGTGCCCGGTGTGA	stx2c in EC970520	750	EC970520
stx2c-r	TTTTCCGGCCACTTTACTG	stx2c in EC970520		
SBC	GCGTGTGACTTCGTCGTCGAAAC	sbcB insertion site forward	1500	K12 (NC_000913)
PBP	CTGGCGTAATGCGATGACTA	sbcB insertion site reverse		EC970520
SBC	GCGTGTGACTTCGTCGTCGAAAC	sbcB insertion site forward	2000	K12 (NC_000913)
pyz8	GAAGATTGGACGGCGGATAAC	sbcB/phage left junction		EC970520
PBP	CTGGCGTAATGCGATGACTA	sbcB insertion site reverse	1700	EC970520
406phage	TCATTTTGATTTTGCTGCAC	sbcB/phage right junction		
wrbA-C	AGGAAGGTACGCATTTGACC	wrbA insertion site reverse	314	Besser *et al.* (2007)
wrbA-D	CGAATCGCTACGGAATAGAGA	wrbaA insertion site forward		
wrbA-C	AGGAAGGTACGCATTTGACC	wrbA insertion site reverse	592	Besser *et al.* (2007)
wrbA-G	ATCGTTCGCAAGAATCACAA	wrbA/phage left junction		
wrbA-D	CGAATCGCTACGGAATAGAGA	wrbaA insertion site forward	506	Besser *et al.* (2007)
wrbA-H	CCGACCTTTGTACGGATGTAA	wrbA/phage right junction		

PCR's were conducted using the following parameters: 30 s denaturation at 95 ºC, followed by 30 cycles of 30 s at 95 ºC and 12min at 68 ºC with a final 7 min 70 ºC extension step. PCR products were visualized under UV light on a 0.6% (w/v) agarose gel stained with ethidium bromide (0.5 µg/ml). Negative controls incorporated all reagents of the PCR except water was used as a template.

2.2.4. Data analysis

Sequencing of cosmids and amplificons were completed at the University of Guelph (University of Guelph Molecular Supercenter, ON). DNA sequences were analyzed by homology searching using Blast (NCBI) and annotated by Kodon^{TM} total genome and sequence analysis software (Applied Maths, TX). A blastn (NCBI) comparison was performed with the EC970520 sequence and other Stx2 phage sequences in order to assess their homology. The similarity percentages were derived from % identity and length of alignment.

2.2.5. PCR identification of genes specific to Lineage I and II in *E. coli* O157:H7

To determine if the isolated Stx2c phage genes were lineage-specific, 5 sets of primers were designed from the completed sequence and used for PCR screening of lineage specific strains from Table 2.2.1.(personal communication, Zhang). A primer set specific for Stx2 from the lineage I strain EDL933 was also PCR amplified in lineage-specific strains.

2.2.6. Integration sites of Stx2 phage

E. coli O157:H7 isolates from Table 2.2.2. were analyzed by PCR for insertion sites using selected primers from Table 2.2.3. Primers for *sbcB* were designed and tested in EC970520 and *E. coli* K12 to determine the site of interruption by the phage genome. Primers were then designed for both the left and right phage-chromosome junctions of the *sbcB* integration sites. To confirm the presence or absence of phage integration into *sbcB* loci, isolates were also screened for intact gene products. It has previously been determined that the integration site for Stx2 phage of EDL933 is *wrbA* (Plunkett *et al.,* 1999) and strains were further assessed by PCR using primers for *wrbA* integration that were previously identified by Shaikh and Tarr (2003).

2.3. Results

2.3.1. Cloning and sequencing of the Stx2c phage genome

A cosmid genomic library was prepared for *E. coli* O157:H7 strain EC970520 (personal communication, Zhang). Cosmid clones containing Stx2 regions were identified from the genomic library by PCR screening using primers with homology to the Stx2 gene (primers VT2F and VT2R Table 2.2.3.). A cosmid clone, #302 was isolated that contained the Stx2 genes (Figure 2.3.1.). However, when sequenced, this clone did not contain the gene sequence of the entire bacteriophage nor the bacteriophage bacterial host DNA junction. Therefore, PCR primers from clone #302 were developed (primers pyz1, pyz2, rstx1, P7 Table 2.2.3.) (personal communication, Zhang) and used to isolate cosmid clones containing upstream and downstream gene sequences. Cosmid clone #406 was isolated using these primers and nucleotide sequencing determined this clone contained sequence downstream of cosmid clone #302 and a possible integration site for the phage into the host genome. Primers specific for the integration site were developed based on the interrupted gene, *sbcB*, in the clone #406. Since the *sbcB* gene may be an integration site for other phage, primers for intact *sbcB* were designed from *E. coli* strain K12 which contains no phage DNA at the *sbcB* gene (Table 2.2.3.). Verification of the intact chromosome-phage right junction was confirmed by amplification of a 1.7 kb fragment using primers 406phage and PBP with DNA from strain EC970520 (Figure 2.3.1.). The intact chromosome-phage left junction primer, SBC, and clone#302 upstream primer, pyz2, was used in a long template PCR to amplify long pieces of phage DNA (>2kb) to isolate the upstream sequence of clone #302 (Figure 2.3.1.) (personal communication,

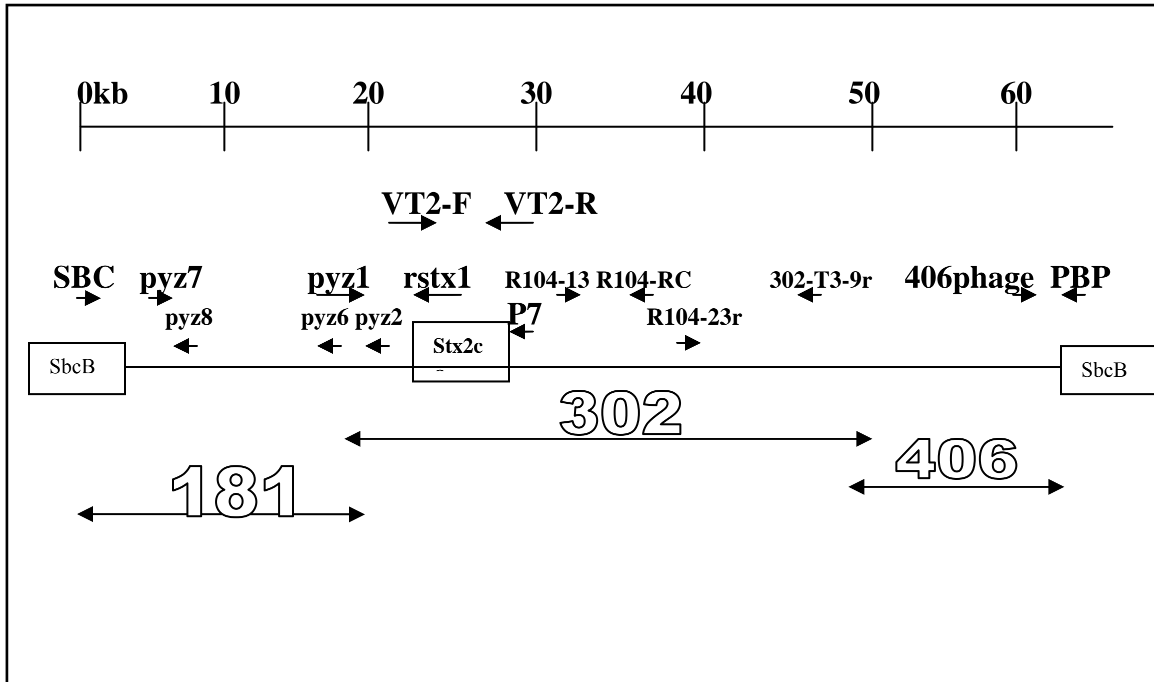


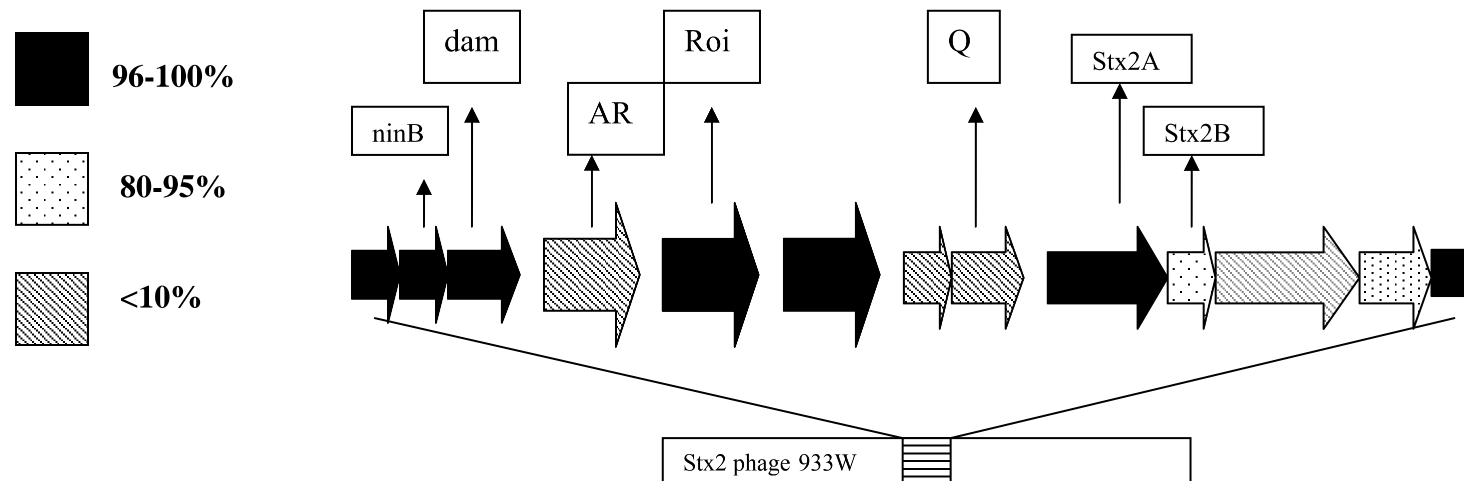
Figure 2.3.1. Relative location of primers found within the Stx2c phage sequence. The numbers 302 and 406 represent the length of cosmid DNA sequences. 181 represents the length of the PCR product (personal communication, Zhang). The *SbcB* gene is interrupted by phage sequence. Total size of the phage genome is 62,147 bp.

Zhang). Since pyz2 yielded only partial upstream sequence, primers pyz6 and pyz7 were designed and used in a long template PCR to complete the upstream sequence. Sequencing of the products termed #181 confirmed the upstream sequence of clone#302 and the *sbcB* integration site (Figure 2.3.1.).

A blastn ORF comparison of the Stx2c from lineage II strain EC970520 to other Stx2 phages 933W, Stx2Φ-I, VT2-Sakai, VT2-Sa showed high sequence similarity (96-100%) to several ORF surrounding the Stx2 genes including *Stx*2A, *Roi*, *dam, ninB* and several other hypothetical proteins (Figure 2.3.2.). However, there was weak similarity between the phages in the *Q* antiterminator and putative anti-repressor proteins in this region (Figure 2.3.2.). Sequence similarity existed within *Stx*2*B* although the % identity (80-95%) was lower in this area than the other highly homologous regions.

2.3.2. Annotation of the Stx2c phage genome sequence

DNA sequence information for the bacteriophage DNA was annotated using Kodon ORF finder (personal communication, Zhang). This program was defined to identify ORFs of at least 100 amino acids. Blastx (NCBI) was used to identify homologous gene segments (Figure 2.3.3.; Table 2.3.1.). The entire DNA sequence of the Stx2c phage contained 62,147 bp. Comparison of the phage sequence to the *E.coli* K12 genome, which contains no Stx2 phage, revealed only homology to the *sbcB* gene, which encodes a penicillin binding protein. However, as previously stated, this region of DNA was interrupted by phage DNA sequence and is the integration site for this Stx2c-



gene	HP	ninB	dam	AR	Roi	HP	HP	Q	Stx2A	Stx2B	HP	HP	HP
ORF location start in EC970520	18923	19224	19745	20753	21586	22314	22916	23578	24715	25686	26252	26819	28939
ORF location end in EC970520	19372	19775	20299	21517	22314	22919	23586	24066	25674	25955	26575	78756	29365
EDL933 (AF125520)													
% Identity	99	98	96	<10	99	97	<10	<10	99	95	<10	96	96
Alignment %	99	100	100	<10	100	99	<10	<10	100	100	<10	84	98
Stx2Φ-I (AP004402)													
% Identity	97	97	97	<10	98	98	<10	<10	96	96	<10	96	96
Alignment %	99	98	96	<10	99	96	<10	<10	99	95	<10	84	98
VT2-Sakai (AP004402)													
% Identity	98	98	98	94	94	94	<10	<10	96	96	<10	96	96
Alignment %	99	99	96	34	90	90	<10	<10	99	95	<10	84	98
VT2-Sa (NC_000902)													
% Identity	98	98	98	98	94	94	<10	<10	96	96	<10	94	94
Alignment %	99	98	96	33	90	96	<10	<10	99	95	<10	84	88

Figure 2.3.2. Blastn homology of EC970520 (LII) to EDL933 (LI), Stx2Φ-I, VT2-Sakai, and VT2-Sa regions surrounding Stx2 genes based on % identity and length of alignment (%).
HP represents hypothetical protein. Arrows represent ORF's found within the Stx2 phage from EC970520 compared to EDL933 Stx2 phage 933W (accession no. AF125520) and labeled based on the percentage of coverage of homologous regions to the EC970520 sequence. HP are not labeled.

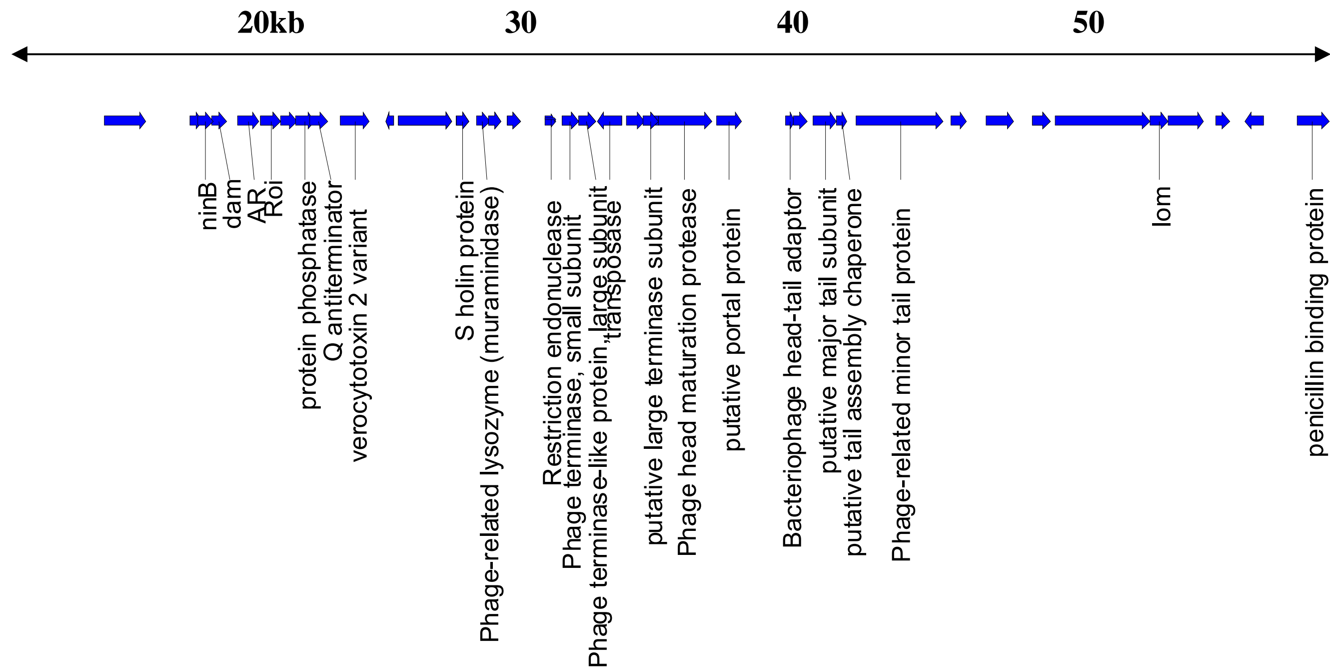


Figure 2.3.3. Annotation of ORF's found within the Stx2c phage within EC970520. Putative functions are based on homologous regions from NCBI blastx searches. The annotation includes cosmids 302 and 406 but not the PCR cloned region. Hypothetical proteins are not shown.

Table 2.3.1. ORF with putative genes and homologs of Stx2 phage identified within *E. coli* O157:H7 strain EC970520 (personal communication, Zhang).

start	end	Gene	orientation	Notes
1	699	*sbcB*	>	sbcB fragment, penicillin binding protein
657	669		>	direct repeat (cgtaatcgtaaaa)
884	2062	*Int*	>	phage integrase family protein
2312	2656		>	putative lipoprotein
2844	3194		>	hypothetical protein within CP-933W
3413	3547		>	N-terminal of Z3371 in EDL933
3544	4059		>	homolog to phage P7
4061	5008		>	hypothetical protein within CP-933W
5325	5606		>	hypothetical protein within CP-933W
5781	5969		>	hypothetical protein within VT2-Sakai
5960	6640	*Exo*	>	putative exonuclease
6637	7422	*Betw*	>	putative bet recombination protein
6985	7461		>	homolog to stx2 II p107
7428	7844	*Gam*	>	host-nuclease inhibitor
7799	8068	*Kil*	>	Kil protein, host killing
7911	8015	*cIII*	>	λ homolog regulatory protein, antitermination
8146	8516		>	λ homolog, single-stranded DNA binding protein
9018	9290		>	ECs1181 in Sakai
9018	9362	*N*	>	phage nil2 homolog, early antiterminator N protein
9277	9450		>	ECs1182 in Sakai
9575	9841		>	homolog to stx1p118
9747	9905		>	ECs1183 in Sakai
10019	10540		>	ECs1184 in Sakai
11042	11695	*cI*	>	phage nil2 homolog, cI repressor
11812	12027	*Cro*	>	phage nil2 homolog, control of repressor
12169	12465	*cII*	>	phage nil2 homolog, repressor
12498	12659		>	phage nil2 homolog
12646	12659	*O*	>	origin binding protein, replication of DNA
12646	13467		>	phage nil2 homolog, replication of DNA
13464	14841		>	APEC 01 homolog, helicase
14947	15348		>	hypothetical protein in ISEC8 (EDL933)
15345	15348		>	hypothetical protein in ISEC8 (EDL933)
15742	17280		>	hypothetical protein in ISEC8 (EDL933)
17369	17980		>	phage HK022 putative endonuclease
18228	18443		>	ORF 69 homolog in phage ST64T
18445	18777		>	gene 48 homolog in phage Sf6
18923	19372		>	ECs2983 in Sakai
19224	19775	*ninB*	>	pfam05772, ninB protein
19745	20299	*Dam*	>	pfam05869, DNA N-6-adenine methyltransferase of phage
20753	21517		>	putative antirepressor protein (AR)
21586	22314	*Roi*	>	pfam003374, putative DNA-binding protein roi of phage
22314	22919		>	phage nil2 homolog
22916	23586		>	2A homolog protein phosphatase
23578	24066	*Q*	>	Q protein, antitermination

start (continued)	end	*Gene*	orientation	Notes
24715	25674	*Stx2cA*	>	verocytotoxin 2 variant 2c subunit A
25686	25955	*stx2cB*	>	verocytotoxin 2 variant 2c subunit B
26252	26575		<	CACO5542 in E.coli
26819	28756		>	CACO5543 In E.coli
28939	29385		>	hypothetical protein in BP-933W
29462	29677	*S*	>	holin protein S homolog
29677	30174		>	phage related lysozyme
30171	30608		>	hypothetical protein in E.coli B171
30811	31308		>	pfam04383, kilA- N domain
31305	31562		>	hypothetical protein in phage HK620
32093	32251		>	ECs1788 in Sakai
32293	32658		>	restriction endonuclease
32950	33513		>	phage terminase, small subunit
33510	34142		<	phage terminase-like protein, large subunit
34186	35073		>	phage BP-4795 homolog, transposase orfA protein
35929	36486		>	phage D3 homolog, putative large terminase subunit
36517	38487		>	pfam05065, phage head maturation protease
38699	40063		>	putative portal protein
40060	41283		>	putative portal protein
41280	41616		>	BP-4795 homolog
41616	41966		>	head-tail adaptor
41963	42409		>	ECs1547 I Sakai
42679	43521		>	BP-4795 homolog, putative major tail subunit
43530	43904		>	putative tail assembly chaperone
43928	44143		>	hypothetical protein in CP-933W
44263	47505		>	phage-related minor tail protein
47498	47839		>	minor tail protein
47839	48537		>	phage minor tail protein
49146	50150		>	antirepressor protein
50204	50785		>	K homolog, putative tail component
50840	51520		>	putative tail component
51846	55259		>	tail component, fibronectin type 3 domain
55326	55925	*Lom*	>	CP-1639 homolog, opacity protein, related surface antigen
55990	57303		>	putative tail fiber
57715	58590		>	hypothetical protein in CA143897
58815	59516		>	hypothetical protein in CP--933W
59450	59734		>	ECs2227 in Sakai
60532	60544		>	direct repeat (cgtaatcgtaaaa)
60532	60580	*sbcB*	>	sbcB n terminal, pencillin binding protein

encoding phage. The entire prophage sequence was also flanked by direct repeats (cgtaatcgtaaaa) which may be sites which facilitate recombination between the phage and the host (ORF location 657…669, 60532…60580, Table 2.3.1.). Integrase *int* and exonuclease *exo* genes downstream of the direct repeats were identified; these genes may encode proteins which enable phage integration and excision from the host genome. The genes *betw* and *gam* are involved in recombination and the *kil* and *cIII* genes are important for host-killing and establishment of lysogeny, respectively. An *N* gene also was identified as the first anti-terminator, which is an important phage regulatory protein and essential for Q protein mediated regulation of late phage genes. The *cI* repressor, a well known λ repressor, follows the *N* gene; this repressor blocks expression of lytic genes and is required for the phage to remain in a lysogenic state. The *cro* and *cII* genes identified are early lytic genes in λ. In phage λ *cI* and *cro* recognize the same promoter, *cI* is expressed in the lysogenic state and *cro* is expressed in the lytic state. The *O* gene codes for an important replication protein in phage λ. An IS element ISEC8 is present after the *O* gene and precedes the recombination genes *ninB* and *dam*, which encode a DNA N-6-adenine methyltransferase. The *roi* gene encodes for a DNA-binding protein and precedes the antiterminator *Q* gene which encodes an important antiterminator responsible for initiating transcription of lysis genes and an antirepressor *AR*. Downstream genes encoding *Stx2cA* and *Stx2cB* subunits found in the Stx2c variant code for these toxins and may be under the control of the Q antiterminator. The *S* gene is a cell lysis holin gene and is found upstream of several structural genes belonging to the phage. Specifically, the *lom* gene encodes a major head protein. Within these structural genes, a transposase found within the IS element IS629 is found. The *sbcB* gene

fragment and a direct repeat located at the end of the sequence marks the beginning of the chromosomal DNA backbone and the phage insertion site.

2.3.3. Identification of Stx2 phage among strains of *E. coli* O157:H7 from different lineages

In order to determine if the Stx2c phage from the lineage II strain EC970520 was related to the Stx2c phage from other lineage II strains, five sets of primers (Table 2.2.3.) representative of the phage sequence from EC970520 were used in PCR analysis of DNA from a collection of *E. coli* O157:H7 lineage I, I/II and II strains of human and bovine origin listed in Table 2.2.1. All of these primer pairs were able to amplify products of expected band sizes in 12/12 lineage II strains but did not amplify the expected bands in 17/18 lineage I and I/II strains (Table 2.3.2.). One lineage I/II strain was positive with primers VT2-F and P7 and for forward and reverse Stx2c primers. In addition, some variations ~2kb smaller than the expected product size were observed for lineage II strains using primers R104-23r/ 302-T3-9r. A primer set targeting Stx2 genes (Table 2.2.3.) designed from EDL933 Stx2 phage amplified DNA from 18/18 lineage I and I/II strains but 12/12 lineage II strains were negative with these primers (Table 2.3.2.). This confirmed that all lineage II strains were only positive for Stx2c genes and that the majority of lineage I and I/II were only positive for Stx2 genes (Figure 2.3.4.). The only exception observed was the strain Zap0046, a lineage I/II strain that produced the 750bp expected band size using the Stx2 primer set (Stx2-f/r) and the Stx2c primer set. Zap0046 also produced a 1.8kb band for the VT2-F/P7 which is the same size as that

Table 2.3.2. Distribution of PCR amplified products obtained using lineage-specific primers from EC970520 and EDL933 Stx2 phage in Lineage I, I/II and Lineage II strains of *E. coli* O157:H7.

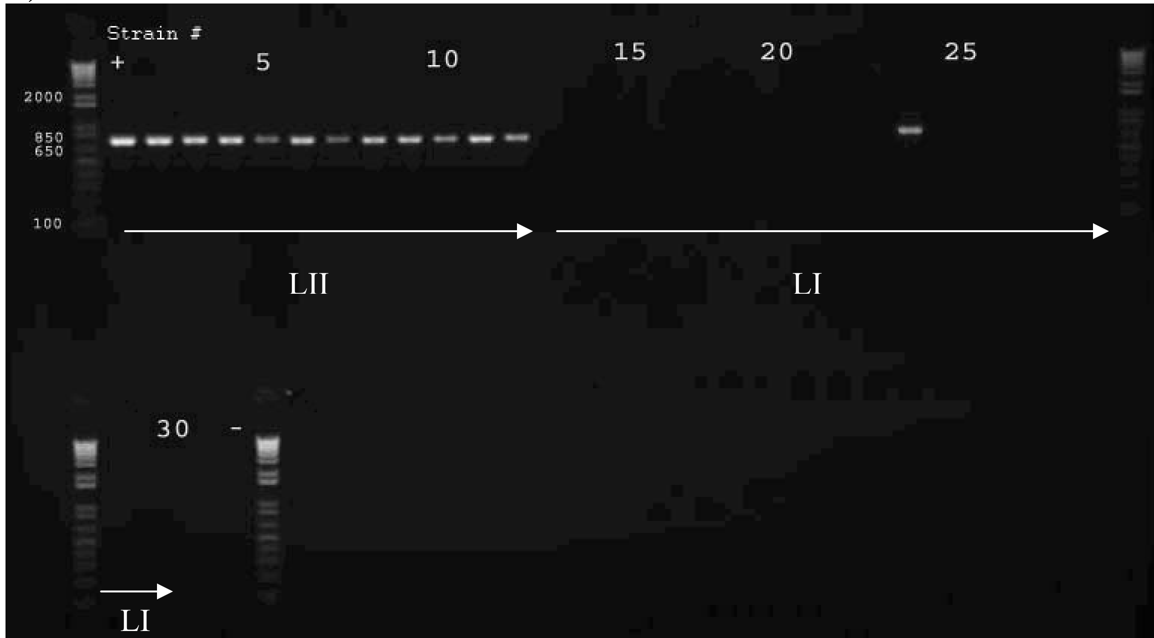
		EC970520					EDL933
Strain	Lineage	R104-RC R104-13[a]	VT2-F P7[b]	R104-23r 302-T3-9r[c]	pyz1 rstx1[d]	Stx2c-F Stx2c-R[e]	Stx2-F Stx2-R[f]
LRH16	I	+*1	-	-	-	-	+
LRH27	I	+*1	-	-	-	-	+
LRH6	I	+*1	-	-	-	-	+
TS97	I	+*1	-	-	-	-	+
F1299	I	+*1	-	-	-	-	+
F5	I	+*1	-	-	-	-	+
EDL933	I	+*1	-	-	-	-	+
63154	I	+*1	-	-	-	-	+
58212	I	+*1	-	-	-	-	+
F1095	I	+*1	-	-	-	-	+
Sakai	I	+*1	-	-	-	-	+
H4420	I	+*1	-	-	-	-	+
E2328	I	+*1	-	-	-	-	+
F1082	I	+*1	-	-	-	-	+
59243	I,II	+*1	-	-	-	-	+
71074	I,II	+*1	-	-	-	-	+
EC20030338	I,II	+*1	-	-	-	-	+
Zap0046	I,II	+*1	+	-	-	+	+
LRH13	II	+	+	+	+	+	-
R1797	II	+	+	+	+	+	-
LS68	II	+	+	+	+	+	-
F12	II	+	+	+	+	+	-
F1081	II	+	+	+	+	+	-
FRIK 920	II	+	+	+	+	+	-
FRIK1999	II	+	+	+	+	+	-
FRIK1985	II	+	+	+*2	+	+	-
FRIK1990	II	+	+	+*2	+	+	-
FRIK2001	II	+	+	+*2	+	+	-
EC970520	II	+	+	+	+	+	-
12491	II	+	+	+*2	+	+	-

Expected band sizes**:** [a]6kb, [b]1.8kb, [c]9.5kb, [d]9kb, [e]750bp, [f]750bp

*1unexpected band sizes of 10kb

*2unexpected band sizes of 7.5-8.0kb

A) Stx2c



B) Stx2

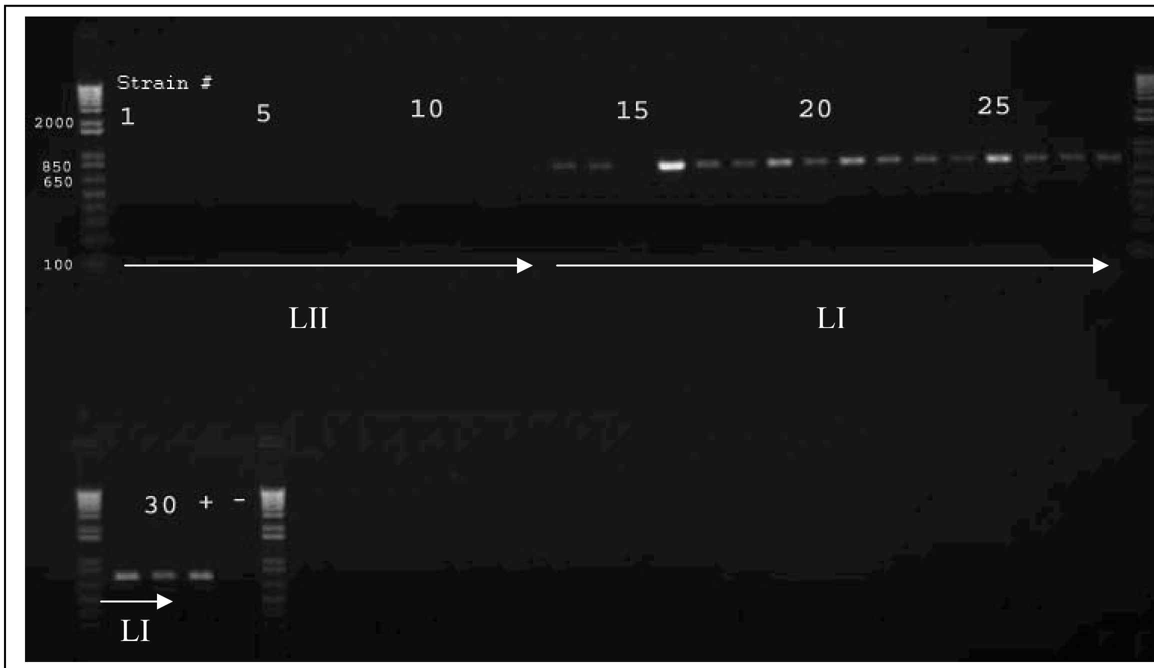


Figure 2.3.4. PCR of A) Stx2c-F/R and B) Stx2-F/R primers.
Expected band sizes were 750bp for each primer set. Positive control was EC970520 for Stx2c for (A) and EDL933 for (B). Lanes 1, 30, 31, 36: 1kb plus ladder. Lanes 2-13: lineage II strains; 14-29, 32-34: lineage I, I/II strains; Lane 35: PCR negative.

Strains listed in Figure 2.3.4. were as follows:

1. EC970520 (II)
2. LRH13 (II)
3. R1797 (II)
4. LS68 (II)
5. F12 (II)
6. F1081 (II)
7. FRIK 920 (II)
8. FRIK 1999 (II)
9. FRIK 1985 (II)
10. FRIK 1990 (II)
11. FRIK 2001 (II)
12. 12491(II)
13. LRH16 (I)
14. LRH27 (I)
15. blank
16. LRH6 (I)
17. TS97 (I)
18. F1299 (I)
19. F5 (I)
20. 71074 (I/II)
21. 59243 (I/II)
22. EC20030338 (I/II)
23. 63154 (I)
24. Zap0046 (I/II)
25. 58212 (I)
26. F1095 (I)
27. H4420 (I)
28. E2328(I)
29. F1082 (I)
30. Sakai (I)
31. EDL933 (I)

+ positive control
- negative control

observed for lineage II strains. However, unlike the other *stx*2c bearing strains, Zap0046 did not yield expected bands for other EC970520 primers sets (R104-RC/13, R104-23r/302-T3-9r, pyz1/ rstx1 Figure 2.3.1.) flank the Stx2c genes, suggesting this phage is distinct from that found in lineage II strains.

2.3.4. Identification of Stx2 phage integration sites within lineage-specific strains of *E. coli* O157:H7

An extended collection of lineage-specific strains of *E. coli* O157:H7 was analyzed by PCR in order to determine if the integration sites of the Stx2 phage within the *sbcB* gene of EC970520 could be identified in other lineage I, I/II or II strains using the oligonucleotide primers described in Table 2.2.3. These primers are specific to the phage-chromosome junctions (SBC/pyz8, PBP/406phage) and were identified within the *sbcB* gene from EC970520 and used to screen each of the strains within the collection. In this experiment, EC970520 was used as a positive control where the phage-chromosome junctions were PCR positive for phage occupation. Consequently K12 was used as a negative control for phage integration as phage-chromosome junctions were PCR negative. Primers designed from K12 were also used to identify intact *sbcB* genes since no phage occupation occurs in this region. The SBC/PBP primers were positive if the gene was intact but negative if the phage was inserted into the *sbcB*. Most lineage I strains 58/59 (98.3%) were negative for *sbcB* chromosome-phage junctions and positive for the intact *sbcB* gene (Table 2.3.3.). Only the Lineage I strain R1388 was shown to contain a *sbcB* chromosome-phage junction and an interrupted *sbcB* gene. In lineage II strains 44/49 (89.8%) were positive for the *sbcB* chromosome junctions, although strains

Table 2.3.3. PCR results for integration of phage in *E. coli* O157:H7 lineage-specific strains.
(+) expected band size present, (–) no band present, (weak) weak band detected. Primers used were as follows: [a]406phage/PBP; [b]pyz8/sbc-j1r; [c]PBP/sbcB-r; [d]wrbA C/G; [e]wrbA H/D; [f]wrbA C/D.

Strain	Lineage	*sbcB*/ phage right junction[a]	*sbcB*/ phage left junction[b]	*sbcB* intact[c]	*wrbA*/ phage left junction[d]	*wrbA*/ phage right junction[e]	*wrbA* intact[f]
F1150	I	-	-	+	+	+	Weak
TS104	I	-	-	+	+	+	Weak
EC20020294	I	-	-	+	+	+	-
R1388	I	+	+	-	-	+	+
E1572	I	-	-	+	-	-	+
107718	I	-	-	+	-	-	+
111570	I	-	-	+	-	-	Weak
R910	I	-	-	+	+	+	Weak
F832	I	-	-	+	+	+	-
F1042	I	-	-	+	+	+	Weak
F1135	I	-	-	+	+	+	-
S2476	I	-	-	+	+	+	Weak
45550	I	-	-	+	-	-	-
72143	I	-	-	+	+	+	Weak
65576	I	-	-	+	+	+	Weak
33336	I	-	-	+	+	+	Weak
AA7821	I	-	-	+	+	+	Weak
TS52	I	-	-	+	+	+	-
LS7	I	-	-	+	+	+	-
LS225	I	-	-	+	+	+	Weak
EC20020290	I	-	-	+	+	+	Weak
TS97	I	-	-	+	+	+	-
LRH6	I	-	-	+	+	+	Weak
LRH16	I	-	-	+	+	+	-
LRH27	I	-	-	+	+	+	-
97701	I	-	-	+	+	+	Weak
F5	I	-	-	+	+	+	-
F1299	I	-	-	+	+	+	-
E1112	I	-	-	+	+	+	-
EC20020292	I	-	-	+	+	+	Weak
EC20020293	I	-	-	+	+	+	Weak
EC20030103	I	-	-	+	+	+	-
R776	I	-	-	+	+	+	Weak
R1277	I	-	-	+	+	+	-
LRH17	I	-	-	+	+	+	Weak
63154	I	-	-	+	+	+	Weak
58212	I	-	-	+	+	+	Weak
F1095	I	-	-	+	+	+	Weak
F1104	I	-	-	+	+	+	Weak
93178	I	-	-	+	+	+	Weak
Sakai	I	-	-	+	+	+	-

Strain continued	Lineage	*sbcB*/ phage right junction[a]	*sbcB* phage left junction[b]	*sbcB* intact[c]	*wrbA*/ phage right junction[d]	*wrbA*/ phage left junction[e]	*wrbA* intact[f]
R904	I	-	-	+	+	+	Weak
EC20020291	I	-	-	+	+	+	Weak
120290	I	-	-	+	+	+	Weak
EC19980119	I	-	-	+	+	+	-
74909	I	-	-	+	+	+	-
LN7264	I	-	-	+	+	+	-
F1082	I	-	-	+	+	+	-
E2328	I	-	-	+	+	+	-
F801	I	-	-	+	-	+	-
F1099	I	-	-	+	+	+	-
94023	I	-	-	+	+	+	-
F1083	I	-	-	+	+	+	-
F1084	I	-	-	+	+	+	-
F1085	I	-	-	+	+	+	-
F1090	I	-	-	+	+	+	Weak
F1097	I	-	-	+	+	+	-
F1106	I	-	-	+	+	+	-
EDL933	I	-	-	+	+	+	Weak
71074	I/II	-	-	+	-	-	+
59243	I/II	-	-	+	-	-	+
EC20020462	I/II	+	+	-	-	-	+
EC20030338	I/II	-	-	+	-	-	+
EC20030282	II	-	-	+	-	-	-
EC20030348	II	-	-	+	-	-	-
EC20030351	II	-	-	+	-	-	+
EC19920026	II	+	+	-	-	-	+
EC19920171	II	+	+	-	-	-	+
EC19920283	II	+	+	-	-	-	+
EC19930086	II	+	+	-	-	-	+
EC19930200	II	+	+	-	-	-	-
F1081	II	+	+	-	-	-	+
EC20030186	II	+	+	-	-	-	+
EC20030216	II	+	+	-	-	-	+
EC20030226	II	+	+	-	-	-	-
EC20030275	II	+	+	-	-	-	+
EC20030277	II	+	+	-	-	-	+
EC20030278	II	+	+	-	-	-	+
EC20030289	II	+	+	-	-	-	+
EC20030340	II	+	+	-	-	-	+
EC20030495	II	+	+	-	-	-	+
LS68	II	+	+	-	-	-	+
FRIK 920	II	+	+	-	-	-	+
FRIK 1999	II	+	+	-	-	-	+
LRH13	II	+	+	-	-	-	+
R1797	II	+	+	-	-	-	+
EC20000623	II	+	+	-	-	-	+
EC19920283	II	+	+	-	-	-	+

Strain continued	Lineage	sbcB/ phage right junction[a]	sbcB phage left junction[b]	sbcB intact[c]	wrbA/ phage right junction[d]	wrbA/ phage left junction[e]	wrbA intact[f]
EC19930086	II	+	+	-	-	-	+
R1797	II	+	+	-	-	-	+
LRH13	II	+	+	-	-	-	+
EC20030528	II	-	-	+	-	-	+
EC20030547	II	+	+	-	-	-	+
EC20030292	II	+	+	+	-	-	-
FRIK 1985	II	+	+	-	-	-	+
R834	II	+	+	+	-	-	+
EC20030154	II	+	+	-	-	-	+
FRIK 1990	II	+	+	+	-	-	+
FRIK 2001	II	+	+	+	-	-	+
EC19920005	II	+	+	-	-	-	+
EC19920192	II	+	+	-	-	-	+
EC19970520	II	+	+	-	-	-	+
EC20030317	II	+	+	-	-	-	+
12491	II	+	+	+	-	-	+
EC20030193	II	+	+	-	-	-	+
EC20030223	II	+	+	+	-	-	+
EC20030281	II	-	+	+	-	-	+
EC20030422	II	+	+	+	-	-	+
EC20011139	II	+	+	+	-	-	+
AA579-2	II	-	-	+	+	+	-
EC20030496	II	-	+	+	-	-	+
EC20030497	II	+	+	+	-	-	+

EC20030281 and EC20030496 were only positive in one *sbcB* phage junction. The *sbcB* gene was interrupted in 33/49 (67.3%) of the lineage II strains but 32.6% (16/49) still contained an intact *sbcB* gene with these primers. Five lineage II strains were similar to lineage I strains as they were negative for *sbcB* chromosome-junctions and contained an intact *sbcB* gene. In the lineage I/II strains, only strain Zap0046 was negative with *sbcB* primers and positive for EC970520 phage junctions at the *sbcB* gene.

Oligonucleotide primers specific to the phage-chromosome junctions and intact gene identified within the *wrbA* gene insertion site identified by Shaikh and Tarr (2003) also were used to screen each of the strains. Most lineage I strains 55/59 (93.2%) were positive for *wrbA* chromosome-phage junctions with the exception of strains F801 and R1388, both of which were only positive for the right side of the insert. Most lineage I strains, 56/59 (94.9%) also were negative or had a weak PCR product for an intact *wrbA* gene. The lineage I strains, E1572 and 107718, were negative for *wrbA* phage junctions and positive for an intact *wrbA* gene. Most lineage II strains, 48/49 (98.0%) were negative for *wrbA* chromosome-phage junctions and most 43/49 (87.8%) were positive for intact *wrbA* genes. Lineage II strain AA579-2 was positive for the *wrbA* chromosome-phage junction, but negative for an intact *wrbA* gene. All lineage I/II strains had intact *wrbA* genes and no phage integration. Overall, most lineage I strains were positive for *wrbA* phage integration and negative for *sbcB* phage integration while lineage II strains were positive for *sbcB* integration and negative for *wrbA* integration (Table 2.3.3.). The Stx2c in lineage I/II strains did not appear to integrate in either *sbcB* or *wrb*A with the exception of Zap0046 which is integrated at *sbcB*.

2.4. Discussion

2.4.1. Sequence of the Stx2c phage genome

The genome of EC970520 Stx2c phage is highly mosiac. It contains similar regions to several bacteriophages indicating that many recombination events may have occurred to create it (Table 2.3.1.). This is consistent with the theory that the majority of phage have common ancestry and undergo an exchange of genetic elements from a common gene pool (Hendrix *et al.*, 1999). This can also be seen in the downstream structural proteins as these genes are composed from a much larger pool of sequences from other bacterial species, which is similar to the Stx2 phage 933W (Plunkett *et al.*, 1999). The entire sequence is flanked by direct repeats which may be involved in phage integration into the bacterial genome. Temperate phage integrate into their hosts by site-specific recombination between short common sequences within the phage (attP) and the bacteria (attB) which generate two flanking sequences within the prophage (attR and attL) (Makino *et al.*, 1999). The presence of these repeats in the Stx2c phage provide additional evidence of the integration site, *sbcB,* within the EC970520 genome.

The Q gene found within the sequence functions as an antiterminator for the expression of late genes downstream including the Stx2 genes. The location of these toxin genes within EC970520 with the late phage genes demonstrates that these genes may only be expressed during the lytic cycle when phage are released from the bacterial genome. Plunkett *et al.* (1999) have suggested that Stx2 in EDL933 may be expressed by a Q-dependent promoter and that this expression would be expected to increase after phage induction. Presence of IS elements represents possible inhibitors to Q-dependent expression present in the EC970520 Stx2c phage. Upstream of the Q gene, an IS element

(ISEC8) is present in EC970520 which may reduce the transcription of Q-dependent expression of downstream lytic genes. In Sakai, an IS element IS629 is found upstream of the lysis genes and this element may have been inserted due to a nonhomologous recombination event or improper excision of bacterial genes after phage transduction and may be responsible for the absence of plaque-forming ability of the phage (Makino *et al.*, 1999; Creutzburg *et al.*, 2005). A transposase homolog found within IS629 was found downstream of the Stx2c genes in EC970520 and the presence of this element may also inhibit the ability of phage release after phage induction. This inability to release Stx2 phage by IS may reduce the virulence of the strain as lytic infection and hence Stx2 expression may be hindered. Not all Stx2 phages contain these IS elements which may explain why some phages are capable of lytic infection whereas others such as Sakai are reported not to be lytic (Sato *et al.*, 2003). It is also possible that some defective proteins involved in lytic induction may be provided by other prophage lysogenized in the same strain although this has not been fully explored (Makino *et al.,* 1999).

The complete sequence for the Stx2c genome from *E. coli* O157:H7 strain EC970520 was 62,147 bp which is larger than those obtained from other Stx2 phage genomes such as Stx2Φ-I (61,765), VT2-Sa (60,942) and 933W (61,663), but smaller than the phage genome VT2-Sakai (62,703) (Sato *et al.*, 2003). Homology searches to these Stx2 phage genomes revealed several conserved and divergent genes surrounding the Stx2c phage genes of EC970520 (Figure 2.3.2.). Similar to other Stx2 variants, EC970520 contained >99% similarity to the A subunit and ~96% identity to the B subunit of Stx2 (Strauch *et al.,* 2004). The low homology of the *AR* and *Q* to other Stx2 genomes may account for differences in Stx2 expression between strains since the Q

protein and the anti-repressor are important regulators of gene activation governing cell lysis. This is similar to Lejeune *et al.* (2004) who reported that a lack of phage-encoded *Q* gene may produce less Stx2. In EC970520, a different type of *Q* gene or *AR* may result in less Stx2 toxin release.

2.4.2. Lineage specificity of Stx2 phage genomes

The Stx2c phage sequence from EC970520 produced similar PCR profiles to Stx2c phage from other lineage II strains. The lineage II strains all carried Stx2c and all were similar in band size for regions surrounding Stx2c in EC970520 although there was a small range of PCR product sizes and therefore variability in regions furthest downstream (Figure 2.3.1.; Table 2.3.2.). The lineage I, I/II strains all contained Stx2 genes found in phage 933W and were divergent from EC970520 in this region except for the downstream Stx2c region where a larger product was amplified in lineage I strains (Figure 2.3.4.; Table 2.3.2.). It is likely that Zap0046 has one copy of Stx2 and one copy of Stx2c in phage which are distinct from EC970520 since both of these genes were amplified but the strain did not contain all genes found in EC970520. A closer examination of more genes from the 933W Stx2 phage will determine if the Stx2 phage is lineage-I and I/II specific.

2.4.3. Integration of the Stx2 phage in *E. coli* O157:H7 strains

Four insertion sites for Stx phage have been determined thus far for *E. coli* O157:H7 strains which include *wrbA*, *yehV*, and *sbcB*, and *yecE* (Ohnishi *et al.,* 2002). The preferred site may depend on the host and availability of the site in the host strain.

The insertion site for EC970520 was determined to be *sbcB* since the gene is found intact in K12 but interrupted by phage sequence and direct repeats at integration sites in EC970520. This is the same integration site of the *E.coli* O157:H7 Stx2 transducing phages found by Ohnishi *et al.*, 2002. Analysis of the *sbcB* integration site determined that most lineage II strains had phage DNA inserted into the *sbcB* site whereas lineage I strains did not (Table 2.2.3.). The lineage I strain, R1388, may contain more than one copy of Stx2 so a Stx2c phage found in lineage II strains may occupy the interrupted *sbcB* site. The strains positive for phage DNA only on one side may have integrated at slightly different sites within *sbcB* or may have been duplicated and improperly excised. A subset of lineage II strains which contain intact *sbcB* genes likely were integrated at a different site. Most lineage I Stx2 phage occupy the *wrbA* sites although there is commonly a faint band for the intact *wrbA* band which is found with DNA from only this lineage. Shaikh and Tarr (2003) proposed the faint intact *wrbA* bands may be due to a subset of bacteria that no longer contain the inserted element; *i.e.*, the phage has been lost. This apparent instability of the Stx2 phage DNA in lineage I strains may increase expression of lysis and toxin genes, promote recombination and the ability to release phage and create new pathogenic strains. No lineage II strains except for AA579-2 were found to have phage DNA inserted into the *wrbA* site. AA579-2 may also be distinct from other lineage II strains due to the presence of multiple copies of Stx similar to Zap0046. It has been suggested that the insertion site occupancy may be determined by the availability of a preferred locus in the host strain (Serra-Moreno *et al.*, 2007). It seems the *wrbA* site is the preferred site for Stx2 phage while *sbcB* is the preferred site for Stx2c phage and *yehV* is used by Stx1 phage. It is important to note that there is an intact *wrbA*

gene in most lineage II strains although these strains still choose *sbcB* and this is opposite for lineage I strains where there is a presence of intact *sbcB* but these strains choose *wrbA* sites so the availability of a locus site may not be the only factor in deciding how these phages integrate. The data suggests that there are Stx phage-specific integration sites. Most lineage I/II strains did not integrate into *sbcB* or *wrbA* as most of these sites appeared to be available in these strains. It is likely that these strains use another integration site not considered in the present study. These lineage I/II strains may have an integration site unique to the Stx2 phage found in these strains. However, it is also interesting to note that the Stx2 phage in lineage I/II strains seem to share a number of phage loci with 933W (see Table 2.3.1.). The phage in lineage I/II strain Zap0046 presents a somewhat confusing situation in that both Stx2 and Stx2c are present in this strain and the *sbcB* site is occupied while the *wrb*A site is not. It is tempting to conclude that the Stx2c phage is occupying the *sbc*B site and the Stx2 phage is present in the undefined lineage I/II-specific Stx2 phage site mentioned above. However, further study will be required to determine the nature of the Stx2 phage in lineage I/II strains and identify the integration site (s). It has been suggested that the attL regions or types of integrase could cause differences in the selection of insertion sites (Serra-Moreno *et al.*, 2007). Phage acquisition and interaction into specific sites and phage stability in bacterial chromosomes may play an important role not only in the pathogenicity of certain strains of *E. coli* O157:H7 and lineages but also aid in understanding the evolution of new pathogenic strains.

2.5. Conclusions

Little is known about phage associated with Stx2c production in lineage II strains. In this study,

1) Sequencing of a Stx2c prophage from the *E.coli* O157:H7 strain EC970520 revealed a 62,147bp genome with an organization similar to that of other Stx-encoding lambdoid phage. This genome is highly mosaic and contained several phage gene homologs implying that these phages have undergone several recombination events over time.
2) Annotation of the genes revealed the integration site of the EC970520 Stx2c phage to be the *sbcB* gene. IS elements found within the genome may interrupt Q-dependent expression and lytic activity of the phage. This inability to release Stx2 phage may reduce the virulence of the strain since Stx2 expression may be hindered.
3) The Stx2c phage are highly lineage-II specific and contain Stx2c phage genes found in strain EC970520.
4) The Stx2 genes from EDL933 are lineage I and I/II specific. Comparison of EC970520 Stx2c and EDL933 Stx2 identified several divergent regions between the lineages including the Q-antiterminator protein. Differences between lineages in this gene may account for in differences in Stx2 expression between the lineages.
5) The Stx2c phage integration site is *sbcB* for lineage II strains and *wrbA* for Stx2 phage in lineage I strains. Most lineage I/II strains integrate at a third undefined site in the host genome. The lineage I Stx2 phage appear to be excised from *wrbA*

in a minority of bacteria in the population. This instability of Stx2 phage may contribute to the level of Stx2 expression and dissemination of this bacteriophage.

The information determined from this study will be used in future studies. First, conserved and variable regions identified within the Stx2 phage genomes will be used in the specific detection of *E. coli* O157:H7 isolates as well as in detecting lineage-specific genetic variations within this serotype. The distribution of *E. coli* O157:H7 Stx2 phage genes among other STEC serotypes will also be studied to determine if any of these phage genes are widely disseminated or restricted to certain pathogenic STEC clones. Further, STEC gene presence/absence data will be used to genotype STEC strains. This study has helped us to understand if genotypic differences in Stx phage observed *in E. coli* O157:H7 strains contribute to phenotypic differences observed between the lineages of this organism.

CHAPTER THREE

3. Detection of Shiga toxin-producing bacteriophage in *E. coli* O157:H7 and other Shiga toxin-producing *E. coli*

3.1. Introduction

Shiga toxin-producing *Escherichia coli* (STEC) including *E. coli* O157:H7 are food- and water borne zoonotic bacterial pathogens capable of causing outbreaks of severe illness in humans (Parry and Palmer, 2002; Herold *et al.,* 2004). *E. coli* O157:H7 is responsible for numerous outbreaks of HC which can result in HUS and is the STEC serotype most frequently isolated from clinical illness in humans in North America (Boyce *et al*., 1995; Johnson *et al*., 1996; Besser *et al*., 1999; Brooks *et al*., 2005). Cattle act as reservoirs of these pathogens and spread the bacteria via contaminated feces, in food and water (Spika *et al.*, 1996). STEC can also be spread to vegetables, fruits and other plants through irrigation water or soil contaminated with fecal material (Meng and Doyle, 1996; Besser *et al.*, 1999). Since *E. coli* O157:H7 is most commonly associated with human disease, it poses a significant risk to human health and the need to develop rapid and specific methods for detecting this pathogen in food and water are needed.

Culture-based techniques used in the detection of STEC can be biased and exclude viable but non-culturable cells (Dunbar *et al.*, 2003). In addition, culture based tests are generic in nature and additional techniques are required to identify the species, serotype and molecular subtype of the organism isolated. This typing information is essential in determining the source of infection in outbreak investigations. Several molecular subtyping and fingerprinting methods have been developed for *E. coli* strains. These include phage typing, plasmid profile analysis, antimicrobial susceptibility testing and ribotyping. However, these methods are not discriminating enough for determining

the epidemiological relatedness of strains (Khakhria *et al.*, 1990; Barrett *et al.*, 1994; Grif, *et al.*, 1998; Strockbine *et al.*, 1996). Pulsed-field gel electrophoresis (PFGE), random amplification of polymorphic DNA (RAPD) and multi-locus enzyme analysis (MLE) also been used to characterize isolates and PFGE is currently considered the "gold standard" among the molecular typing methods (Prager *et al.*, 2002; Grif *et al.*, 1998). However, many of these techniques are also time-consuming, expensive, require multiple tests and are unable to give us specific information about the gene content of the organism or phenotype of the strain of interest. Planar microarrays provide large amounts of specific genetic information but can be time-consuming, expensive and laborious. New methodologies such as bead-based suspension microarrays may provide an alternative to current molecular *E. coli* typing methods such as PFGE, with similar or greater discrimination, more efficiently, more quickly and at a lower cost.

The Bioplex suspension array technology uses beads which promote 3-D hybridizations to nucleic acids which are more advantageous than traditional planar microarrays allowing faster and cheaper detection of genes (Spiro *et al.*, 2000). The bead suspension array incorporates up to 100 different types of microspheres (beads) with each bead containing an internal fluorescent dye with its own spectral address. This spectral address is specific to a given probe, nucleic acid, protein or otherwise, and assays can be carried out in a single reaction vessel (Biorad, CA). A reporter dye in conjunction with hybridized beads is interrogated by two lasers (red and green) in the Bioplex and digitally processed based on the reported fluorescence signals and quantified. The technology can be used to target PCR amplified products and has great multiplexing potential as it is able to target up to 100 different analytes in any given reaction which reduces time and cost of

the assay. Direct DNA hybridizations, in particular using multiplexed PCR DNA in which several genes are amplified in a single reaction, have lead to many applications of the technology such as genotyping, multi-virus detection assays and bacterial gene detection (Smith *et al.*, 1998; Deregt *et al.*, 2006; Wilson *et al.*, 2005). Multiplexed assays which target agents such as HIV, hepatitis C and herpes simplex virus have enabled detection of these agents in patients susceptible to multiple infections (Smith *et al.*, 1998). An eight probe multiplex for animal pestivirus which used asymmetric PCR to allow strand-biased amplification for increased signal detection and a 10-plex PCR assay for biothreat agents have been developed for simultaneous detections of probes and can be expanded for use in genotyping strains of a specific virus (Deregt *et al.*, 2006; Wilson *et al.*, 2005).

Gannon *et al.* (1997) have developed a multiplex PCR assay for specific identification of *E. coli* O157:H7 based on the genes eaeO157, VT1, VT2 and FlicH7. The eaeO157 gene encodes the protein intimin which is responsible for attaching and effacing of microvilli found on intestinal cells of the host. Stx's 1 and 2 (VT1 and VT2) are phage-encoded toxins released by the bacteria which damage human microvascular endothelial cells and lead to blockage of the vascular cells and tissue damage in the colon, kidneys and central nervous system (Brussow, *et al.*, 2004). The FlicH7 gene encodes a flagellar antigen and was included to increase specificity of the multiplex PCR (Gannon *et al.*, 1997). Based on this multiplex, the same gene targets would be expected to provide specific detection of *E. coli* O157:H7 in the Bioplex suspension array. The Bioplex technology can subsequently be used to identify genetic variations within *E. coli* O157:H7 strains. Within *E. coli* O157:H7 strains, octamer based genome scanning has

revealed the presence of two lineages; lineage I (LI) is associated with human and bovine isolates while lineage II (LII) is associated with mainly bovine isolates (Kim *et al.*, 1999). As LI strains appear to be more virulent in humans than LII strains, the presence of these Lineage-specific phage genes may also provide insight into their potential for production of human disease in other STEC strains. The seropathotype (SP) scheme has been developed to facilitate identification of the rate of occurrence of strains responsible for human disease (Karmali *et al.*, 2003). The presence of LI or LII related phage genes found within *E. coli* O157:H7 may also be related to SP strains and higher frequencies of human disease occurrence.

In this study we detect Stx toxin-producing bacteriophage variation among *E. coli* O157:H7 isolates and STEC and consider the evolution of these bacteriophage. The objectives of this study were:

1) Specific detection of genes found within *E. coli* O157:H7 and STEC Stx-encoding bacteriophage
2) An examination of the variation in specific genes within *E. coli* O157:H7 bacteriophage encoding Stx2 and Stx2c.
3) An examination of the variation in specific genes in Stx-encoding bacteriophage found in STEC strains from different seropathotypes.

3.2. Materials and Methods

3.2.1. Bacterial Strains

In this study, thirty *E. coli* O157:H7 strains from Zhang *et al*. (2007) previously described in chapter two were used (Table 2.2.1.). These strains were isolated from different sources and contained several different phage types (PT) and lineage-specific polymorphism types (LPSA). Lineage I/II strains were treated as subgroups often within lineage I strains but were identified as L I/II strains. Forty-six *E. coli* strains (Table 3.2.1.) classified based on seropathotype (SP) by Karmali *et al*. (2003) were used for the SP studies as described below.

3.2.2. Nucleic Acid Probe and Primer Design

The Bioplex suspension array system (Bio Rad, CA) was used for identifying *E. coli* O157:H7 strains and screening lineage-specific and SP collections for strains possessing phage genes. All probes and primers were purchased from Integrated DNA technologies (IDT). Each probe contained a 5'amino group modified with a C12 spacer to allow coupling to carboxylated microspheres. The reverse primers were coupled to a 5' biotin group to allow interaction with the reporter dye, streptavidin (Figure 3.2.1.). Probes for detection of *E. coli* O157:H7 were designed from DNA sequences targeting *E. coli* O157:H7 regions of the Shiga toxin 1 (VT1), Shiga toxin 2 (VT2), intimin (eaeO157) and Flagella (FlicH7) previously described in the multiplex PCR by Gannon *et al.* (1997) (Table 3.2.2.). Upstream phage genes for the probes were chosen from conserved and variable regions found within EC970520 and EDL933. Each

Table 3.2.1. *E. coli* strain seropathotype collection previously used by Karmali *et al.*, 2003.

Strain #	SEROTYPE	HOST	SOURCE	SEROPATHOTYPE
OK1	O157:H7	Human	T.Whittam	A
93111	O157:H7	Human	T.Whittam	A
Sakai	O157:H7	Human	Osaka,Japan	A
EDL933	O157:H7	Human	J.Kaper	A
E32511	O157:NM	Human	H.Smith	A
R82F2	0111:NM	Human	LFZ	B
N01-7015	0103:H2	Human	BCCDC,NLEP	B
CL9	026:H11	Human	LFZ	B
CL1	026:H11	Human	LFZ	B
NO2-1626	0103:H2	Human	BCCDC,NLEP	B
N00-6496	0145:NM	Human	BCCDC,NLEP	B
N01-2454	0103:H2	Human	BCCDC,NLEP	B
Z3F1	0121:H19	Human	LFZ	B
CL106	0121:H19	Human	LFZ	B
CL101	0111:NM	Human	LFZ	B
NO1-2051	0145:NM	Human	BCCDC,NLEP	B
N02-5149	0145:NM	Human	BCCDC,NLEP	B
C69F1	0111:NM	Human	LFZ	B
CL4	O26:H11	Human	LFZ	B
N99-4390	0121:NM	Human	BCCDC,NLEP	C
N89-0541	0113:H121	Human	APLPH	C
EC7-181	091:H21	Human	LFZ	C
N90-0657	0113:H121	Human	CPL	C
B2F1	091:H21	Human	LFZ	C
G5506	0104:H121	Human	T.Whittam	C
N00-4541	05:NM	Human	BCCDC,NLEP	C
N00-4067	05:NM	Human	BCCDC,NLEP	C
N99-3504	0113:H121	Human	BCCDC,NLEP	C
EC6-936	091:H21	Human	L.Beutin	C
EC6-990	091:H21	Human	S.Alessic	C
CL3	0113:H121	Human	LFZ	C
N00-4859	0103:H25	Human	LFZ	D
N02-2616	0103:H25	Human	BCCDC,NLEP	D
N02-4495	0117:H7	Human	BCCDC,NLEP	D
EC7-821	069:H11	Human	LFZ	D
N02-1625	0146:H21	Human	BCCDC,NLEP	D
EC6-484	0172:NM	Bovine	LFZ	D
EC2-032	0171:H2	Bovine	LFZ	D
EC6-371	0113:H4	Bovine	LFZ	D
EC2-265	O119:H25	Human	LFZ	D
EC9-377	098:H25	Bovine	LFZ	E
EC6-626	06:H34	Bovine	APLPH	E
EC4-453	088:H25	Bovine	LFZ	E
EC2-293	039:H49	Bovine	LFZ	E
EC2-020	0156:NM	Bovine	LFZ	E
EC2-211	0113:NM	Bovine	LFZ	E

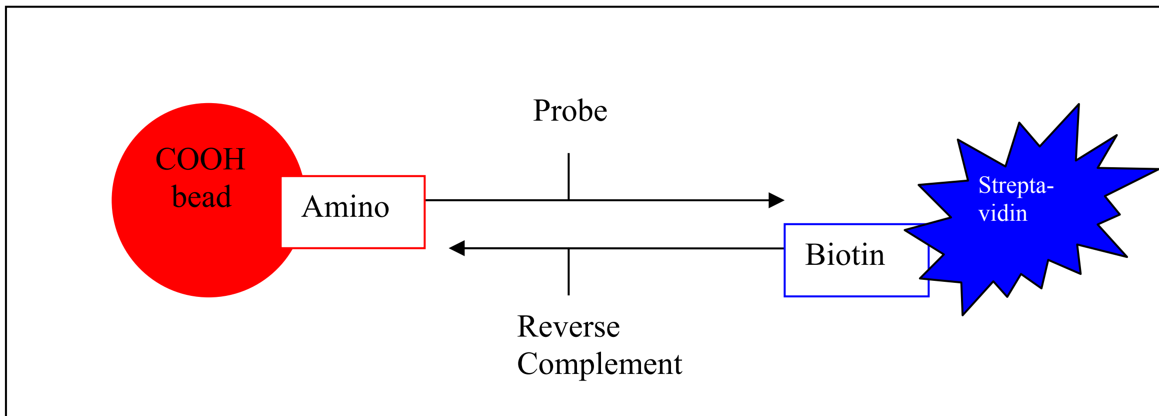


Figure 3.2.1. Hybridization of probes with their reverse complement.
Beads with carboxy (COOH) attachments interact with the amino modified probe and the reporter dye streptavidin interacts with the biotin of the reverse complement. Under optimal conditions, the probe and reverse complement hybridize.

Table 3.2.2. Nucleotide probes used in Bioplex suspension arrays for identification of *E. coli* O157:H7 and upstream phage genes.
All probes were designed with a 5'amino group which was coupled to a C12 spacer linked to a fluorescently tagged microsphere.

Probes	Probe Sequence with 5'amino modified C12 (5'-3')	Size (bp)	Melting Temperature (˚C)
VT1	AGTATACAAAATATAATGATGACGATACCTTTACA	35	59.9
VT2	ATAAAAATAAACAATACATTATGGGAAAGTAATAC	35	60.0
eaeO157	TAAGGATGCTATTAAATATACTGTAAAAGTTATGA	35	59.9
FlicH7	CTTTAACGTAAATGGTAAAGGTACTATTACCAAC	34	61.6
AR-933	GGCAAATAACTATGAGGATGTTACATGGTC	30	57.4
AR-970520	GTTCTTTAATCTCGATTACGACAAAGAAAT	30	54.3
Q-933	AAATCGCATATAGCACTATTAGTTTTCTAAATATT	35	54.3
Q-970520	TATGACAAGGCCAATGTGTAATGATGATGATGGAA	35	60.9
Stx2B-933	AGATACTCGTTTTAATAATCGAAATCATTTTAACC	35	55.0
Stx2cB-970520	CGATGATTAAAACACTTATCTGTGATATTTCCTGT	35	57.2
Roi	GTCAATACAAATCACGGAATACAGATATCG	30	55.7
Stx2A	TACTTTCTACCGTTTTTCAGATTTTACACATATAT	35	55.4

probe was designed using the primer design program, Primer 3, with probe lengths 30-35bp, melting temperatures 54-61 °C and checked for secondary structures using the IDT oligo analyzer program (Table 3.2.2.).

3.2.3. PCR for multiplexes and Bioplex assays

PCR's were designed using the primers from Table 3.2.3. Genomic DNA templates were amplified in 50 μl reaction volumes which contained 200 μM of each deoxynucleoside triphosphate (Qiagen), each primer at 10 pmol, 1X PCR buffer (Qiagen), 1 U DNA Taq polymerase (Qiagen) and 1μl template (100 ng/μl). Negative control incorporated all reagents of the PCR except water was used as a template. The reactions were amplified using a 9600 Gene Amp thermocycler (Applied biosystems Inc, CA). PCR was conducted as follows: 3min denaturation at 94 °C, followed by 25 cycles of 45 s at 94 °C, 30 s at 55 °C and 90 s at 72 °C with a final 10min 72 °C extension step. PCR products were visualized under UV light on a 1.0% (w/v) agarose gel stained with ethidium bromide. DNA molecular 1kb plus ladders (Invitrogen) were included in each gel electrophoresis run. To increase detection, asymmetric PCR was used to preferentially amplify the reverse biotinlyated strand more than the forward strand since the probe and reporter dye only interact with the reverse strand to produce a signal and therefore should increase the signal intensity. The amount of reverse primer was varied

Table 3.2.3. Primers used in Bioplex suspension arrays for identification of *E. coli* O157:H7 and upstream phage genes.
All reverse primers were designed with a 5'Biotin which was coupled to the reporter dye, streptavidin-R-phycoerythrin.

Probes	**Forward primer (5'-3')**	**Reverse primer with 5'Biotin (5'-3')**	**Expected Size (bp)**	**Source sequence**
VT1	AGCTGCATCGCTTTCATTTT	TGCGCACTGAGAAGAAGAGA	171	H19B
VT2	CCATGACAACGGACAGCAGTT	CCTGTCAACTGAGCACTTTG	779	Gannon *et al.* (1997)
eaeO157	AATAGTGCCAAAACGGATGC	TTACCGTTGAACATCCCAAA	296	EDL933
FlicH7	TTGATGAAATTGACCGCGTA	GCGCCAGCAGAAGTTAAATC	229	E32511
AR-933	ATCAGACCGAGGTAGCCAGA	CGGGGATCAGTCCCTTAAAT	200	EDL933
AR-970520	AATCACATTCCCCTGGTTCA	GGCAAGAGCTTTAGCCAGAA	200	EC970520
Q-933	CGGAGGGGATTGTTGAAGGC	GCAAAATGCTGTTACGCAAA	700	Lejeune *et al.* (2004)
Q-970520	AATTCATGGAGAGCGTGGAG	AGGTCTTGCGACGCTATGAT	197	EC970520
Stx2B-933	TCATAGCAGGGGCCTTTTTA	TAAAATAAGGAGCGGGCTGA	200	ECL-933
Stx2cB-970520	GACACCGGACTGAACTCACC	CAACGTGGCCCCATTTAATA	245	EC970520
Roi	GATCGCGGCTATTTCACAGT	ATTAAGCAGCCTCCCCTGTT	157	EC970520
Stx2A	AATTTATATGTGGCCGGGTTC	TGACGACTGATTTGCATTCC	176	EC970520

but the rest of the PCR remain unchanged. A multiplex PCR assay for all lineage-specific genes, including the conserved genes was created under the same conditions to determine if the phage patterns produced by the multiplex assays also could be found in other strains of *E. coli* O157:H7.

3.2.4. Coupling of beads to Nucleic Acid probes

Beads with carboxy (COOH) attachments for coupling to amino group modified probes were purchased from Biorad and selected using the bead table guide provided by the Luminex Corp. (Luminex, TX). Beads (1.0×10^6) were Vortexed and sonicated for 30s, pelleted at 10,000x g for 3min and resuspended in 10 µl of 0.1 M MES (2[N-Morpholino] ethanesulfonic acid) (Sigma) pH 4.5. Nucleic acid probes (0.10 nmol) were added to the resuspended beads with 2.5 µl of fresh 10 mg/ml EDC (1-ethyl-3-[3dimethylaminopropyl] carbodiimide hydrochloride) (Sigma) and incubated at room temperature for 30 min in the dark. A second addition of fresh EDC solution was added to the mix and incubation repeated. Coupled microspheres were washed with 0.02% Tween-20 (Sigma) followed by a wash with 0.1% SDS in TE (10 mM Tris HCl [pH8.0], 1mM EDTA). The beads were pelleted and resuspended in 20 µl TE. The suspension was enumerated using a hemacytometer and diluted to 150 beads/µl in 4.5 M Tris-HCl (pH 8.0), 75 mM EDTA (pH 8.0), 0.15% Sarkosyl. Coupled beads were stored in the dark at 4 °C.

3.2.5. Direct Hybridization

All hybridizations were done using 96-well PCR plates (Costar, MD). Stored beads were Vortexed and then sonicated for 30 s. A total of 24µl of beads (150beads/µl) was added to each well with 24 µl 1.5X TMAC (Tetramethyl ammonium chloride) (Sigma) hybridization solution (4.5 M TMAC, 4.5 M Tris-HCl (pH 8.0), 75 mM EDTA (pH 8.0), 0.15% Sarkosyl) in a 96-well Costar PCR plate. PCR product or complementary oligo was added to selected wells except for the background control and the plate was sealed and Vortexed. Hybridization was performed in a thermocycler programmed for a denaturation at 95 °C for 5min followed by a 20 min 50 °C hybridization. While the plate was still in the thermocycler, 1 µl of the reporter dye, streptavidin-R-phycoerythrin (0.1 mg/ml) (Invitrogen), was added to each well followed by a quick Vortex and incubated for another 20 min. Samples were mixed by pipetting up and down to prevent bead aggregation and analyzed in the Bioplex 200 (Biorad, CA). The platform temperature was set at 50 °C with a bead count of 100. Assay conditions for coupling beads and hybridizations were optimized. To determine coupling efficiencies, reverse complements of the probe sequences were designed with 5'biotin modifiers for coupling to reporter dye, streptavidin-R-phycoerythrin. Coupling efficiencies for each probe were initially tested using reverse complement sequences.

3.3. Results

3.3.1. Specific detection of genes found within *E. coli* O157:H7 in STEC strains by multiplex PCR and O157 Bioplex assay

An O157 Bioplex assay was developed from a multiplex PCR using Stx 1 (VT1), Stx2 (VT2), FlicH7 and eaeO157 specific genes found within *E. coli* O157:H7. To test the O157 Bioplex assay for reproducibility and accuracy in detecting *E. coli* O157:H7, a collection of 30 *E. coli* O157:H7 strains (Table 2.2.1.) were assayed with the Bioplex following three different PCR protocols as well as testing the reproducibility of the Bioplex by testing replicates obtained from a single PCR experiment. All strains (30/30) that produced a target-specific PCR product on agarose gels also produced positive signals for their respective Bioplex probes (Table 3.3.1.). *E. coli* strains contain different combinations of Stx genes and the O157 Bioplex assay was able to accurately differentiate among strains which produced Stx1, Stx2 and both toxins. Most importantly, the O157 Bioplex assay was able to detect eaeO157 PCR products from all strains (30/30). An *E. coli* K12 and a blank DNA sample were used as negative controls and both produced negative signals. Forty-six non-O157 *E. coli* strains (Table 3.2.1.) were tested to determine the ability of the O157 Bioplex assay to detect specific genes found in other STEC strains. A positive isolate for *E. coli* O157:H7 contained eaeO157, FlicH7, and either Stx1, Stx2 or both. As expected, all (5/5) of *E. coli* O157:H7 strains were positive for these markers in the O157 Bioplex assay (Table 3.3.2.). All 46/46 STEC strains that produced a positive or negative Stx1 PCR band also produced their corresponding absolute positive or negative Stx1 signal on the Bioplex. Most Stx2 PCR positives were also detected in the Bioplex except for Stx2 genes from strains of

Table 3.3.1. Bioplex assay (O157) mean detection of *E. coli* O157:H7 specific genes in *E. coli* O157:H7 strains.
Mean detection in 1:1:2:3 (VT1:VT2:eaeO157:FlicH7) reverse primer ratio PCR multiplex assay. PCR results prior to O157 Bioplex assay are displayed. K12 and PCR water were used as negative controls.

Strain	Serotype	PCR positives	VT1	VT2	eaeO157	FlicH7
LRH16	O157:H7	VT1, VT2, eaeO157, FlicH7	+++	+	++	-+
LRH27	O157:H7	VT1, VT2, eaeO157, FlicH7	+++	+	++	+
LRH6	O157:H7	VT2, eaeO157, FlicH7	-	+	++	+
TS-97	O157:H7	VT1, VT2, eaeO157, FlicH7	+++	+	++	+
F1299	O157:H7	VT1, VT2, eaeO157, FlicH7	+++	-+	++	+
F5	O157:H7	VT1, VT2, eaeO157, FlicH7	+++	+	++	+
EDL933	O157:H7	VT1, VT2, eaeO157, FlicH7	+++	+	++	+
63154	O157:H7	VT1, VT2, eaeO157, FlicH7	+++	+	+++	+
58212	O157:H7	VT1, VT2, eaeO157, FlicH7	+++	+	++	+
F1095	O157:H7	VT1, VT2, eaeO157, FlicH7	+++	+	++	+
Sakai	O157:H7	VT1, VT2, eaeO157, FlicH7	+++	+	++	+
H4420	O157:H7	VT1, VT2, eaeO157, FlicH7	+++	+	++	+
E2328	O157:H7	VT1, VT2, eaeO157, FlicH7	+++	+	++	+
F1082	O157:H7	VT1, VT2, eaeO157, FlicH7	+++	++	++	+
59243	O157:H7	VT2, eaeO157, FlicH7	-	+	++	+
71074	O157:H7	VT2, eaeO157, FlicH7	-	+	++	+
EC20030338	O157:H7	VT2, eaeO157, FlicH7	-	+	++	+
Zap0046	O157:H7	VT2, eaeO157, FlicH7	-	+	+++	++
LRH13	O157:H7	VT1, VT2, eaeO157, FlicH7	+++	+	++	+
R1797	O157:H7	VT1, VT2, eaeO157, FlicH7	+++	+	++	+
LS68	O157:H7	VT1, VT2, eaeO157, FlicH7	+++	+	++	+

F12	O157:H7	VT1, VT2, eaeO157, FlicH7	+++	-+	++	+
F1081	O157:H7	VT1, VT2, eaeO157, FlicH7	+++	+	++	+
FRIK 920	O157:H7	VT1, VT2, eaeO157, FlicH7	+++	-+	++	+
FRIK 1999	O157:H7	VT1, VT2, eaeO157, FlicH7	+++	+	++	+
FRIK 1985	O157:H7	VT1, VT2, eaeO157, FlicH7	+++	+	++	+
FRIK 1990	O157:H7	VT1, VT2, eaeO157, FlicH7	+++	-+	++	+
FRIK 2001	O157:H7	VT2, eaeO157, FlicH7	-	+	+++	+
EC970520	O157:H7	VT1, VT2, eaeO157, FlicH7	+++	+	++	+
12491	O157:H7	VT2, eaeO157, FlicH7	-	+	+++	+
K12			-	-	-	-
PCR negative			-	-	-	-

- negative signal (MFI 0-500)
-+ weak signal (MFI 500-1000)
+positive signal (MFI 1000-3000)
++strong positive signal (MFI 3000-5000)
+++absolute positive signal (MFI 5000+)

Table 3.3.2. Bioplex assay (O157) mean detection of *E. coli* O157:H7 specific genes in STEC strains.
Mean detection in 1:1:2:3 (VT1:VT2:eaeO157:FlicH7) reverse primer ratio PCR multiplex assay. PCR results prior to O157 Bioplex assay are displayed. K12 and PCR water were used as negative controls.

***E. coli* strain**	**Serotype**	**PCR positives**	**VT1**	**VT2**	**eaeO157**	**FlicH7**
OK1	O157:H7	VT1,VT2,eaeO157,FlicH7	+++	+	++	+
93111	O157:H7	VT1,VT2,eaeO157,FlicH7	+++	+	++	+
Sakai	O157:H7	VT1,VT2,eaeO157,FlicH7	+++	+	++	+
EDL933	O157:H7	VT1,VT2,eaeO157,FlicH7	+++	+	++	+
E32511	O157:NM	VT2,eaeO157,FlicH7	-	+	++	+
R82F2	0111:NM	VT1,eaeO157	+++	-	-	-
N01-7015	0103:H2	VT1	+++	-	-	-
CL9	026:H11	VT1	+++	-	-	-
CL1	026:H11	VT1	+++	-	-	-
NO2-1626	0103:H2	VT1	+++	-	-	-
N00-6496	0145:NM	VT1,VT2,eaeO157	+++	+	+	-
N01-2454	0103:H2	VT1	+++	-	-	-
Z3F1	0121:H19	VT2	-	+	-	-
CL106	0121:H19	VT2	-	+	-	-
CL101	0111:NM	VT1,eaeO157	+++	-	-	-
NO1-2051	0145:NM	VT2,eaeO157	-	+	+	-
N02-5149	0145:NM	VT1,VT2,eaeO157	+++	+	++	-
C69F1	0111:NM	VT1,eaeO157	+++	-	-	-
CL4	O26:H11	VT1	+++	-	-	-
N99-4390	0121:NM	VT1,FlicH7	+++	-	-	+
N89-0541	0113:H121	VT2	-	-	-	-
EC7-181	091:H21	VT2	-	+	-	-
N90-0657	0113:H121	VT2	-	-+	-	-
B2F1	091:H21	VT2	-	+	-	-
G5506	0104:H121	VT2	-	-+	-	-
N00-4541	05:NM	VT1	+++	-	-	-
N00-4067	05:NM	VT1	+++	-	-	-
N99-3504	0113:H121	VT2	-	-+	-	-
EC6-936	091:H21	VT2	-	+	-	-
EC6-990	091:H21	VT2	-	-+	-	-
CL3	0113:H121	VT2	-	+	-	-
N00-4859	0103:H25	VT1,eaeO157	+++	-	-	-
N02-2616	0103:H25	VT1,eaeO157	+++	-	-	-

E. coli strain continued	Serotype	PCR positives	VT1	VT2	eaeO157	FlicH7
N02-4495	0117:H7	VT1,FlicH7	+++	-	-	+
EC7-821	069:H11	VT1	+++	-	-	-
N02-1625	0146:H21	VT1	+++	-	-	-
EC6-484	0172:NM	VT2	-	-	-	-
EC2-032	0171:H2	VT2	-	-+	-	-
EC6-371	0113:H4	VT1,VT2	+++	-	-	-
EC2-265	O119:H25	VT2	-	++	-	-
EC9-377	098:H25	VT1	+++	-	-	-
EC6-626	06:H34	VT2	-	-	-	-
EC4-453	088:H25	VT2	-	+	-	-
EC2-293	039:H49	VT1,VT2	+++	-+	-	-
EC2-020	0156:NM	VT2	-	+	-	-
EC2-211	0113:NM	VT2	-	++	-	-
K12			-	-	-	-
PCR –ve			-	-	-	-

- negative signal (MFI 0-500)
-+ weak signal (MFI 500-1000)
+positive signal (MFI 1000-3000)
++strong positive signal (MFI 3000-5000)
+++absolute positive signal (MFI 5000+)

serotypes 0113:H121, 0172:NM, 0113:H4 and 06:H34. The eaeO157 Bioplex probe was PCR and Bioplex positive for all O157:H7 strains and all three 0145 strains as well as an EPEC O55:H7 strain. PCR products for eaeO157 were generated from two 0103 and three 0111 strains and produced the expected band size; however, they were not positive in the Bioplex assay. The PCR and Bioplex FlicH7 probe was able to detect all H7 bearing STEC strains.

3.3.2. Variation in Stx2 and Stx2c-encoding bacteriophage genes in EC970520 and EDL933

Primers specific to the Stx2 upstream genes found in the LI strain EDL933 and Stx2c upstream genes found in LII strain EC970520 were developed to examine variation of the phage-specific genes within *E. coli* strains using the Bioplex assay. PCR primers specific to genes coding for a putative anti-repressor protein (AR-933), an anti-terminator Q protein (Q-933) and B subunit found in Stx2 (Stx2B-933) were chosen to identify genes found within the Stx2 phage from EDL933. Other PCR primers specific to Stx2c variant found in EC970520 also were developed: *i.e.*, gene segments specific to a putative anti-repressor protein (AR-970520), an anti-terminator Q protein (Q-970520) and B subunit found in Stx2c (Stx2B-970520). Primers specific for conserved Stx2 phage genes coding for a DNA-binding protein (Roi) and A subunit in Stx2 were chosen as positive controls which would produce amplified products in both EDL933 and EC970520. The EDL933 PCR primers (AR-933, Q-933 and Stx2B-933) were able to amplify gene segments when DNA from *E. coli* O157:H7 strain EDL933 was used as a template but not EC970520 (Figure 3.3.1.). Similarly, EC970520 primers (AR-970520,

Q-970520, Stx2B-970520) were able to amplify gene segments when DNA from *E. coli* O157:H7 strain EC970520 was used as a template but not EDL933 (Figure 3.3.1.). Expected bands were amplified in both strains EDL933 and EC970520 for the conserved Stx2A and Roi targets. No bands were amplified in *E. coli* K12 or distilled water, both of which were PCR negative controls.

3.3.3. Identification of genes specific to Stx2 and Stx2c-encoding phage in *E. coli* O157:H7 strains in the Stx2 phage Bioplex assay

A Stx2 phage Bioplex assay was developed to determine if the variation of phage genes from LI strain EDL933 and LII strain EC970520 can be used to identify these gene targets in other *E. coli* O157:H7 strains. Multiplexes for each gene from both lineages were prepared for use with the PCR products in the Stx2 phage Bioplex assay. Multiplex PCR product detection was compared to uniplex PCR's in the Bioplex assay to ensure that the products produced were the same for both and was found to be useful in discriminating products between primer sets when the PCR products were similar in size (Figure 3.3.2.; Figure 3.3.3.). The multiplex and uniplex PCR products produced similar signals in the Bioplex assay, therefore, multiplex PCR assay products were employed in the Bioplex assay in subsequent experiments. In all LII strains, 12/12 strong positive signals were obtained in the Bioplex assay for all EC970520 Stx2-encoding bacteriophage upstream genes and negative signals were obtained for all EDL933 Stx2-encoding bacteriophage upstream genes (Table 3.3.3.). For LI *E. coli* O157:H7

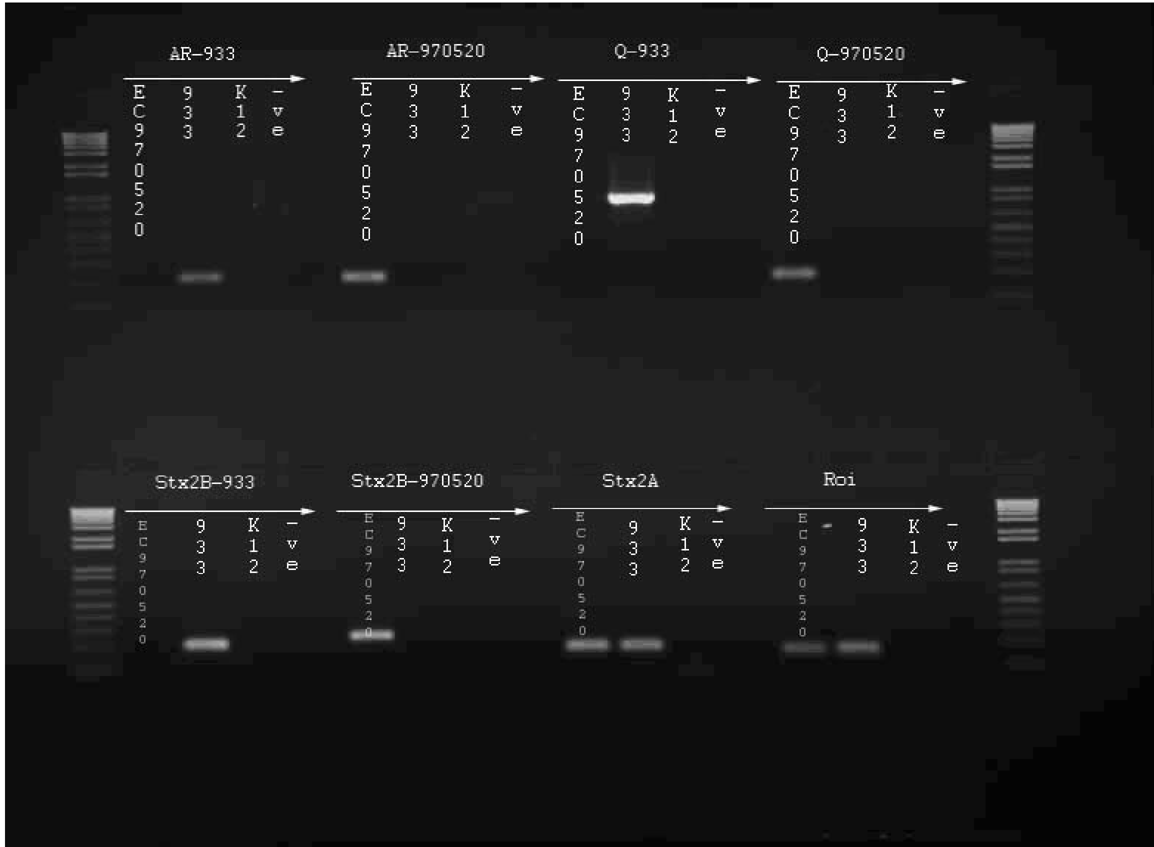


Figure 3.3.1. Expected band sizes using primers based on sequences of Stx2 (EDL933) and Stx2c (EC970520) upstream phage genes.
Each primer set: AR-933; AR-EC970520; Q-L 933; Q-970520; Stx2B-933; Stx2B-970520; Stx2A; Roi was screened for PCR products using the strains EC970520 (LII), EDL933 (LI), K12 (no Stx phage), and PCR negative (water).

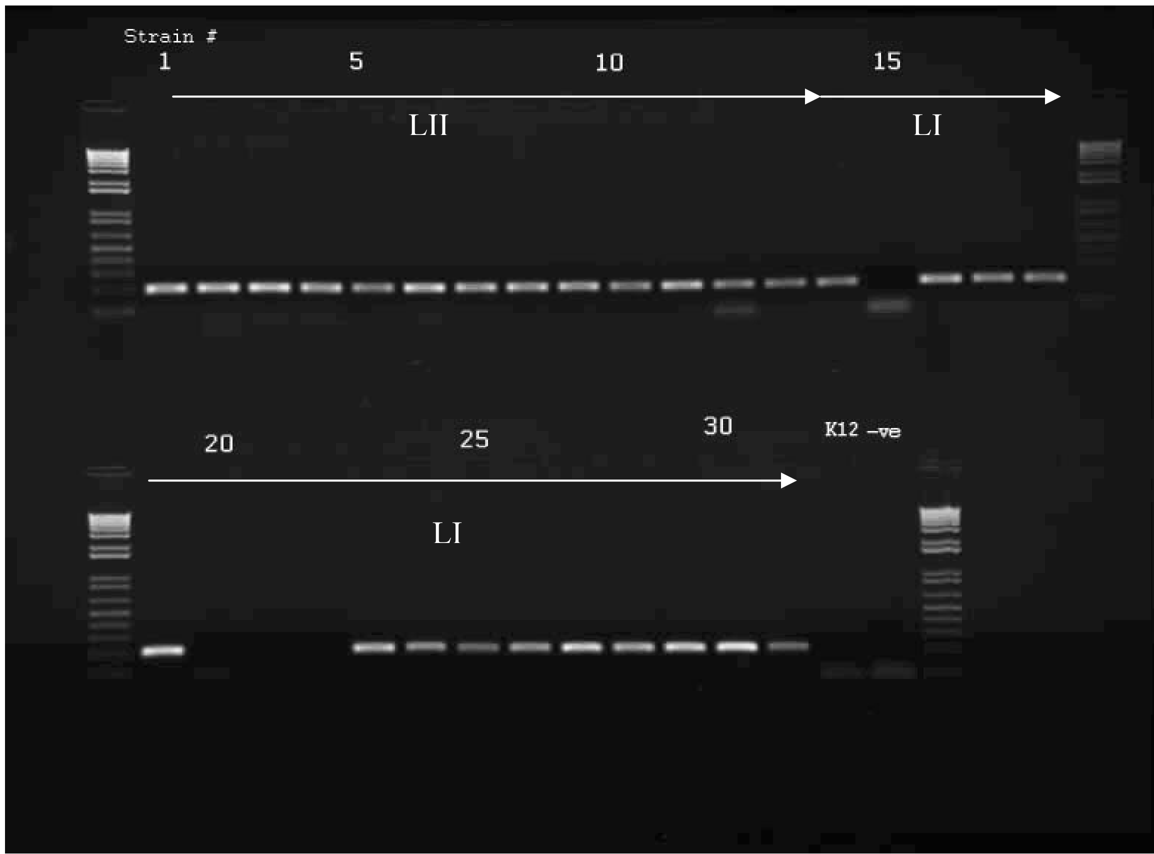
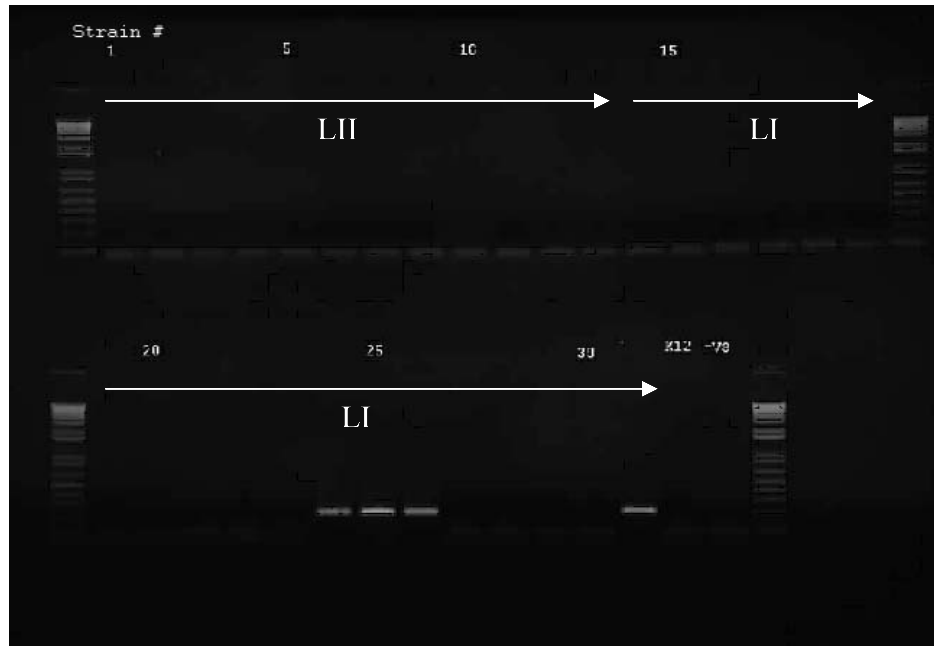


Figure 3.3.2. Expected band sizes for AR-933/ AR-EC97020 multiplex PCR in *E. coli* O157:H7 lineage-specific strains.
This multiplex was used as a representative example of several multiplexes developed for phage Bioplex assay. Lanes 2-13: lineage II strains; 14-15, 17-19, 22-34: lineage I, I/II strains; 16: blank; 35: K12; 36: water; 1, 20, 21, 37:1 kb plus ladder.

A) AR-933



B) AR-EC970520

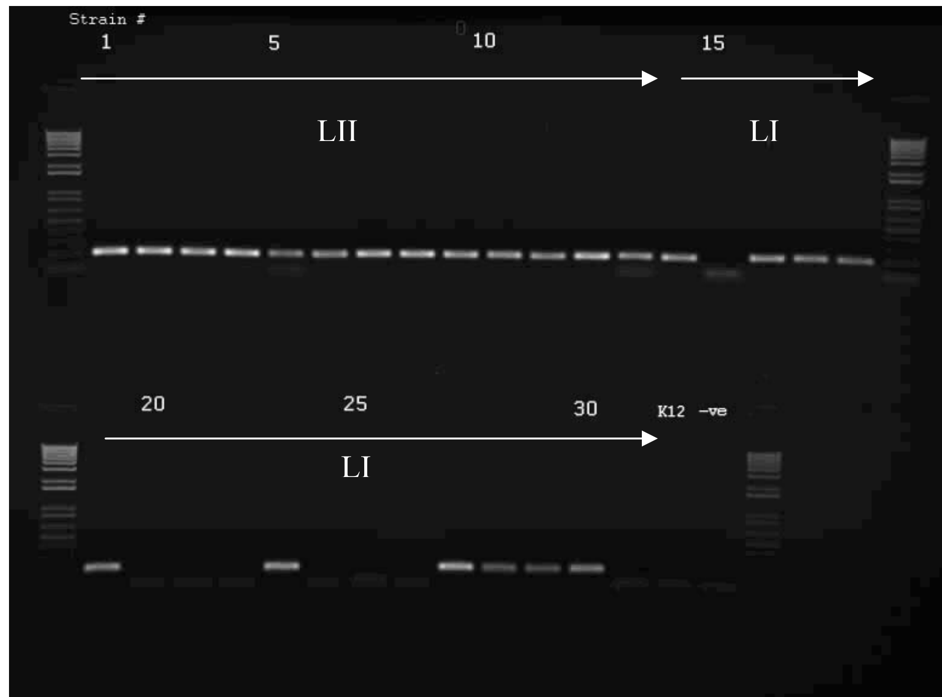


Figure 3.3.3. Expected band sizes in *E. coli* O157:H7 lineage-specific strains using A) AR-933 and B) AR-970520 PCR.
These uniplexes were used as a representative example of PCR's developed for phage Bioplex assay. Lanes 2-13: lineage II strains; 14-15, 17-19, 22-34: lineage I, I/II strains; 16: blank; 35: K12; 36: water; 1, 20, 21, 37:1 kb plus ladder.

Strains listed in Figure 3.3.2. and Figure 3.3.3. were as follows:

1) EC970520 (II)
2) LRH13 (II)
3) R1797 (II)
4) LS68 (II)
5) F12 (II)
6) F1081 (II)
7) FRIK 920 (II)
8) FRIK 1999 (II)
9) FRIK 1985 (II)
10) FRIK 1990 (II)
11) FRIK 2001 (II)
12) 12491 (II)
13) LRH16 (I)
14) LRH27 (I)
15) blank
16) LRH6 (I)
17) TS-97 (I)
18) F1299 (I)
19) F5 (I)
20) 59243 (I/II)
21) 71074 (I/II)
22) EC20030338 (I/II)
23) Zap0046 (I/II)
24) 63154 (I)
25) 58212 (I)
26) F1095 (I)
27) H4420 (I)
28) E2328 (I)
29) F1082 (I)
30) Sakai (I)
31) EDL933 (I)
32) K12
33) PCR -ve

Table 3.3.3. Bioplex phage assay mean detection of Stx2 (933) and Stx2c (970520) genes in *E. coli* O157:H7 strains. K12 was used as a negative control.

Strain	Serotype	lineage	AR-933	Q-933	Stx2B-933	AR-970520	Q-970520	Stx2B-970520	Stx2A	Roi
LRH16	O157:H7	I	-	-+	++	+++	-	-	+++	+++
LRH27	O157:H7	I	-	-+	++	+++	-	-	+++	++
LRH6	O157:H7	I	-	+	+++	++	-	-	+++	+++
TS-97	O157:H7	I	-	-+	++	+++	-	-	+++	++
F1299	O157:H7	I	-	-+	+++	+++	-	-	+++	++
F5	O157:H7	I	-	+	+++	+++	-	-	+++	++
EDL933	O157:H7	I	++	+	+++	-	-	-	+++	+++
63154	O157:H7	I	++	-+	++	-	-	-	+++	++
58212	O157:H7	I	++	+	++	-	-	-	+++	++
F1095	O157:H7	I	++	+	++	-	-	-	+++	++
Sakai	O157:H7	I	-	+	+++	+++	-	-	+++	+++
H4420	O157:H7	I	-	+	++	+++	-	-	+++	+++
E2328	O157:H7	I	-	+	+++	+++	-	-	+++	++
F1082	O157:H7	I	-	+	++	+++	+	-	+++	+++
59243	O157:H7	I/II	-	-+	+++	-	-	-	+++	-
71074	O157:H7	I/II	-	+	+++	-	-	-	+++	-
EC20030338	O157:H7	I/II	-	+	+++	-	-	-	+++	-
Zap0046	O157:H7	I/II	-	-	+++	+++	++	+++	+++	++
LRH13	O157:H7	II	-	-	-	+++	++	+++	+++	+++
R1797	O157:H7	II	-	-	-	++	++	++	+++	+++
LS68	O157:H7	II	-	-	-	+++	++	+++	+++	+++
F12	O157:H7	II	-	-	-	+++	++	++	+++	++
F1081	O157:H7	II	-	-	-	+++	++	+++	+++	+++
FRIK 920	O157:H7	II	-	-	-	+++	++	+++	+++	++
FRIK 1999	O157:H7	II	-	-	-	+++	++	+++	+++	+++
FRIK 1985	O157:H7	II	-	-	-	+++	+++	+++	+++	++
FRIK 1990	O157:H7	II	-	-	-	+++	++	+++	+++	++
FRIK 2001	O157:H7	II	-	-	-	+++	++	+++	+++	++
EC970520	O157:H7	II	-	-	-	+++	++	+++	++	+++
12491	O157:H7	II	-	-	-	++	+++	++	+++	++
K12			-	-	-	-	-	-	-	-

- negative signal (MFI 0-500)
-+ weak signal (MFI 500-1000)
+ positive signal (MFI 1000-3000)
++strong positive signal (MFI 3000-5000)
+++absolute positive signal (MFI 5000+)

strains, only 4/14 (28.6%) which include 63154, 58212, F1095 and EDL933 produced a positive signal for all upstream genes for EDL933 and negative EC970520 genes. All LI strains (14/14) and most L I/II strains 3/4 (75%) produced positive signals for Q-933 and Stx2B-933 genes, however, 10/14 (71%) of the LI strains also produced positive AR-970520 signals. Neither AR-933 nor AR-970520 genes were found in three of the four L I/II strains (59243, 71074 and EC20030338). The L I/II strain Zap0046 produced positive signal for all LII EC970520 upstream genes in addition to the Stx2B-933 gene. No signal was obtained for DNA from *E. coli* K12.

3.3.4. Identification of gene specific to Stx2 and Stx2c-encoding phage in seropathotype strains in phage Bioplex assay

The phage Bioplex assay was used to determine if the genes found in *E. coli* O157:H7 LI strain EDL933 and LII strain EC970520 were present in any of the other STEC strains belonging to different serotypes and seropathotypes (SP). *E. coli* O157:H7 strains OK1 (SPA) and EDL933 (SPA) had positive signal for all LI EDL933 genes and negative for EC970520 LII genes (Table 3.3.4.). *E. coli* O157:H7 E32511 (SPA) and EC970520 produced positive signal for all of its own primers, however, E32511 also contained EDL 933 genes. The EDL933 genes were found in all SPA 5/5 (100%) strains but in only 9/41 (22.0%) SPB to SPE strains. The EC970520 phage genes were distributed throughout strains of SPA to SPE and occurred in 27/46 (58%) of all strains. When the sizes of bands obtained using a multiplex PCR with the two sets of phage primers (EDL933 or EC970520) were compared on agarose gels, three strains, OK1

(SPA), 93111 (SPA) and EC6-371 (SPD) had a pattern of bands the same as EDL933 and 13/46 (28%) in SP collection had the same pattern as LII EC970520 pattern.

Table 3.3.4. Bioplex phage assay mean detection of Stx2 (933) and Stx2c (970520) genes in SP strain collection. K12 was used as a negative control.

Strain	Serotype	Sero-pathotype	AR-933	Q-933	Stx2B-933	AR-970520	Q-970520	Stx2B-970520	Stx2A	Roi
OK1	O157:H7	A	++	-+	++	-	-	-	+++	+
93111	O157:H7	A	-	+	+	++	-	-	-	+
Sakai	O157:H7	A	-	-+	+++	+++	-	-	+++	++
EDL933	O157:H7	A	+++	+	++	-	-	-	+++	+
E32511	O157:NM	A	-	+	+	++	++	++	+	+
R82F2	0111:NM	B	-	-	-	-	-	-	-	+
N01-7015	0103:H2	B	-	-	-	+++	-	-	-	++
CL9	026:H11	B	-	-	-	+++	-	-	-	++
CL1	026:H11	B	-	+	+++	+++	-	-	+++	++
NO2-1626	0103:H2	B	-	-	-	+++	-	-	-	++
N00-6496	0145:NM	B	-	-	-	-	-	-	+++	+
N01-2454	0103:H2	B	-	-	-	+++	-	-	-	++
Z3F1	0121:H19	B	-	-	-	+++	-	-	-	++
CL106	0121:H19	B	-	-+	+++	+++	-	-	+++	++
CL101	0111:NM	B	-	-	-	-	-	-	-	++
NO1-2051	0145:NM	B	-	-+	-	-	-	-	+++	-
N02-5149	0145:NM	B	-	-	-	-	-	-	+++	+
C69F1	0111:NM	B	-	-	-	-	-	-	+++	+++
CL4	O26:H11	B	-	-	-	+++	-	-	-	++
N99-4390	0121:NM	C	-	-	-	+++	++	-	-	++
N89-0541	0113:H121	C	-	-	-	-	-	+++	+++	-
EC7-181	091:H21	C	-	-	-	-	-	-	+++	-
N90-0657	0113:H121	C	-	-	-	-	-	-	+++	-
B2F1	091:H21	C	-	-	-	-	-	+++	+++	-
G5506	0104:H121	C	-	-	-	-	-	+++	+++	-
N00-4541	05:NM	C	-	-	-	+++	-+	-	-	+
N00-4067	05:NM	C	-	-	-	+++	-+	-	-	++

Strain continued	Serotype	Sero-pathotype	AR-933	Q-933	Stx2B-933	AR-970520	Q-970520	Stx2B-970520	Stx2A	Roi
EC6-936	091:H21	C	-	-	-	-	-	-	+++	-
EC6-990	091:H21	C	-	-	-	-	-	-	+++	-
CL3	0113:H121	C	-	-	-	-	-	-	+++	-
N00-4859	0103:H25	D	-	-	-	-	-	-	-	++
N02-2616	0103:H25	D	-	-	-	-	-	-	-	++
N02-4495	0117:H7	D	-	-	-	++	++	-	-	++
EC7-821	069:H11	D	-	-	-	+++	-	-	-	++
N02-1625	0146:H21	D	-	-	-	-	-	-	-	-
EC6-484	0172:NM	D	-	+	++	++	-	-	+++	++
EC2-032	0171:H2	D	-	-	-	-	-	+	+++	-
EC6-371	0113:H4	D	++	+	-	+++	-	++	+++	+
EC2-265	O119:H25	D	-	++	+	-	-	-	+++	+
EC9-377	098:H25	E	++	-	-	+++	-	-	+	++
EC6-626	06:H34	E	-	-+	+++	+++	-	-	+++	+
EC4-453	088:H25	E	-	-	-	-	-	++	++++	+
EC2-293	039:H49	E	-	-	-	-	-	-	+++	-
EC2-020	0156:NM	E	-	-	-	-	-	+++	+++	-
EC2-211	0113:NM	E	-	++	-	-	-	-	+++	+
EC970520	O157:H7		-	-	-	++	-+	++	+++	+
K12			-	-	-	-	-	-	-	-

- negative signal (MFI 0-500)
-+ weak signal (MFI 500-1000)
+ positive signal (MFI 1000-3000)
++strong positive signal (MFI 3000-5000)
+++absolute positive signal (MFI 5000+)

3.4. Discussion

3.4.1. Specific detection of genes found within *E. coli* O157:H7 in STEC strains by multiplex PCR and O157 Bioplex assay

The O157 Bioplex assay developed based on genes specific to *E. coli* O157:H7: e.g., shiga toxins (VT1, VT2), flagella (FlicH7) and intimin (eaeO157); accurately and consistently identified 30/30 (100%) *E. coli* O157:H7 strains and identified the type(s) of Stx found in each strain. Not only did the PCR and Bioplex assays allow specific detection of *E. coli* O157:H7, they were also able to identify *E. coli* O157:H7 genes in 46 STEC from other serotypes. The virulence of the STEC strains may be related the type of Stx produced and presently, severe human diseases such as HUS have been linked to strains possessing Stx2 (Freidrich *et al.*, 2002). Therefore, the ability to detect those *E. coli* O157:H7 and STEC strains that possess Stx2 may be important in identifying highly pathogenic *E. coli* O157:H7 strains. The Bioplex assay may also contain detection probes more specific to *E. coli* O157:H7 strains than the multiplex PCR. This was evidenced by the fact that some eaeO157 and Stx2 (VT2) PCR positive strains from non-O157 serotypes failed to produce a signal in the O157 Bioplex assay (Table 3.3.2.). This is likely due to low homology of these genes to the detection probe. As reported by Gannon *et al*. (1997), eaeO157 primers also amplified DNA from 0145:NM strains, however, FlicH7 and VT genes aid in the discrimination among strains and increased the specificity of the Bioplex assay in the identification of *E. coli* O157:H7.

3.4.2. Variation in Stx2 and Stx2c-encoding bacteriophage genes from EC970520 and EDL933

The upstream gene primers were found to be specific to LI EDL933 or LII EC970520 phage gene. The Stx2A and Roi genes were present in both lineages (Figure 3.3.1.). The Stx2c phage genes from EC970520 were present in all LII *E. coli* O157:H7 strains tested. In contrast, only 4 strains including EDL933 contained all of the same upstream Stx2 phage genes from EDL933. It seems that only a subset of LI strains contain these EDL933 Stx2 phage genes. Several LI strains were missing the AR-933 gene but contained the AR-970520 gene. These LI strains may have been missing the AR or contain a different type of AR. Sequencing of *E. coli* O157:H7 strains have identified several cryptic prophages within the genomes although most do not contain the full complement of genes needed for lytic phage release (Perna *et al.,* 2001; Ohnishi *et al.,* 2002). These phage are highly heterogeneous but may have shared a common ancestor and derive new genes from related phage (Hendrix *et al.,* 1999). Therefore these genes may be somewhat randomly distributed throughout phage genomes. The Stx1 phage is the most likely to contain similar genes to the Stx2 phage since the Stx2 is about 55% similar to Stx1 at an amino acid level (Gamage *et al.*, 2004). The L I/II strains were AR negative differing from other LI and LII strains and this gene heterogeneity may be responsible for unique properties associated with this intermediate lineage. A unique distribution of phage genes was also seen in the L I/II strain Zap0046.

3.4.3. Upstream Stx2 phage assay and multiplex patterns in SP strains

STEC serotypes have been classified into SPA to SPE based on the frequency and severity of human disease with which they associated (Karmali *et al.*, 2003). SPA are most frequently associated with outbreaks of disease and the sometimes fatal HUS and this group consists only of *E. coli* O157:H7 strains. SPB, C and D are also pathogens but are associated with less serious disease. SPB are serotypes occasionally associated with outbreaks and cases of HUS; SPC serotypes are associated with sporadic cases of human illness and rarely with HUS; SPD serotypes are associated with diarrhea; and SPE serotypes have been isolated from animals but have never been implicated in human disease. The benefit of this system is that it allows identification of STEC strains of specific serotypes which have been implicated in human disease of varying frequency and severity and provides a framework in which to identify virulence traits responsible for the specific epidemiological and clinical characteristics of these strains. Since Stx2 phage carry important virulence factors, characterization of these phage in different seropathotypes may help us to determine if these phage are associated with higher occurrences of severe disease (HC, HUS and death) and disease outbreaks.

In the phage Bioplex assay of upstream genes of Stx2 phage, only one other strain, OK1 (SPA), had the same complement of genes as *E. coli* O157:H7 strain EDL933. An important trend in the Bioplex assay data was that the EDL933 genes regions were found in SPA strains and less commonly in the strains of other SPs. The EC970520 genes were found in E32511 (SPA) but this strain also contains Stx2c genes and therefore both phages. The EC970520 genes were more randomly distributed throughout the SP and not specifically associated with a particular SP.

The multiplex PCR patterns also determined that 93111 (SPA) and EC6-371 (SPD) appear to be similar to the EDL933 Stx2 phage and that 28% of strains were closely related to the EC970520 Stx2 phage. It is clear that the Bioplex assay was able to more accurately distinguish between similar genes found within Stx2 phage than simple PCR assays. It is evident that many more Stx2 phage contain gene variants not present in EDL933 and EC970520. While the phage probe sets used in the Bioplex assays in this study were valuable for detection of Stx2 phage genes and in the differentiation among Stx2 phage types found in *E. coli* O157:H7 strains and other STEC, the use of more genes targets in Bioplex assays would be valuable in the identification of other variants of these Stx2 phages.

3.4.4. Applications of *E. coli* O157:H7 and phage gene Bioplex assays

The development of these assays can be used for a number of applications. Individuals living in rural areas in Canada are at particular risk where high agriculture activity occurs and there is direct contact with cattle populations which can act as reservoirs for the disease, contaminating groundwater (Spika *et al.*, 1996). The assay can be developed to identify contamination of *E. coli* O157:H7 and STEC strains in food and water samples. Wilson *et al*. (2005) have developed a suspension array for multiplexed PCR detection of biothreat pathogens using simulated environmental samples demonstrating the suspension array as a viable and cost-effective technology for this application. Environmental multiplexed suspension arrays have also been developed to detect *Enterococcus* spp. in river samples, marine recreational water and beach sand but there has been no development of an environmental assay to detect specific pathogenic *E.*

coli strains (Baums *et al.,* 2007). The assay can also be used to identify strains that may have previously gone undetected by other methods. Current culturing techniques to identify *E. coli* O157:H7 in water sources do not detect viable but non-culturable cells encouraging the need to develop efficient techniques to identify these strains. This assay may have the capability to identify strains of this nature since detection is determined by the genetic composition of the organism, not the ability for these cultures to grow on selective media. Bacterial strains are highly diverse organisms and this assay can also be developed to detect diversity within *E. coli* O157:H7 strains. The addition of Stx2 and Stx2c probes specific to bovine and human strains can be used to identify sources of infection and the pathogenic capabilities of strains within *E. coli* O157:H7 and other STEC strains in the medical and agricultural industries.

3.4.5. Future tests

A significant limitation to the current Bioplex assay developed here is the use of PCR amplified product for detection. The use of genomic DNA in the suspension array would remove the PCR step improving efficiency and decreasing costs but genomic DNA suspension arrays have been difficult to develop since signal intensity has not been adequate for detection. Borucki *et al*. (2005) have increased genomic DNA detection by using DNA dendrimers hybridized to the bead-DNA complex to increase target labeling therefore increasing signal to accurately detect the pathogen *Listeria monocytogene*. This option may improve the efficiency of the *E. coli* O157:H7 assay and should be investigated. The *E. coli* O157:H7 assays developed here are sensitive and specific enough to detect *E. coli* O157:H7 as well as those genes found within other STEC strains

and are useful in identifying genetic variations between STEC human and bovine isolates.

3.5. Conclusions

We detected variation in Stx toxin-producing bacteriophage among *E. coli* O157:H7 and STEC. In this study,

1) The Bioplex O157 assay developed was able to specifically detect *E. coli* O157:H7 and the types of Stx(s) found within each strain. These genes were also identified in STEC which shows that this method is useful for identifying those STEC strains with pathogenic potential. The Bioplex assay may also contain detection probes more specific to *E. coli* O157:H7 strains than the multiplex PCR.
2) Phage genes of Stx2c found in EC970520, were lineage II specific but only a subset of lineage I strains contained the Stx2 phage genes found in EDL933. Lineage I/II strains contain phage genes different from both lineages that may contribute to its unique properties.
3) The Stx2 phage genes identified by the phage Bioplex assay were found to be distributed mainly in SPA whereas the Stx2c phage genes were distributed throughout the SP. The phage Bioplex assay may be more specific than multiplex PCR as the Bioplex was able to distinguish differences within specific genes.

These assays can be used for screening environmental samples, viable but non-culturable cells and identifying sources of infection. Future testing to improve Bioplex assay signal can be useful in developing more assays to specifically identify more genetic variations within human and bovine isolates of *E. coli* O157:H7 which is important for determining the source of infections and identifying evolving strains with pathogenic potential.

CHAPTER FOUR

4. Phenotypic differences of Shiga toxin 2-encoding bacteriophage between *E. coli* O157:H7 lineage I and II strains

4.1. Introduction

Escherichia coli (E. coli) O157:H7 is a food and water borne bacterial pathogen that is associated with outbreaks of gastrointestinal illness such as hemorrhagic colitis (HC) and a sometimes fatal systemic toxemia termed hemolytic uremic syndrome (HUS) (Karmali *et al.,* 1983; Obrig *et al.,* 1998; Ochea and Cleary, 2003). These pathogens colonize the bowel and create attaching and effacing (A/E) lesions in epithelial cells of the intestinal mucosa. The adherence to the intestine is aided by a type-III secretion system which is encoded on an ~45 kb pathogenicity island termed the locus of enterocyte effacement (LEE) (Perna *et al*., 1998). This island also produces several secreted proteins including intimin, an outer membrane protein required for intimate adherence. Once bacteria have adhered to the mucosa they produce one of the most important virulence factors in *E. coli* O157:H7, the potent cytotoxins termed Shiga toxins (Stx) which damage the large bowel. These toxins have N-glycosidase activity which target 28s rRNA resulting in inhibition of protein synthesis and cell death (Karmali, 2004; Law, 2000). These toxins are thought to directly lead to systematic damage of other organs such as the brain, pancreas and lungs resulting in development of HUS in infected individuals (Obrig *et al*., 1988; Monnens *et al*., 1998). There are two major types of Stx termed Stx1 and Stx2. A Stx-producing *E. coli* (STEC) strain can produce either Stx1 or Stx2 or both toxins. Sequencing of several Stx phages has shown that these genomes are highly heterogeneous in nature. Several variants of Stx2 have been

identified (Stx2c, Stx2d, Stx2e, and Stx2f) and virulence of STEC strains including *E. coli* O157:H7 may be related to the type of toxin produced; e.g., severe human disease associated with HUS has been linked to bacterial strains producing Stx2 or the variant Stx2c rather than Stx1 (Friedrich *et al.*, 2002; Herold *et al.*, 2004).

Stx genes found in *E. coli* O157:H7 and other STEC are located in temperate lambdoid bacteriophages. During lysogeny, the bacteriophage DNA is inserted into the bacterial chromosome and in this inactive state the phage DNA is referred to as a prophage. Upon induction, the integrated bacteriophage enters the lytic cycle and phage progeny are assembled with the help of the host's machinery resulting in death of the host, and spread of the phage to new *E. coli* host bacteria. The Stx genes in *E. coli* O157 strains are present in the late region of the phage genome and are expressed only in the lytic stage of the phage life cycle (Gamage, 2004). Induction of lysogenic phage into the lytic cycle can occur after exposure to DNA damaging agents such as UV light or the mutagen mitomycin C, following exposure to neutrophil activation products such as hydrogen peroxide or after treatment with antibiotics such as ciprofloxacin. These treatments activate a cellular SOS response which results in removal of inhibitors of the lytic cycle, derepression of phage lytic genes and increased Stx synthesis and release (Goerke *et al.*, 2006; Smith and Walker, 1998; Muhldorfer *et al.*, 1996; Zhang *et al.*, 2000).

The type of phage, the ability to enter a lytic cycle and Stx release differs among *E. coli* O157:H7 strains. Stx phages from clinical isolates have been shown to be highly variable and differ in the level of toxin production (Gamage *et al*., 2004). High resolution genotyping methods have shown that human and bovine isolates of *E. coli*

O157:H7 are not randomly distributed among lineage I and lineage II (Kim *et al.*, 1999). Lineage I strains are found in human and bovine isolates whereas lineage II are most commonly associated with cattle. This indicates that one of the lineages may be less virulent to humans or may not be efficiently transmitted to humans from a bovine source (Kim *et al.*, 1999). Ziebell *et al.* (2008) have also determined that Stx2 type and the level of Stx production are specific to these lineages.

In Chapter two of the thesis, genetic relationships among different STEC strains of human and bovine origin were examined. It was determined that in *E. coli* O157:H7, lineage I strains of both human and bovine origin produced Stx2 whereas lineage II strains produced the variant Stx2c and were mainly of bovine origin. In this chapter, lineage-specific differences in the Stx2 bacteriophage and toxin production by lineage I and II strains are examined. Specific objectives of this study were to:

1) Determine if Stx2 phage is released in a lineage-specific manner after exposure to specific chemical and physical inducers
2) Determine if relative toxin release following induction by strains is lineage-specific

4. 2. Materials and Methods

4.2.1. Bacterial strains

A collection of 18 *E. coli* O157:H7 lineage-specific strains which were isolated from different sources and contained several different phage types (PT) were used in this study (Table 4.2.1.) (Khakria *et al.*, 1999; Kim *et al.*, 1999). Lineage-specific polymorphism assay (LPSA) types of the bacteria were previously determined by microarray-based comparative genomic hybridization (Zhang *et al.,* 2007). LI/LII strains were treated as subgroups within lineage I but were identified as lineage I/II. EDL933 and EC970520 were used as representative examples of lineage I and lineage II strains respectively. These bacteria contain Stx2 phage with known sequence and lineage-specificity (Plunkett *et al.,* 1999; Zhang *et al.*, 2007). *E. coli* K12 strain MG1655 was used as a negative control because it contains no intact Stx bacteriophage (Blattner *et al.*, 1997). Several *E. coli* non-pathogenic strains, LE392, DH5α, C600 and ML:1061OR:H48 were used as bacteriophage-free, plating bacteria.

4.2.2. Induction and isolation of bacteriophage from *E. coli* O157:H7 strains

Bacteriophage production was induced with ciprofloxacin, mitomycin C or UV light. Bacteria were grown from single colonies in 50 ml Luria-Bertani (LB) Broth and placed in a 37 °C shaker overnight at 200 cycles/min (Orbital shaker D52-500-2, VWR). Inductions were performed by making a 1/10 dilution of the overnight culture after which 0.15 μg/mL ciprofloxacin (Biochemika) or 0.5 μg/mL mitomycin C (Sigma) was added and the mixture shaken at 37 °C for 3 hr. Bacteriophages were isolated from the induced cultures by pelleting the cells and lysed debris at 8000 rpm in a (Thermo IEC centrifuge,

Table 4.2.1. *E. coli* O157:H7 lineage-specific strains used in this study. Strains were previously used and characterized for LPSA type by Zhang *et al.* (2007) from microarray-based comparative genomic hybridizations.

Strain #	**Serotype**	**Phage**	**Source**	**LPSA type**	**Lineage**	**Stx1**	**Stx2**
53722	O157:H7	14	Human	IIIIII	I	+	+
F1299	O157:H7	14	Bovine	IIIIII	I	+	+
EDL933	O157:H7	21	Human	IIIIII	I	+	+
63154	O157:H7	31	Human	IIIIII	I	+	+
58212	O157:H7	31	Human	IIIIII	I	+	+
F1095	O157:H7	31	Bovine	IIIIII	I	+	+
Sakai	O157:H7	32	Human	IIIIII	I	+	+
F1082	O157:H7	87	Bovine	IIIIII	I	+	+
59243	O157:H7	2	Human	2IIIII	I/II	-	+
71704	O157:H7	2	Human	2IIIII	I/II	-	+
LRH13	O157:H7	23	Human	222222	II	+	+
R1797	O157:H7	23	Human	222222	II	+	+
L568	O157:H7	23	Bovine	222222	II	+	+
F1081	O157:H7	23	Bovine	222222	II	+	+
FRIK 1999	O157:H7	23	Bovine	222222	II	+	+
R834	O157:H7	54	Human	222212	II	-	+
EC970520	O157:H7	67	Bovine	222222	II	+	+
12491	O157:H7	74	Human	222222	II	-	+

Micromax) for 30 min. The supernatant was filtered through a 0.45 μm-pore size membrane (Micro Filtration Systems) and stored at 4 ºC with 5 μL chloroform (Sigma). For UV light inductions, overnight cultures were centrifuged at 8000 rpm for 30 min, resuspended in 10 ml saline and then exposed to UV light for 10 min. A 1/10 dilution of saline solution in LB was placed into a 37 °C shaker for 3 hr. Induced cultures were centrifuged, filtered and stored with chloroform at 4 ºC as previously described.

4.2.3. Measurement of cells and lysates

The optical density of bacterial cultures was measured at 600 nm (OD_{600}) using a spectrometer prior to and after bacteriophage induction to monitor bacterial lysis (DU800 spectrometer, Beckman Coulter). Control and induced cultures were compared in each experiment. Strains EDL933 and EC970520 were measured prior to and after every hour to measure the rate of phage production post induction. Measurements were repeated three times for each isolate.

4.2.4. Plating of bacteriophage

Bacteriophages were plated using the protocol described by Sambrook, (1986). Bacteriophage-free *E. coli* were used to assess the presence or absence of phage from different strains of bacteria which were capable of plaque production. A single colony of plating bacteria was inoculated in 50mL of LB broth with 0.2% maltose and placed in a 37 °C shaker overnight at 200 cycles/min. The culture was pelleted by centrifugation at 10,000 rpm for 30 min and re-suspended in 0.01 M $MgS0_4$ until an OD_{600} of ~2.00 was obtained. Tenfold serial dilutions of induced bacteriophage were prepared in an SM

medium consisting of 2.9 g NaCl, 1 g $MgSO_4$ $7H_2O$, 1 M Tris-Cl (pH 8.0) and 2% gelatin. In a sterile tube, 100 μL of plating bacteria plus 100 μL of induced bacteriophage supernatant were mixed and incubated together for 15 min with either 1 μl ciprofloxacin or mitomycin C at room temperature (RT). No treatment or chemical was added to the UV induced cultures. The mixture was added to 4 mL of 65 °C LB agar, mixed gently and poured onto prewarmed (37 ºC) LB agar plates and incubated overnight at 37 ºC. Plates were examined for plaque formation the following day. K12 and Sakai were used as negative controls since K12 does not contain viable phage and Sakai has been shown previously to lack plaque-forming ability (Blattner *et al*., 1997; Makino *et. al*, 1999).

4.2.5. DNAse treatment of bacteriophage DNA

Bacterial cultures containing Stx2 bacteriophage released into the supernatant during a lytic cycle were identified using PCR. Prior to amplification, template DNA from the bacteriophage supernatants were treated with DNAse (Ambion) to ensure that the PCR product was the result of amplification of bacteriophage DNA and not chromosomal DNA from integrated prophage. DNAse treatment of phage supernatants has been utilized previously to digest the nucleic acids liberated from lysed bacteria without disturbing phage nucleic acids which are protected by a phage protein coat (Yokoyama *et al.*, 2000). The supernatant was incubated at 37 ºC for 30 min with 2U DNAse and 1X DNAse buffer and inactivated at 95 ºC for 10 min. A PCR was performed using 3 μL of bacteriophage DNA as template. The PCR was replicated for ciprofloxacin, mitomycin C and UV induced cultures.

4.2.5.1.Confirmation of Stx2 bacteriophage lysates by PCR

PCR of DNAse treated phage supernatants for identification of Stx2 phage were prepared in 50 μl reaction volumes. Each contained 10 pmol each of primer VT2-F (5'CCATGACAACGGACAGCAGTT3') and VT2- R (5'CCTGTCAACTGAGCACTTTG3'), 200 μM of each deoxynucleoside triphosphate, 1X PCR Buffer (Qiagen) and 1 U of Taq DNA polymerase (Qiagen). The PCR was conducted as follows: 3 min denaturation at 94 ºC, followed by 25 cycles of 45 s at 94 ºC, 30 s at 55 ºC and 90 s at 72 ºC with a final 10 min extension step at 72 ºC. The same conditions were applied for Stx1 identification except that the primers used in the PCR reaction were VT1-F (5'CATTGTCTGGTGACAGTAGCT) and VT1-R (CCCGTAATTTGCGCACTGAG). The VT1 and VT2 primers were designed from conserved regions in STEC strains which identify both Stx's and their variants and previously used in the *E. coli* O157:H7 assay from Gannon *et al.* (1997). PCR products were visualized under UV light on a 1.0% (w/v) agarose gel stained with ethidium bromide (0.5 μg/ml). Negative controls incorporated all reagents of the PCR except water was used as a template.

4.2.6. Stx2 plaque hybridization

A method to identify bacteriophage plaques by Wang and Cutler (1992) which uses nitrocellulose membrane hybridization and PCR to characterize plaques was used to identify the number of Stx2 plaques present on plates. Nitrocellulose membrane (Transblot) discs were prepared using a glass Pasteur pipette to puncture small holes in the membrane and placed on plaques from EDL933 and EC970520 for ~30 s. The discs

were then used as a template for PCR. Discs were treated with 2U DNAse (Ambion) in 1X DNAse I Buffer for 30 min at 37 ºC and deactivated for 10min at 95 ºC prior to PCR. All transfers were made with sterile tweezers to prevent cross-contamination of discs. K12-induced plated cultures were used as a negative control. All results were replicated.

4.2.7. Evaluation of toxin production

To determine the relative amount of toxin produced by induced and non-induced cultures of lineage-specific strains, an enzyme-linked immunosorbent assay (ELISA) was performed. A Premier EHEC kit (Meridian Bioscience Inc.) was used for detection of Stx produced by enterohemorrhagic *E. coli* (EHEC) according to manufacturer's instructions. Prior to detection, overnight cultures were diluted 1/10, and half of the cultures were induced with ciprofloxacin for 3 hrs at 37 ºC shaking as previously described. The cultures were then diluted with LB to an OD_{600} of 0.2. All samples and readings were done in triplicate.

4.2.8. Toxin production of phage after cellular lysis in LE392

The ability of bacteriophage to propagate, undergo cellular lysis and release Stx in LE392 was tested using the infection procedure described in the Expand cloning kit manual (Roche Applied Science). A single colony of plating bacteria (LE392) was inoculated in 20 mL of LB broth with 0.2% maltose and 10mM $MgSO_4$ and placed in a 37 °C shaker at 200 cycles/min until an OD_{600} of 0.8-1.0 was obtained. Plating bacteria were centrifuged at 3500 rpm for 5 min and resuspended in 10 mM $MgSO_4$ and adjusted to an OD_{600} of 0.8-1.0. In a sterile tube, 400 µL of plating bacteria and 200 µL of

induced bacteriophage supernatant obtained as previously described were incubated in a 37 ºC shaker. Toxin production was measured by ELISA.

4.3. Results

4.3.1. Bacteriophage production in response to antibiotic, mutagen or UV light treatment

Both *E. coli* O157:H7 lineage I strain EDL933 and *E. coli* O157:H7 lineage II strain EC970520 displayed a similar response to treatments with antibiotic (ciprofloxacin), chemical mutagen (mitomycin C) and UV light (Figure 4.3.1.; Figure 4.3.2.). The OD_{600} in the untreated cultures increased in density steadily over time to ~0.55. After UV treatment the OD_{600} increased over time but the density was lower than that obtained for non-induced cultures; *i.e.*, the cell density increased to a maximum of ~0.4 for EC970520 and to ~0.45 for EDL933 by 180 min post-treatment. Treatment with mitomycin C and ciprofloxacin resulted in smaller increases in cell density; *i.e.*, when both *E. coli* O157:H7 strains EDL933 and EC970520 were treated with mitomycin C, cell density rose from ~0.10 to 0.25 and 0.20, respectively, by 120 min post-induction. While values remained similar in EC970520 up to 180 min (Figure 4.3.2.), they decreased to ~0.15 by 180 min in EDL-933 (Figure 4.3.1.). Treatment with ciprofloxacin had the greatest effect on OD_{600} for both strains of bacteria. In *E. coli* O157:H7 strain EDL933 treatment with ciprofloxacin caused values to only increase from 0.09 to 0.11 by 60 min and remained constant up to 180 min (Figure 4.3.1.). For *E. coli* O157:H7 strain EC970520 values increased from 0.05 to 0.16 by 60 min after which the OD_{600} only increased to 0.19 by 180 min (Figure 4.3.2.).

The OD_{600} of eight randomly selected lineage I, I/II and lineage II strains after a 3 hr induction was compared to that of untreated cultures to determine if any lineage-specific

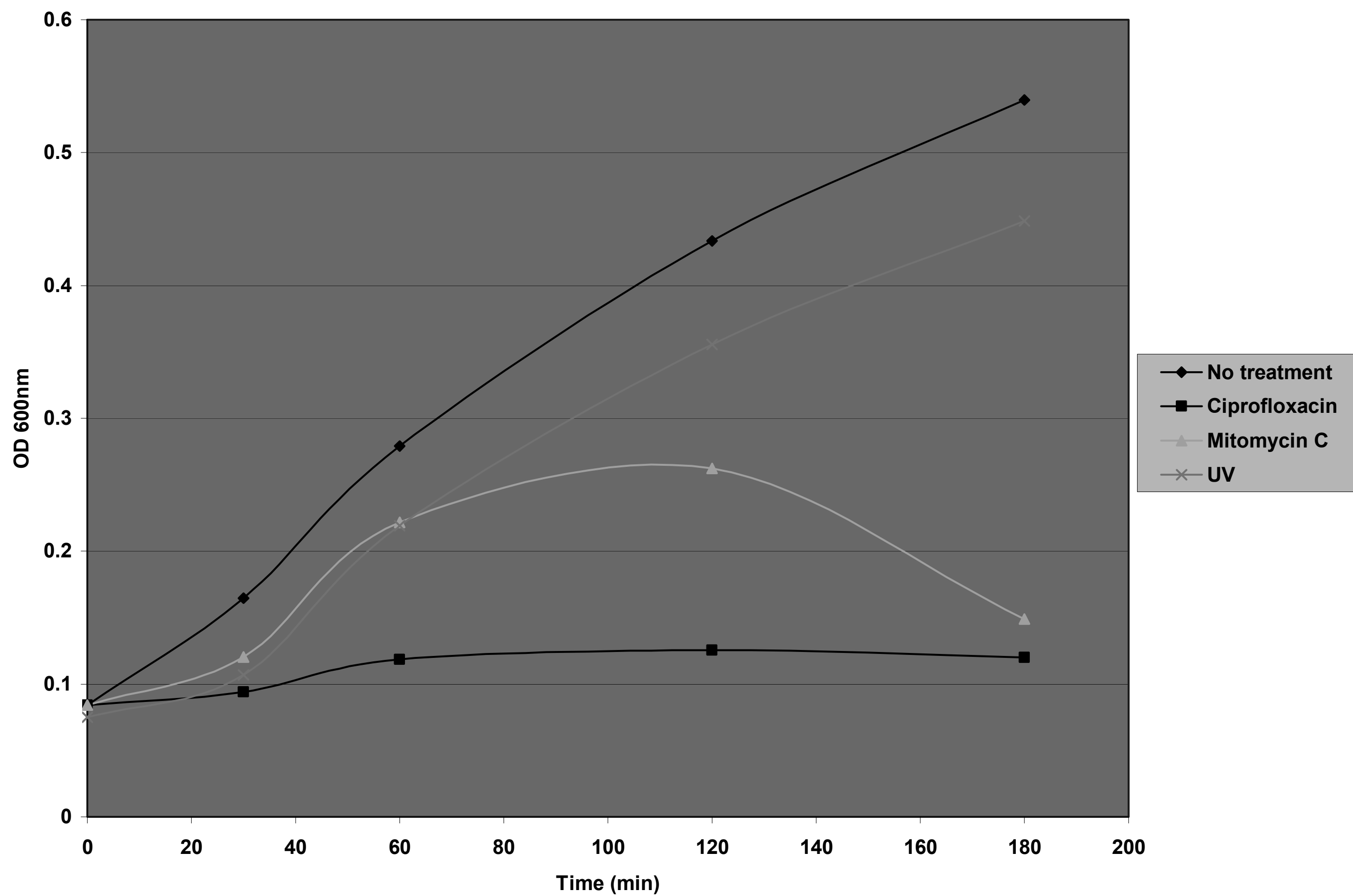


Figure 4.3.1. The mean OD_{600} of EDL933, a representative strain of lineage I, over 3hr after addition of ciprofloxacin, mitomycin C or exposure to UV light verses no treatment.
Standard deviations (values < 0.01) are not shown.

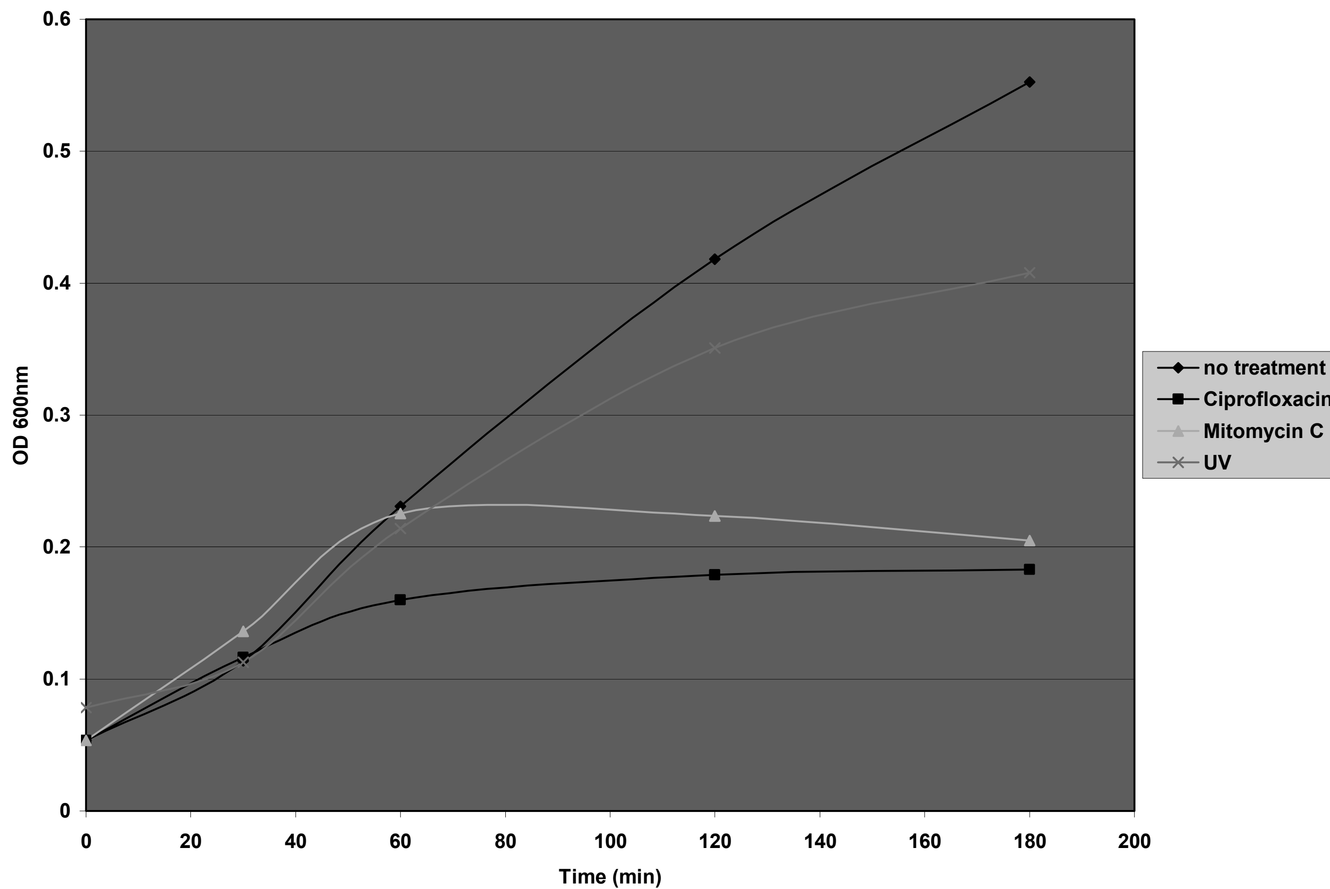


Figure 4.3.2. The mean OD_{600} of EC970520, a representative strain of lineage II over 3hr after addition of ciprofloxacin, mitomycin C or exposure to UV light verses no treatment.
Standard deviations (values < 0.01) are not shown.

differences were evident. Few differences in the OD_{600} were observed among the lineages (Figure 4.3.3.; Figure 4.3.4.; Figure 4.3.5.). In each case, the observed cell density was lower for bacteria treated with ciprofloxacin, followed by mitomycin C, and little difference in densities was observed for UV light treatment compared to the uninduced control cultures (Figure 4.3.6.). *E. coli* K12 was used as a negative control in each case and displayed less of a difference in OD_{600} between induced and non-induced cultures than the *E. coli* O157 strains.

4.3.2. Plaque production after induction of bacteriophage

To determine if plaque formation after induction was lineage-specific, supernatants of the induced cultures from above were mixed and plated with *E. coli* K12 strain LE392. Filtered supernatants of 3 hr ciprofloxacin-induced *E. coli* O157:H7 EDL933 cultures were used as positive controls (Plunkett *et al.,* 1999) (Figure 4.3.6.). For *E. coli* O157:H7 EDL933, a decrease in bacterial growth was evident when undiluted phage supernatants were plated with *E. coli* LE392 compared to the negative control plate where an even lawn of bacterial growth was observed (Figure 4.3.7.). Isolated plaques were also present in the 1/100 dilution for induced cultures of *E. coli* O157:H7 EDL933. For the lineage II strain, *E. coli* O157:H7 EC970520, bacterial growth was sparse and several large spots were evident when the undiluted EC970520 supernatant was plated (Figure 4.3.8.). As the dilution increased, no plaques or clearing were seen and an even layer of bacterial growth was observed similar to the negative control. With mitomycin C and UV light similar results were obtained for *E. coli* O157:H7 EDL933

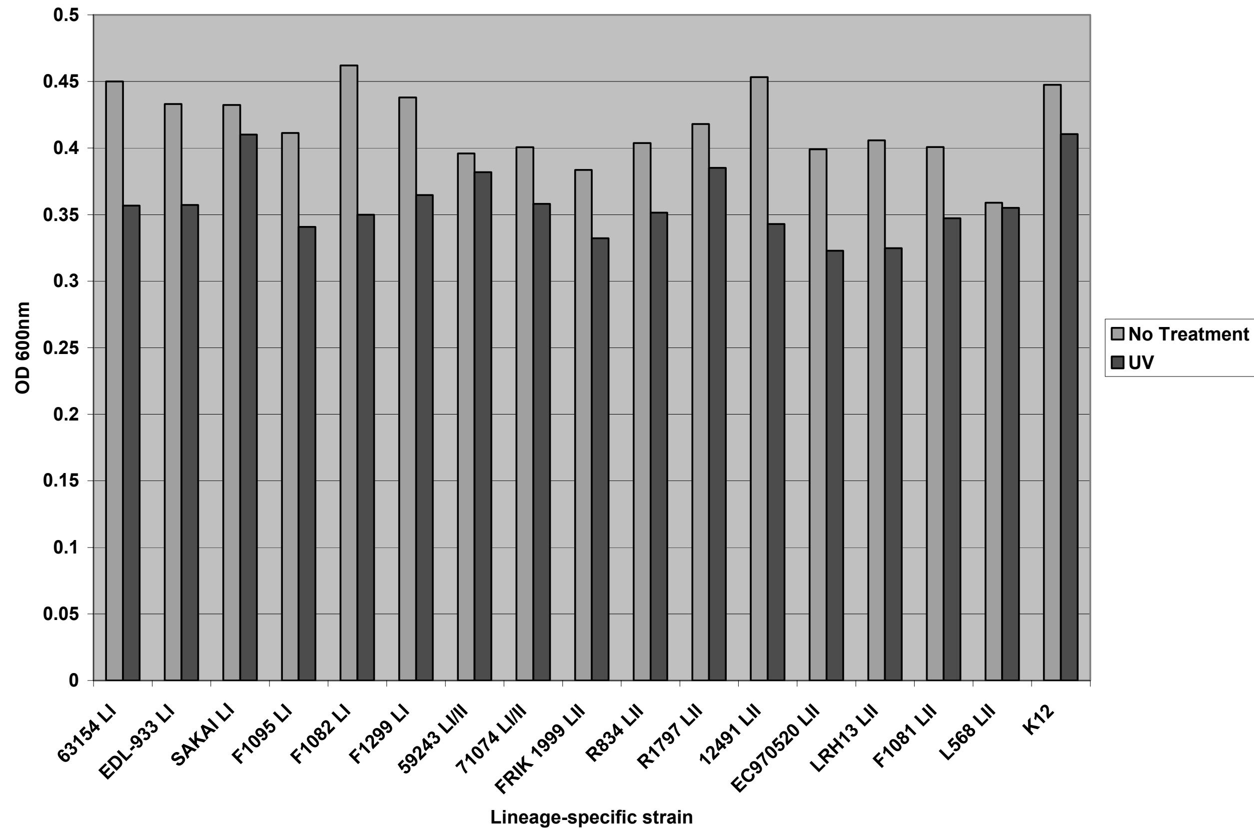


Figure 4.3.3. The mean OD_{600} of lineage I, I/II and lineage II strains of UV induced and non-induced cultures after 3 hr. Measurements were repeated three times for each isolate. Standard deviations are not shown (<0.01). The strains are 63154, EDL933, Sakai, F1095, F1082, F1299, 59243, 71074, FRIK 1999, R834 R1797, 12491, EC970520, LRH13, F1081, L568 and K12.

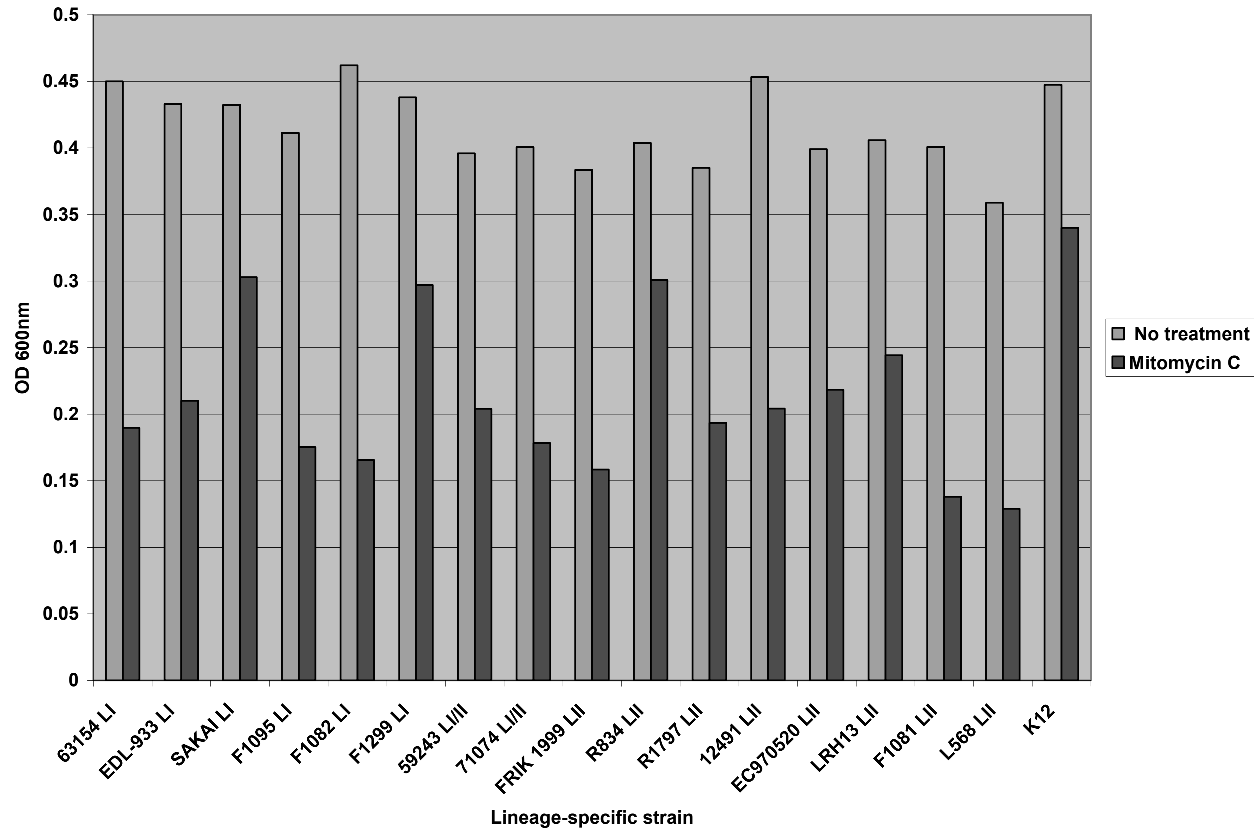


Figure 4.3.4. The mean OD_{600} of lineage I, I/II and lineage II strains of mitomycin C induced and non-induced cultures after 3 hr. Measurements were repeated three times for each isolate. Standard deviations are not shown (<0.01). The strains are 63154, EDL933, Sakai, F1095, F1082, F1299, 59243, 71074, FRIK 1999, R834, R1797, 12491, EC970520, LRH13, F1081, L568 and K12.

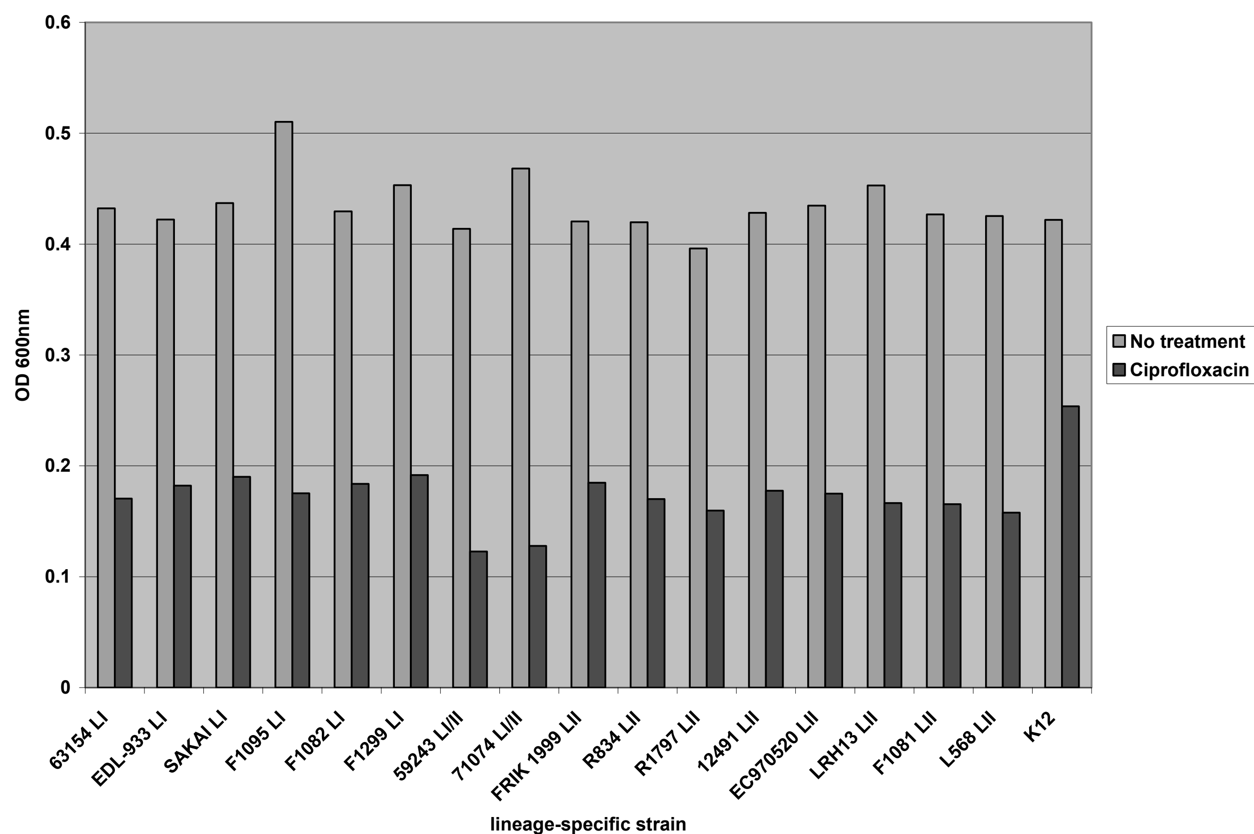


Figure 4.3.5. The mean OD_{600} of lineage I, I/II and lineage II strains of ciprofloxacin induced and non-induced cultures after 3 hr. Measurements were repeated three times for each isolate. Standard deviations are not shown (<0.01). The strains are 63154, EDL933, Sakai, F1095, F1082, F1299, 59243, 71074, FRIK 1999, R834, R1797, 12491, EC970520, LRH13, F1081, L568 and K12.

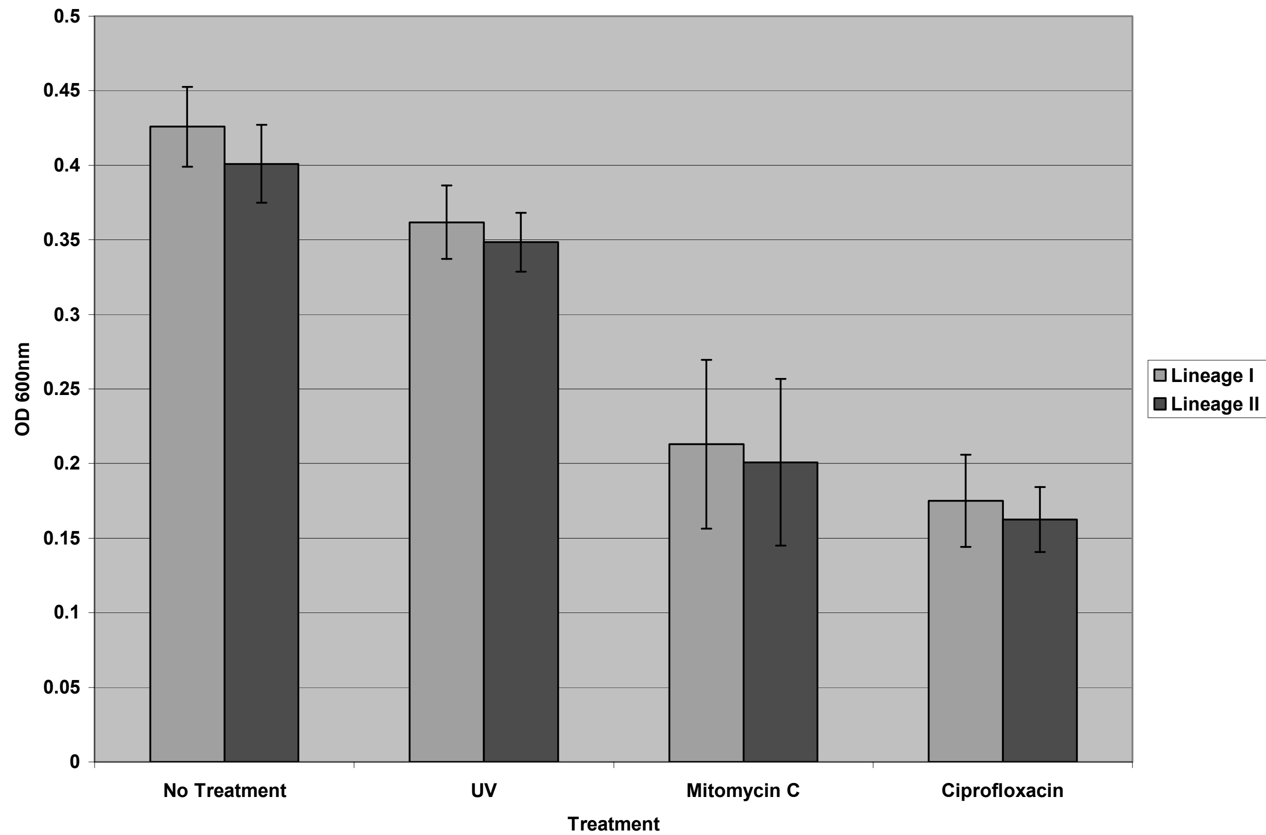
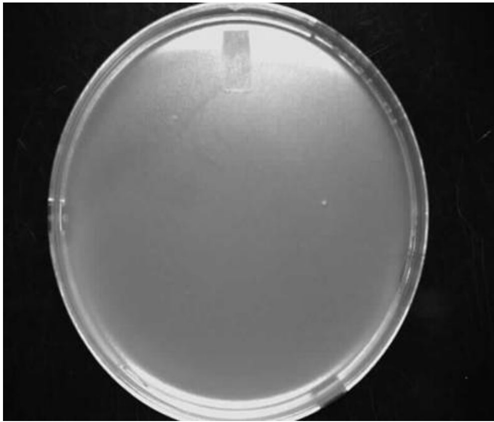
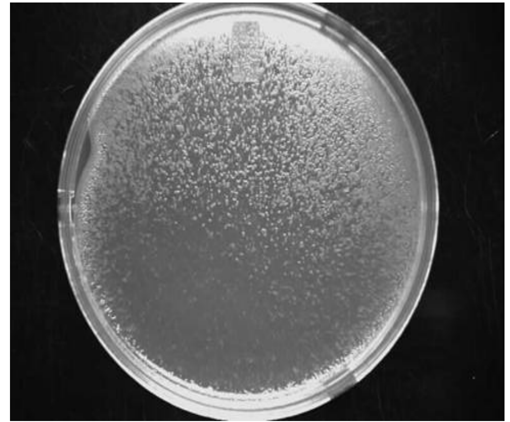


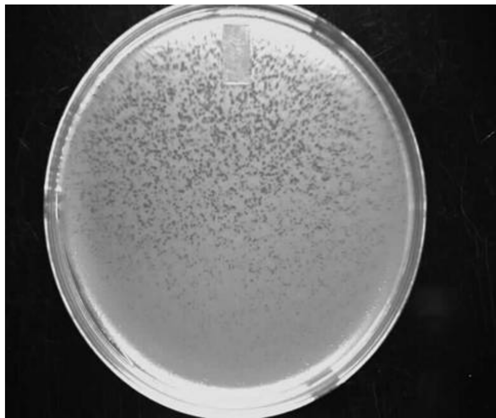
Figure 4.3.6. The mean OD_{600} of lineage I, (I/II) and lineage II strains of UV light, mitomycin C, and ciprofloxacin induced cultures verses non-induced cultures after 3 hr.
Error bars represent standard deviations.



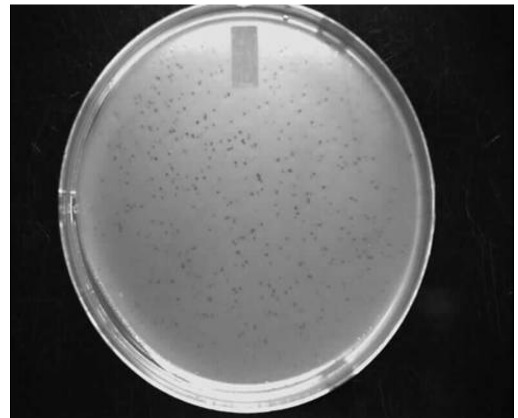
A) LE392: no phage



B) EDL933/LE392: no dilution

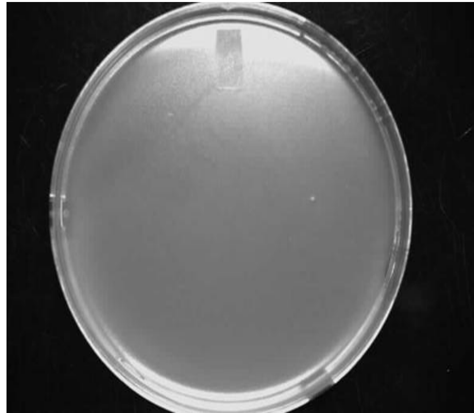


C) EDL933/LE392: 1/10 dilution

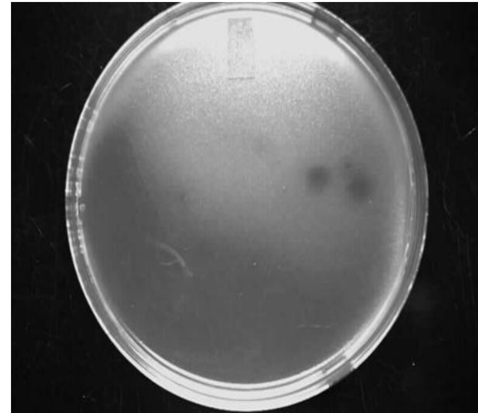


D) EDL933/LE392: 1/100 dilution

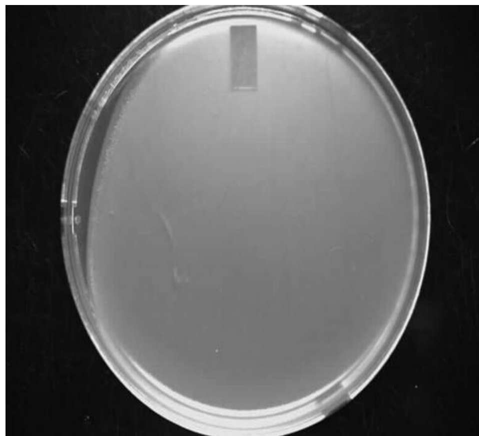
Figure 4.3.7. Plating ciprofloxacin induced bacteriophage from EDL-933 on bacteriophage-free *E. coli* strain, LE392.
A) no induced phage; B) undiluted induced EDL933 phage; C) 1/10 dilution of EDL933 phage; D) 1/100 dilution of EDL933.



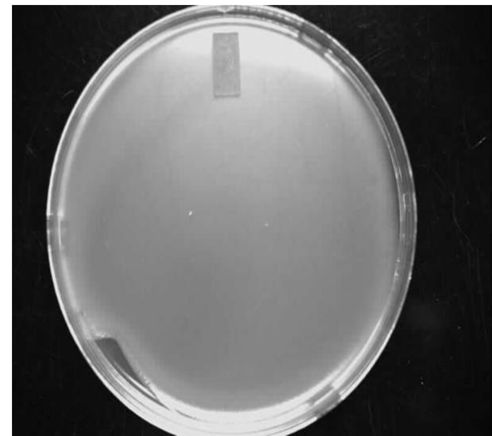
A) LE392: no phage



B) EC970520/LE392: no dilution



C) EC970520/LE392: 1/10 dilution



D) EC970520/LE392 :1/100 dilution

Figure 4.3.8. Plating ciprofloxacin induced bacteriophage from EC970520 on bacteriophage-free *E. coli* strain, LE392.
A) no induced phage; B) undiluted induced EC970520 phage; C) 1/10 dilution of EC970520 phage; D) 1/100 dilution of EC970520.

and EC970520 cultures except that plaques were very small for EDL933. *E. coli* K12 and *E. coli* O157:H7 Sakai induced cultures did not produce plaques.

To determine if *E. coli* O157:H7 strain EC970520 and EDL933 were representative of the lineages in terms of plaque formation, one additional lineage I, two lineage I/II and three additional lineage II strains were compared in their ability to form plaques on *E. coli* LE392 following induction with ciprofloxacin (Table 4.3.1.). The other lineage I strain (F1299) and lineage I/II strains (59243) and (71074) produced plaques similar to those produced by *E. coli* O157:H7 EDL933 following induction. Like *E. coli* O157:H7 EC970520, the three other lineage II strains' undiluted supernatants inhibited growth of *E. coli* LE392 but did not produce clearly defined plaques. Similar results were obtained from tests of the supernatants of these induced lineage I, I/II and II *E. coli* O157:H7 strains using three other *E. coli* test strains including ML:1061OR:H48, C600 and DH5α (Table 4.3.1.). Lineage I and I/II strains produced plaques similar to EDL933 on most *E. coli* strains whereas lineage II strains did not. However, lineage II strain R834 produced a few plaques when plated on *E. coli* DH5α. It was also observed that some lineage I and I/II strains produced plaques when no inducer was present (Table 4.3.1.).

4.3.3. Presence of Stx2 producing-bacteriophage

To determine if Stx2-encoding bacteriophage were induced but not detected in *E. coli* K12 plaque assays, the supernatants from induced cultures were treated with DNAse

Table 4.3.1. Plating ciprofloxacin induced bacteriophage from lineage specific (I, I/II or II) *E. coli* O157:H7 strains on bacteriophage-free *E. coli* strain, ML:1061OR:H48, LE392, C600, or DH5α..
Plaques (**P**):+,++,+++,++++ - increasing number of plaques; Bacteria (**B**) (No plaques present) +,++,+++,++++ - increasing layer of bacteria.

Lineage	Strain	ML:1061OR:H48	LE392	C600	DH5α	No chemical-LE392
I	EDL933	**P**++++ plaques touching	**P**++++ plaques touching	**P**+++ few plaques touching	**P**++++ plaques touching	**P**++++ plaques touching
I	F1299	**P**+++ plaques touching	**P**+++ few plaques touching	**P**+ few individual plaques	**P**++++ big, touching plaques	**P**++ spots, not well defined plaques
I/II	59243	**P**+++	**P**++ single plaques	**B**++++	**P**+++ plaques touching	**B**+++
I/II	71074	**P**++++ plaques touching	**B**++++ even growth	**B**++++ even growth	**P**++++ plaques touching	**P**+++ plaques touching
II	EC970520	**B**+++ even layered but clearer	**B**+++ clear corner with spots	**B**++ clearing with big spots	**B**+++ corner clearing with big spots	**B**++++ almost even layer
II	F1081	**B**+++ even layer but clearer	**B**++ clearing with big spots	**B**++ clearing with big spots	**B**++ half cleared with spots	**B**++++ even layer but slightly clearer
II	L568	**B**+++ even layer but clearer	**B**+ clearing with spots	**B**+++ big spots	**B**++ half cleared with spots	**B**++++ even layer but slightly clearer
II	R834	**B**+++	**B**++++	**B**+++	**P**+	**B**+++

to destroy any free chromosomal DNA, DNAse was inactivated by heat treatment, and a Stx2-specific PCR was performed. Most lineage I and I/II bacterial strains were positive for the Stx2 gene target following use of at least one induction method including *E. coli* O157:H7 Sakai (Table 4.3.2.A). *E. coli* O157:H7 strain F1299 was an exception; although belonging to lineage I and producing plaques (Table 4.3.1.), its culture supernatant tested negative for the Stx2 bacteriophage. By contrast, culture supernatants of lineage II strains were mostly negative for the Stx2 except for *E. coli* O157:H7 strain R834 which tested positive following both mitomycin C and ciprofloxacin but not UV treatment (Table 4.3.2.A).

All lineage II strain culture supernatants were negative for the Stx1 gene whereas lineage I strains were positive for Stx1 genes following use of at least one induction method (Table 4.3.2.B). However, supernatants from both lineage I/II strains, 71074 and 59243, were Stx1 negative. It was also observed that following treatments with UV light supernatants from most strains were Stx1 gene negative.

Since several bacteriophage are present in *E. coli* O157:H7 strains, DNA was extracted from plaques and Stx2 PCR assays were carried out to identify and quantify plaques associated with Stx2-encoding bacteriophage (Perna *et al.*, 2001). Plaques containing the bacteriophage were treated with DNAse to ensure that the amplified product was not from chromosomal prophage DNA. Replicate PCR results from 40 ciprofloxacin induced plaques of EDL933 revealed that 37/40 plaques (92.5%) were Stx2 positive. When the large spots from EC970520 were tested, 3/5 (60.0%) and 3/7 (42.8%)

Table 4.3.2. PCR detection of A) VT2 and B) VT1 amplified products for Stx phage detected in lineage-specific *E. coli* O157:H7 strains after induction with ciprofloxacin, mitomycin C or UV light and DNAse treatment of the supernatant.
(+): presence of expected band size. (-): no band. All results were replicated and shown in table.

A) VT2

Strain	Lineage	Ciprofloxacin	Mitomycin C	UV light
F1299	I	-, -	-, -	-, -
EDL933	I	+, +	+, +	+, +
63154	I	+, +	+, +	+, +
58212	I	+, +	+, +	+, +
F1095	I	+, +	+, +	+, +
Sakai	I	+, +	+, +	+, +
59243	I/II	-, -	+, +	+, +
71074	I/II	-, -	+, +	+, -
LRH13	II	-, -	-, -	-, -
R1797	II	+, -	-, -	-, -
L568	II	-, -	-, -	-, -
F1081	II	-, -	-, -	-, -
FRIK 1999	II	-, -	-, -	-, -
R834	II	+, +	+, +	-, -
EC970520	II	-, -	-, -	-, -
12491	II	+, -	-, -	-, -

B) VT1

Strain	Lineage	Ciprofloxacin	Mitomycin C	UV light
F1299	I	+, +	+, +	-, +
EDL933	I	-, +	+, +	-, -
63154	I	+,+	+, +	-, -
58212	I	+,+	+, +	-, -
F1095	I	+,+	+, +	-, -
Sakai	I	-, -	+, +	-, -
59243	I/II	-, -	-, -	-, -
71074	I/II	-, -	-, -	-, -
LRH13	II	-, -	-,-	-, -
R1797	II	-, -	-,-	-, -
L568	II	-, -	-,-	-, -
F1081	II	-, -	-,-	-, -
FRIK 1999	II	-, -	-,+	-, -
R834	II	-, -	-,-	-, -
EC970520	II	-, -	-,-	-, -
12491	II	-, -	-,-	-, -

were Stx2 positive. In all cases *E. coli* K12 strains tested negative for the Stx2 bacteriophage.

4.3.4. Assessment of Stx production

An *E. coli* Stx-specific ELISA was performed to determine the relative toxin production in lineages I, I/II and II strains before and after induction. After ciprofloxacin treatment, toxin production increased in all lineage I, I/II and lineage II strains (Figure 4.3.9.). The average ratio of induced Stx production (ciprofloxacin induction: no treatment) was similar for both lineages; *i.e.* the ratio was 4.2 for lineage I and 4.7 for lineage II strains. However average toxin production for lineage I strains was higher in both untreated (0.437) and in ciprofloxacin induced cultures (1.13) than the average observed for lineage II strains in untreated (0.058) and in ciprofloxacin induced cultures (0.293). Exceptions to this observation were seen for the lineage I strain F1299 which did not produce increased amounts of toxin after induction and for *E. coli* O157:H7 strain Sakai which did not show an increase in toxin production following induction equal to that of other lineage I and I/II strains. Lineage II strain LRH13 exhibited higher levels of toxin production relative to other lineage II strains after ciprofloxacin treatment. *E. coli* K12 did not produce any Stx in either the induced or uninduced cultures.

4.3.5. Stx-encoding bacteriophage and toxin release following infection of *E. coli* LE392

In order to determine if induced bacteriophage from *E. coli* O157:H7 strains were able to produce Stx in other *E. coli,* LE392 was used as a recipient. Culture supernatants

from *E. coli* O157:H7 strain EDL933 were inoculated into broth cultures of *E. coli* LE392. Stx production was measured by ELISA assays. There was significant toxin production, however, there was no difference in toxin production in supernatants between the ciprofloxacin-induced cultures and those that were untreated (Figure 4.3.10.). No toxin production was observed in induced and uninduced *E .coli* O157:H7 EC970520 or control *E. coli* K12 culture supernatants.

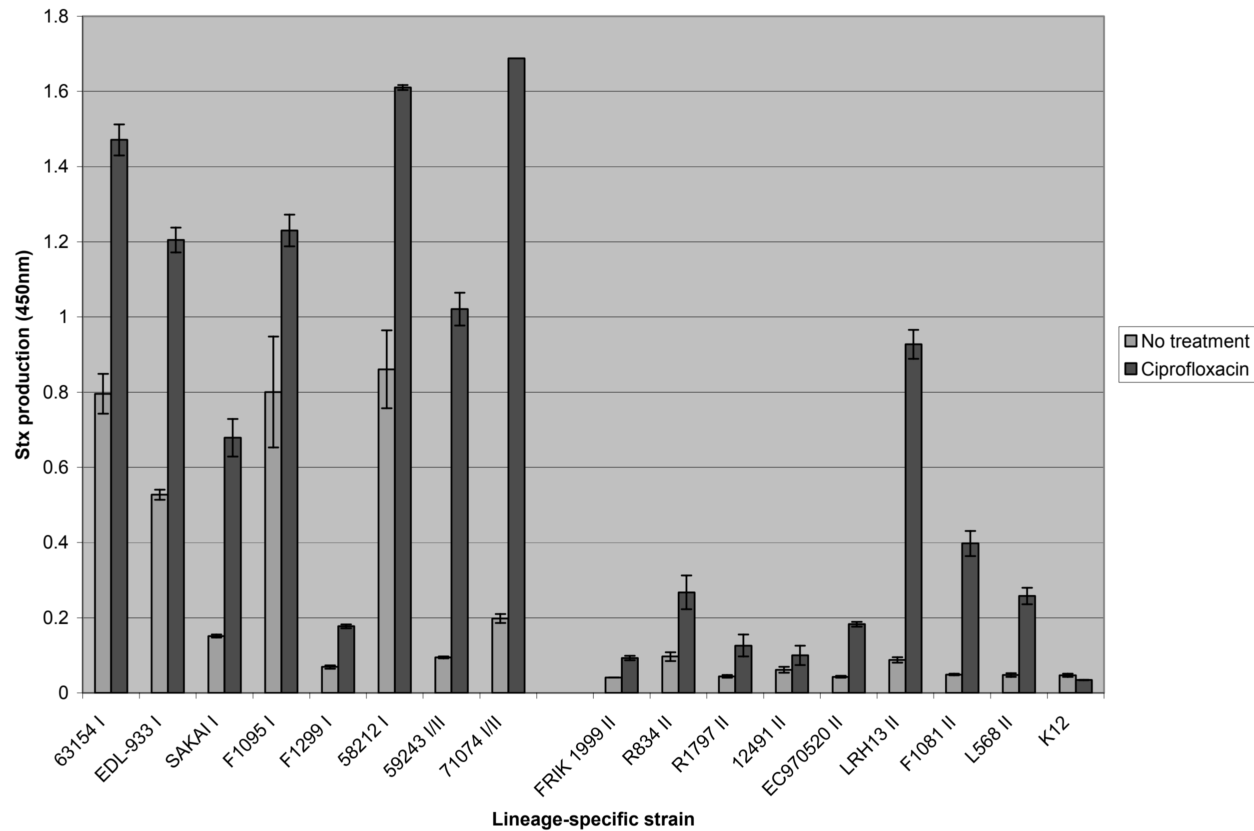


Figure 4.3.9. ELISA Stx production for lineage I, I/II and lineage II *E. coli* O157:H7 strains treated with ciprofloxacin verses no treatment.
Standard deviations are shown by error bars. The strains are 63154, EDL933, Sakai, F1095, F1299, 58212, 59243, 71074, FRIK 1999, R834, R1797, 12491, EC970520, LRH13, F1081, L568, and K12.

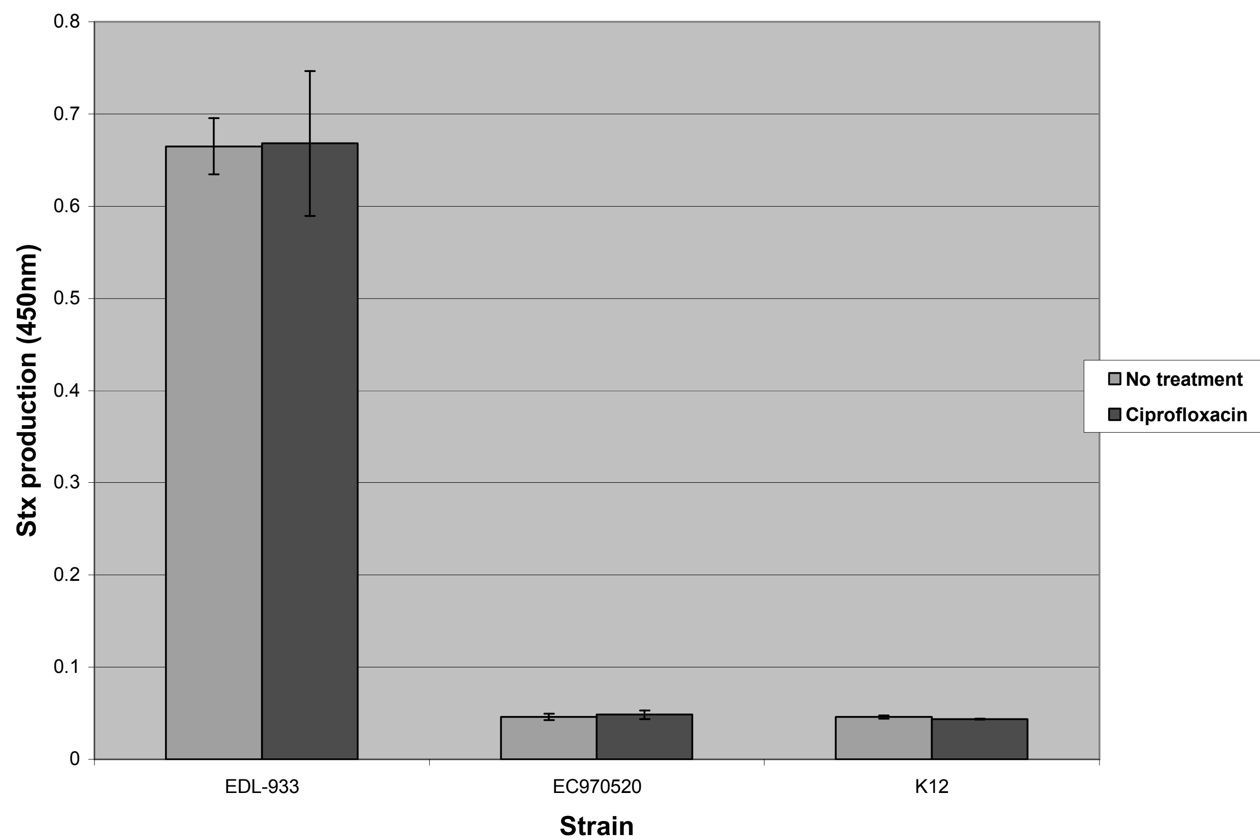


Figure 4.3.10. ELISA Stx production after transfer of supernatant induced EDL933, EC970520 and *E. coli* K12 to an *E. coli* LE392 recipient strain before and after ciprofloxacin induction.
Errors bars represent standard deviations.

4.4. Discussion

4.4.1. Phage release due to exposure to inducers

Exposure of *E. coli* cells to agents that damage or interfere with DNA replication can lead to induction of the lytic cycle resulting in phage release and toxin production. Cellular lysis of *E. coli* associated with phage release has been monitored by other works by measuring cellular optical density at 600 nm after exposure to inducers (Muniesa *et al*., 2003). In this study, the OD_{600} of cultures induced with ciprofloxacin, mitomycin C and UV light were measured and compared to non-induced cultures to determine the relative effectiveness of these inducers on phage release from *E. coli* O157:H7. It has also been determined that phage induction typically results in an initial increase in OD followed by a decrease, as phage are produced and bacterial lysis occurs (Gamage, 2004). Muniesa *et al*. (2003) have shown that in some *E. coli* O157 strains these lower OD_{600} values resulting from bacterial cell lysis correspond to higher levels of phage release. The results of this study suggest that ciprofloxacin treatment resulted in the highest levels of phage release; decreases in OD_{600} over time were greatest for ciprofloxacin, followed by mitomycin C (Figure 4.3.6.). UV light was not a strong inducer of bacteriophage production as the OD_{600} was closer to the non-induced treatments than the other inducers 3 hr post treatment. Each of the methods triggers phage induction in a slightly different manner; UV light and the mutagen, mitomycin C, directly damage DNA while the quinolone antibiotic, ciprofloxacin inhibits DNA gyrase (Lejeune *et. al*, 2004; Schmidt *et. al*, 1999; Gamage, 2004). It is possible that inhibition of DNA gyrase induces the SOS response and subsequent phage release more efficiently than DNA damage-

mediated induction effects of UV or mitomycin C. For this reason, ciprofloxacin also was used in most of our experiments.

It was observed that the OD_{600} from both lineages responded in a similar fashion before and after induction; there was a significant decrease in the OD_{600} following exposure to each of the inducers (Figure 4.3.3.; 4.3.4.; 4.3.5.). Muniesa *et al*., (2003) has concluded that induced cultures with a similar absorbance likely contain similar amounts of bacteriophage. However, previous studies have shown that the amount of extracellular toxin released by infected cells does not always correspond to the decrease in bacterial growth observed; *i.e.*, some bacterial strains may produce different amounts of toxin (Grif *et al.*, 1998).

The results of this study also showed that *E. coli* K12 cultures decreased in OD_{600} after treatment with inducers, but not as much as the *E. coli* O157:H7 strains examined. This suggests that some of the decrease in cellular density observed in our study might be attributed to bacterial death caused by the inducer and not just from phage lysis. In the present study *E. coli* O157:H7 strains Sakai and F1299 occasionally grew to higher cell densities suggesting that the phages in these strains may be less inducible. This observation concurs with studies by Makino *et al*. (1999) who reported that the Stx-encoding prophage in *E. coli* O157:H7 strain Sakai was not inducible.

The importance of linking bacteriophage release to the induction process is critical as the risk of toxemia may be enhanced after exposure to agents such as ciprofloxacin and mitomycin C when they are used as therapeutics. These toxins may be expressed after induction and could result in more severe disease such as HUS. Treatment of STEC patients with antibiotics is therefore controversial as many of these

agents inhibit DNA synthesis in the infected bacteria leading to induction of the phage lytic cycle, an increase in bacteriophage release and toxin production. Mitomycin C is used as a chemotherapeutic agent, but also is a potent bacteriophage inducer which is often associated with HUS. It has been reported that between 2 and 10% of mitomycin C-treated patients come down with HUS (Wu *et al.*, 1997). In addition, UV light which is currently used as a source for disinfection of bacterial pathogens when aseptically filling liquid foods such as milk, beer and fruit juices, in the ripening of cheese and dry sausages, and in water treatment may be of concern. Phage released from *E. coli* O157:H7 strains have been shown to infect and produce Stx toxin in non-pathogenic *E. coli* strains and this source of toxin may contribute to illness associated with *E. coli* O157:H7 (Gamage, 2004). In addition, to the possibility of increased phage release, these phage are more UV resistant than bacterial cells and therefore may survive bactericidal food processing procedures (Meng and Doyle, 2001). Techniques which allow proper monitoring of phage release need to be developed and will be helpful in identifying those strains of *E. coli* with pathogenic potential to cause disease in humans.

4.4.2. Induction and release of bacteriophage

Since Stx genes may only be expressed in the lytic cycle, phage release by different strains of *E. coli* O157:H7 was assessed in this study by their ability to form plaques. Lineage I and I/II strains of *E. coli* O157:H7 were able to produce plaques after induction but most lineage II strains were not. This suggests that phage in lineage I and I/II strains are much more readily able to enter the lytic cycle and produce viable phage particles that are able to infect other bacterial strains after their induction than lineage II

strains. However, these experiments also left the possibility that the lineage II strains produce a different type of phage that does not form plaques on the *E. coli* test strains used in this study. This may be evidenced by the different plaque morphologies observed for lineage I and I/II and lineage II strains in this study (Figure 4.3.7.; Figure 4.3.8.). The ability of lineage II *E. coli* O157:H7 to produce large zones of clearing on *E. coli* K12 lawns may be a result of the production of relatively few phage particles since these zones disappeared when the culture supernatants were diluted. In some cases, the frequency of plaque formation might also be attributed to the presence of Stx1 phage in addition to Stx2 phage in the bacteria; e.g., *E. coli* O157:H7 strains 71074 and 59243 lack Stx1 and did not always produce plaques. In addition, *E. coli* O157:H7 strain R834 may be more inducible than other lineage II strains as it was able to produce a small number of plaques on *E. coli* DH5α while other lineage II strains could not. It also is interesting to note that *E. coli* O157:H7 strain R834 although from lineage II was isolated from a human with clinical disease and this strain may be more pathogenic than other lineage II strains.

Some plaque formation was observed in the absence of a chemical inducer and may be attributed to spontaneous induction. Spontaneous activation of lytic phage occurs once in every 10^4 cell divisions and can be due to sporadic DNA damage such as stalled replication forks or decreased repressor (Calendar, 2006). However, spontaneous induction of plaques was only observed in lineage I and I/II strains. The small undefined plaques produced in response to UV light and mitomycin C treatments could result from lower levels of phage release.

Bacterial strains frequently contain defective or cryptic prophages in addition to complete ones (Calendar, 2006). Deletions, mutations and insertions within prophage genomes over time contribute to the presence of these defective phages. Lack of plaque formation may indicate an inability of the phage to produce lytic infection. In Chapter Two of the thesis, the presence of IS elements in the *E. coli* O157:H7 strain EC970520 were noted; these elements may contribute to production of a defective Stx2c lytic phage. Moreover, this defect may be present in all lineage II bacteria.

4.4.3. Presence of Stx2 bacteriophage

Sequencing of *E. coli* O157:H7 strains has revealed the presence of several prophages and phage-related elements within the genome. However, the extent to which these prophages are capable of producing viable phage is unknown. Many of these prophages appear to be defective and incapable of entering the lytic cycle. In contrast, most Stx2 producing bacteriophage have been shown to be capable of entering the lytic cycle and subsequently being released as infective phage particles (Ohnishi *et al.*, 2002). In this study, PCR of DNAse-treated culture supernatants and DNA extraction was used to show that most lineage I and I/II *E. coli* O157:H7 strains are able to produce Stx2 phage and that most lineage II strains are not (Table 4.3.2.A). The ability for *E. coli* O157 strains to produce viable Stx2 producing phage may contribute to the virulence of lineage I and I/II *E. coli* O157:H7 strains since phage release from these bacteria has been associated with higher levels of toxin production and more severe disease (Muniesa *et al.*, 2003; Ziebell *et al*., 2008). An exception to this was the lineage II strain, R834, which was positive for the Stx2 phage and produced plaques when plated onto *E. coli* K12.

However, this strain's Stx2 phage does not seem to be as sensitive to induction as those found in lineage I strains since it did not produce plaques consistently and did not produce high levels of toxin after induction similar to lineage I and I/II strains. The lineage I strain F1299 did not produce Stx2 phage, however, it did produce plaques; this likely is due to the presence of Stx1-encoding phage in the bacteria (Table 4.3.2.B). The toxin levels in F1299 were also low, similar to lineage II strains. This may make this bovine strain less virulent than other lineage I strains.

Use of PCR to examine culture supernatants from induced bacteria for the Stx1 gene revealed that most lineage I strains were positive for Stx1 but not lineage II strains. The lineage I/II strains 71074 and 59243 were as expected, PCR negative for Stx1 phage lysogens. UV light was unable to induce production of any Stx1 carrying phage. However, this may be due to the lower inducibility of the UV method. Optical density testing revealed very little cell lysis with the UV method. This also suggests that Stx2 producing phage may be more sensitive to UV induction than Stx1 producing phage. As severe disease has been linked to strains producing Stx2; this may be due to their ability to readily release phage and produce more phage-encoded Stx2 than those strains containing only Stx1 (Gamage, 2004). Similarly, the lack of phage induction in most lineage II strains and an accompanying lower toxin production may explain their infrequent association with human disease relative to lineage I strains.

Nitrocellulose membrane transfers were used to demonstrate that plaques contained Stx2. Most of the plaques in EDL933 were Stx2 positive (92.5%) but only a subset of the cleared zones found in the lineage II strain EC970520 contained Stx2 (60.0%, 42.8%). The presence of Stx2 in the EC970520 may be due to low rates or

occasional phage release, or chromosomal leakage. It is also possible that *E. coli* K12 could aid in producing functional Stx2 phage in EC970520 resulting in a different morphology of plaque. *E. coli* K12 contains remnant phage DNA and may be able to rescue mutations in related bacteriophage either by recombining with them to produce functional genes (Blattner *et al.*, 1997) or by complementing gene deficiencies within the related strains.

4.4.4. Relative toxin production in lineage I and II *E. coli* O157:H7

Toxin production was monitored by an ELISA to determine if the quantity of toxin released was related to the ability of lineage-specific strains to make viable Stx2 gene carrying phage. Lineage I, I/II and lineage II strains produced toxin following treatment with ciprofloxacin. Lineage I and I/II strains produce larger amounts of toxin than lineage II strains. The lineage I strain, F1299 did not produce very much toxin before or after induction with ciprofloxacin; and toxin production is likely only attributable to Stx1 (Table 4.3.2.B). This also demonstrates that strains which cannot produce viable Stx2 phage do not produce as much Stx, even if they are capable of producing viable Stx1 phage. It can also be noted that Stx2-producing phage strains 71074 and 59243 which were missing Stx1, were still able to produce high toxin levels (Figure 4.3.9.). The Sakai strain produced slightly less toxin than other lineage I and I/II strains and it is possible that this may be due to its lack of plaque-forming ability (Makino *et al.*, 1999) (Figure 4.3.9.). This strain may be capable of releasing Stx2 but not releasing Stx2-encoding phage like other lineage I and I/II strains. Lineage II strains produced very little toxin with the exception of LRH13 which produced similar levels of

toxin to the lineage I and I/II strains (Figure 4.3.9.). The reason why this strain is able to produce high levels of toxin is unknown. However, it is interesting to note that this lineage II strain was isolated from a human source. However, in contrast another human lineage II strain R834 did not produce high levels of toxin even though Stx2 producing phage were induced from this strain (Figure 4.3.9.). It should be noted that plaques were not consistently produced with R834 on bacteriophage-free strains and were uninducible with UV light (Table 4.3.1.; Table 4.3.2.).

4.4.5. Bacteriophage viability and toxin production

The ability of bacteria containing bacteriophage to produce viable phage, lyse the recipient strain LE392 and produce toxin was verified by ELISA. EDL933 filtered supernatants were able to transfer the ability to produce toxin to LE392; but filtered supernatants from lineage II EC970520 were not (Figure 4.3.10.). Future experiments with strains including F1299, Sakai, and R834 may aid us in understanding why these strains appear to differ from other strains of the same lineage and determine if these strains are producing viable Stx-encoding bacteriophage. The ability of lineage I strains to produce viable phage that are capable of converting strains that are otherwise non-pathogenic into toxin-producing strains capable of cellular lysis allows toxin production to be increased or amplified 1000-fold (Gamage *et al*., 2004) and could also contribute to the evolution of pathogenic organisms from non pathogens. It has been proposed that bacteriophages are part of a mobile common gene pool which can enable the creation of new phage with new properties at high frequencies (Hendrix *et al.*, 1999). The co-evolution of both virus and host can result in generation of mutants that also contribute to

the diversity of newly emerging pathogens by changing their fitness and host range (Weitz *et al.*, 2008). The mobility of inducible phage can cause recombination between genomes and generate diversity both within species and with other members of the intestinal flora creating the possibility for expansion of the bacterial host range of Stx2-encoding bacteriophage. The ability of lineage I strains to produce viable Stx2 phage that can infect other strains may contribute to the evolution of more highly pathogenic species capable of causing more severe human infections.

4.5. Conclusions

Little is known about genotypic differences between Stx2-encoding phages from different *E. coli* O157:H7 lineages and to what extent they contribute to phenotypic differences between the lineages. In this study,

1) Ciprofloxacin treatment was shown to result in the greatest decrease in cell density over time. The effect of mitomycin C treatment was intermediate and UV light treatment was the lowest in terms of cell density. Most lineage I and I/II strains were capable of plaque production while most lineage II bacteria were not or produced few phage with a different plaque morphology. Lineage I and I/II strains were capable of producing Stx2 phages whereas lineage II strains produce none or low amounts of Stx2 phage. Spontaneous phage induction was shown to occur in lineage I and I/II strains. Stx2 phage appear to be more sensitive to UV induction than Stx1 producing phage.

3) Lineage I and I/II strains produce higher levels of toxins than lineage II strains. Strains that cannot produce viable Stx2 phages appear to produce less toxin, and strains without Stx1 but with Stx2 produce high toxin levels. Some lineage II human strains produce more Stx2 than others and this may make them more virulent than other lineage II strains. The ability to produce viable Stx2-encoding phage and by so doing increase toxin expression and dissemination of phage may be important in the evolution of highly pathogenic *E. coli* O157:H7 strains.

CHAPTER FIVE

5. Implications and Future Studies

The Stx2 phage genomes within *E. coli* O157:H7 are highly heterogeneous and this level of heterogeneity suggests that many recombination events have occurred resulting in the formation of new strains with varying pathogenic potentials. The EC970520 Stx2 phage was selected for sequencing because microarray and PCR studies suggested that lineage II Stx2 phages are distinct from those found in *E. coli* 057:H7 lineage I strains. The Stx2 phage in EC970520, like the majority of other lineage II strains, contains the Stx2c variant. Lineage II strains consist mainly of bovine isolates whereas lineage I strains consist of both human and bovine isolates. While the Stx2c variant has been shown to be produced by strains associated with human disease, strains which produce this toxin variant may not be as virulent as strains which produce Stx2, such as the outbreak associated lineage I strain EDL933. This decreased virulence may be associated with the toxin type (Stx2c versus Stx2) or simply by the amount of Stx2 produced. Since toxin production in these phages appears to be closely linked to induction of the Stx2 phages into the lytic cycle, it is possible that genetic differences between Stx2 phage such as the presence of an IS element in the lineage II strains may inhibit the activation of crucial lytic genes. Several lineage-specific genes such as those for the Q anti-terminator protein upstream of the Stx2 genes in the phage sequence may be vital for Stx2 expression. Integration of Stx2 phages occurs in the *sbcB* region in lineage II strains whereas integration of this phage in lineage I strains occurs in the *wrbA* region. This difference in integration sites is likely associated with a difference in genetic mechanisms which allow these phage to integrate in a site-specific manner.

Differences in the ability of Stx2 phage present in each lineage to enter the lytic cycle and integrate into specific regions of the genome may contribute to the bacteria's virulence for humans and biased host distribution. Based on the results of this study, lineage I strains exhibit much higher inducer-mediated and spontaneous rates of phage release than lineage II strains. Triggering of the lytic cycle is clearly associated with increased Stx2 production. Following lysis, the phage released are also capable of infecting commensal *E. coli* to increase toxin production further. The phage may also integrate into the genomes of these other *E. coli* strains and allow a new STEC strain to emerge. The characterization of Stx-producing phage in human and bovine isolates will aid in identifying these phage in other bacterial strains and help us to determine how these phage are evolving and creating new potential pathogens. These data are also useful for genotyping strains and understand phenotypic differences observed among *E. coli* O157:H7 and STEC isolates from other seropathotypes.

A multiplex Bioplex assay was developed and it was able to specifically identify *E. coli* O157:H7 and the types of Stx(s) found within each strain. The Bioplex assays may be more specific than simple PCR for some loci. This assay format may be useful for detection of *E. coli* O157:H7 contamination in food and water samples. Several probes for the Bioplex assay were designed based on genetic sequences from Stx2 phage from EC970520 and EDL933. These probes were used to detect variation in the Stx2 phage present in human and bovine *E. coli* O157:H7 strains. The EC970520 Stx2 phage genes were lineage II-specific but only a subset of EDL933 Stx2 phage genes were lineage I-specific. The presence of several Stx2 phage gene combinations within a collection of lineage I strains confirms the heterogeneity of these phage and their highly

recombinogenic nature that allows the production of new phage variants. When characterizing these Stx2 phage genes in a collection of STEC strains from a variety of serotypes and seropathotypes, Stx2c phage genes from lineage II strain EC970520 were found to be more widely distributed throughout these strains than those from the Stx2 phage from lineage I strain EDL933. Certain EDL933 Stx2 phage genes appear to be markers for a subset of the most virulent strains from seropathotype A. These Stx2 probes are also helpful in differentiation among STEC strains and may be helpful for "molecular risk assessment" and epidemiological typing of strains to identify sources of infection.

We wished to determine if genotypic differences in Stx2 phage in *E. coli* O157:H7 strains was associated with their ability to produce viable phage and toxin. Ciprofloxacin treatment resulted in the greatest decrease in *E. coli* O157:H7 cell density over time followed by Mitomycin C and UV treatments. This decrease in cell density was associated with plaque formation and Stx2 production by most lineage I *E. coli* O157:H7 strains. Therefore treating human STEC infections with antibiotics such as ciprofloxacin or disinfection of food by UV light may trigger toxin production and phage release. In contrast, lineage II *E. coli* O157:H7 strains produce fewer plaques of distinct morphology from lineage I strains following treatment with these inducers. It is possible that the IS element found in lineage II EC970520 may also contribute to defective Stx2 phage production in other lineage II strains. Lineage I strains were also capable of spontaneous Stx2 phage release. The greater association of lineage I strains with human illness may be directly related to higher toxin production by both induced and non-induced cultures compared with those of most lineage II strains. Interestingly, one

lineage II strain with relatively high levels of induced and non-induced toxin production was isolated from a human with clinical disease. In addition, strains which possessed the Stx2 phage but lacked the Stx1 phage appeared to produce as high a level of toxin as strains which produced both Stx1 and Stx2.

The ability of Stx2 phage to produce viable Stx2 phage capable of producing plaques and therefore able to leave their hosts and integrate into the genomes of other bacteria is important in the evolution of new pathogenic bacteria. Detection of these phage in bacterial species and identification of their genetic components could aid in understanding how these strains have emerged, to trace sources of exposure in outbreaks of disease and how to prevent and treat disease associated with them. It is evident from this study that Stx phages are critical components in the evolution of these important pathogenic microorganisms.

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