STRAIN SPECIFIC DIFFERENCES IN THE SHIGA TOXIN-2 EXPRESSION AND AMPLIFICATION IN ESCHERICHIA COLI O157:H7 WHEN COINCUBATED WITH NON-PATHOGENIC ESCHERICHIA COLI STRAINS

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by
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Escherichia coli O157:H7 is a zoonotic pathogen that has been implicated in foodborne outbreaks linked to various food vehicles, including fresh produce and ground beef. They are notorious due to a low infectious dose of 10-100 CFU. Upon ingestion, the symptoms progress from watery diarrhea, to bloody diarrhea and severe abdominal cramps. In some cases, the infected patients develop hemorrhagic colitis (HC) or hemolytic uremic syndrome (HUS). There is great genotypic diversity within *E. coli* O157:H7. Clade classification based on single nucleotide polymorphisms (SNPs) identified a hyper-virulent subset, designated clade 8. Compared to other clades, strains clustered within clade 8 show increased hospitalization rate and higher incidence of HUS.

Among the virulence factors associated with *E. coli* O157:H7, expression of Shiga toxins (Stx) is an important determinant of pathogenicity. The *stx* genes are encoded within lamboid prophages which, when induced by DNA damaging agents, lyse the bacterial host as they switch from lysogenic to lytic life cycle. Examples of DNA damaging agents include antibiotics that function by targeting DNA replication, UV light, hydrogen peroxide, neutrophils and DNase colicins. In addition to induction by DNA damaging agents, non-pathogenic bacteria can also enhance Stx2 expression. Previous work on interactions between *E. coli* O157:H7 and non-pathogenic *E. coli* strain C600 demonstrated that Stx2 expression increased from basal levels when the two were co-cultured. The phage spontaneously induced from *E. coli* O157:H7 infects susceptible *E. coli* strains, resulting in increased phage and Stx accumulation. Apart from phage-mediated lysis, the non-pathogenic strains can also enhance Stx2 production by secretion of factors, such as DNase colicins.
Our lab investigated the diversity of Stx2-converting phage genomes by sequencing 22 inducible phage from different E. coli O157:H7. This led to the identification of 9 phage sequence types (PSTs), which grouped into 3 clusters. A notable cluster, termed PST2, grouped phage from two clade 8 E. coli O157:H7 strains, PA2 and PA8, with phage from prominent non-O157 outbreak strains, such as E. coli O104:H4, E. coli O103:H25 and E. coli O103:H2.

The Stx2-converting phage genome of PA2, when compared to prototypical strains Sakai and EDL933, showed differences in the early regulatory and replication region. To test the phenotypic manifestations of the genetic variations seen between Stx2-converting phage, we studied the kinetics of phage and Stx2 production upon induction with DNA damaging agents, as well as when co-cultured with E. coli C600. When induced with an antibiotic ciprofloxacin, PA2 produced the lowest amount of phage and Stx2, as compared to EDL933 and Sakai. However, when co-cultured with non-pathogenic C600, PA2 showed 20-fold higher Stx2 than when present alone. The Stx2 quantified from PA2 co-cultured with C600 was also significantly higher than C600 coincubated with Sakai and EDL933. Similarly, the other clade 8 strain, PA8 that also clustered within PST2 showed increased Stx2 expression with C600. Twelve non-pathogenic E. coli strains were co-cultured with O157:H7 to test Stx2 amplification. Four of the 12 strains showed increased Stx2 accumulation when co-cultured with the three E. coli O157:H7 strains. One of the 4 commensals expressed factors that enhanced Stx2 expression. Four categories of non-pathogenic E. coli strains; three of which amplified Stx2, while the fourth did not, were recognized. The Stx2 levels recorded when co-culturing the three O157:H7 with 12 commensals showed that PA2 corresponded with higher cumulative fold amplification, compared to EDL933 and Sakai.
Our results show strain specific variations in the fold amplification of Stx2 when susceptible *E. coli* are co-cultured with three *E. coli* O157:H7 strains. It is notable that PA2, showing highest fold amplification in co-culture, is a low producer of phage and Stx2 when present alone. This phenotype is also seen with PA8, another strain from the PST2 cluster. Additionally, we identified 4 categories of commensals that alter Stx2 levels by different mechanisms, which suggest that the host microbiota may alter the course of an *E. coli* O157:H7 infection. These findings may explain why some *E. coli* O157:H7 are more pathogenic.
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Chapter 1: Problem Statement

*E. coli* O157:H7 is a notorious foodborne pathogen, implicated in life-threatening diseases, such as hemorrhagic colitis and hemolytic uremic syndrome (HUS) that follows ingestion of the bacterial doses, as low as 10-100 CFU. There is great genotypic diversity within the *E. coli* O157:H7. SNP-based typing of numerous O157:H7 strains lead to the identification of a hypervirulent subset, designated clade 8. Among the strains tested, those clustering within clade 8 showed increased hospitalization rates and higher incidence of hemolytic uremic syndrome (HUS), compared to other clades.

Pathogenicity of O157:H7 is due to the presence of multiple virulence factors; most prominent of which is the production of Shiga toxin (Stx). The *stx* genes are encoded within prophages, integrated into the O157:H7 genome. Prophages replicate quiescently with the host and are maintained in a lysogenic state due to phage repressors. When conditions become unfavorable, repression is relieved and the phages switch from the lysogenic to the lytic state. This process of induction results in both phage and Stx expression. DNA damaging agents, including antibiotics, hydrogen peroxide and UV light induce Stx2-converting phages from lysogenic to the lytic cycle, ultimately causing release of virions and Stx as part of the expression of late genes in the phage operon.

Another cause for altered Stx2 expression is co-culture with non-pathogenic *E. coli* strains. When an *E. coli* strain is grown with O157:H7, it may increase or decrease the Stx2 levels. The increase could be attributed to phage-mediated lysis, which occurs when spontaneously induced phages from O157:H7 infect the susceptible commensal *E. coli*. The phages directly go into the lytic cycle, and as a result, more phage and Stx2 accumulates. According to this model, the increase in Stx2 expression would depend on the amount of phage
and Stx2 expressed by the O157:H7 alone. In the study done by Gamage et al., a high Stx2 and phage producer amplified the toxin expression when coincubated with the phage susceptible E. coli strain C600. The Stx2 levels after co-culturing susceptible C600 with E. coli O157:H7 producing lower levels of Stx2 is not known. Moreover, the strain specific difference in Stx2 levels after coincubation, especially when clade 8 E. coli O157:H7 is co-cultured with C600, has not been characterized. The same study showed 10% of human commensal strains have increased Stx2 expression when co-cultured with O157:H7 phage, but did not elaborate on the mechanism. The increase in Stx2 levels could be attributed to mechanisms other than phage-mediated amplification. Other independent studies have demonstrated increased Stx2 expression when DNase-colicin-producing commensals were coincubated with O157:H7. Characterization of mechanisms by which commensal E. coli increase Stx2 levels also needs to be investigated.

Our lab previously clustered the phage genomes from 22 O157:H7 strains and identified three phage sequence type (PST) clusters, namely PST1, PST2 and PST3. The strains belonging to PST2 were most remarkable, as not only did they contain two clade 8 strains, but also clustered with other non-O157:H7 strains implicated in the devastating 2011 German outbreak that caused over 50 casualties. In our study, the overarching objective was to investigate the differences between the prototypical strains and strain PA2 from the PST2 cluster. We compared the genotype and phenotype of PA2 to the prototypical strains Sakai and EDL933, both of which have well characterized genomes and have been implicated in prominent outbreaks. Clustering of phage genomes had highlighted that there are differences in the Stx2-converting phage of PA2, EDL933 and Sakai. The aim was to investigate the phenotypic manifestations of these genotypic differences. Our first objective was to study the kinetics of phage and Stx2 expression upon administering a DNA damaging antibiotic ciprofloxacin to the three strains. This approach would allow the characterization of PA2, when present alone, and how it compares to other prototypical strains.
The next objective was to test the strain specific differences in Stx2 expression when the three O157:H7 strains were co-cultured with a phage susceptible non-pathogenic *E. coli* strain, C600. Based on the diversity of O157:H7, our objective was to examine how strains will vary in their ability to alter Stx2 expression upon co-culturing. The cell counts for both O157:H7 and C600 strain were done to profile the population dynamics during the course of coincubation. As part of this objective, the Stx expression was studied both *in vitro* and *ex vivo*. The *ex vivo* analysis was done by developing a model that uses cattle intestine as a growth medium.

The third objective was to investigate *E. coli* other than C600 for their ability to alter Stx2 levels. We wanted to investigate the host range of O157:H7; especially how different *E. coli* strains would alter Stx2, when co-cultured with the O157:H7. The aim was also to compare the cumulative fold increase in Stx2 levels between the three O157:H7, and identifying strain specific differences in Stx2 amplification. Finally, Stx2 expression upon co-culturing C600 with clade 8 O157:H7 strains, including the strains belonging to PST2 cluster was tested. This is to characterize the fold increase, if any, in the clade 8 strains. The understanding of strain specific differences in Stx2 accumulation when co-culturing *E. coli* O157:H7 with other *E. coli* will help characterize the Stx2 amplification phenotype.
Chapter 2
Literature review

*Escherichia coli*

*E. coli* is a member of the family Enterobacteriaceae, encompassing strains that are mostly associated as normal gut flora of humans and other warm-blooded animals (1, 2). Some strains, however, acquired virulence genes and attained the ability to cause disease, thus becoming pathogenic. Based on the type of infection, the pathogenic subset of *E coli* is further classified into intestinal (diarrheagenic) and extra-intestinal (3). Extra-intestinal pathogenic *E. coli* (ExPEC) are implicated in diseases including septicemia (SEPEC), urinary tract infections (UPEC), and neonatal meningitis (NMEC) (3, 4). On the other hand, intestinal *E. coli* is divided into six pathotypes based on the mechanisms through which they cause disease and the virulence factors present. These pathotypes are enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteropathogenic *E. coli* (EPEC), enteroheemorrhagic *E. coli* (EHEC), and diffusely adherent *E. coli* (DAEC). A new pathotype was proposed after the German outbreak in 2011. Upon sequencing, it was revealed that the strains carried two plasmids, both of which were characteristic of EAEC. The first plasmid housed aggregative fimbrial adhesion operon, while the other carried a β-lactamase gene. In addition to these, the strains also bore prophages that encode Shiga toxins. Thus the strains were designated entero aggregative hemorrhagic *E. coli* (EAHEC) (5).
Phylogroup classification and distribution of *E. coli*

*E. coli* strains have a diverse and heterogeneous genetic substructure (6). To characterize the diversity, multilocus enzyme electrophoresis (MLEE) was performed using 38 enzymes on 72 strains from the *E. coli* reference (ECOR) collection. Four phylogroups, namely A, B1, B2 and D were identified, and each group showed variation in the enzyme-encoding genes. They also differed in distribution, with group-A strains being most commonly isolated from humans, while B1 contained strains from non-primate mammals. On the other hand, B2 strains, originated from humans and other primates (7). Another study expanded their work by using 202 *E. coli* strains from 81 mammalian and avian species and revealed that distribution is based on geographic origin and host taxonomic order (8). Clermont et al (4, 6) developed a quick triplex-PCR based screen to replace MLEE. Studies using the modified approach showed that phylogroups B2 and D also include extra-intestinal *E. coli* strains, while A are mostly commensal strains. Overall, the distribution within species was also characterized, as humans were shown to have a higher abundance of group A strains (40.5%) as compared to B2 (25.5%) or B1 (17%) and D (17%) (9).

**E. coli O157:H7**

The serotype of EHECs, which are most commonly implicated in HUS related cases are O157:H7. Phenotypically, O157:H7 cannot ferment sorbitol and are β-glucuronidase negative. This trait allows O157:H7 to be distinguished from other *E. coli*. The nomenclature O157:H7 is based on this serotype possessing the 157th somatic or Ohne (O) antigen and 7th flagellar Hauch (H) antigen (2). The serotype was first recognized as a major pathogen in 1983 by Riley et al. (10), when the group studied two outbreaks linked to consumption of undercooked hamburgers and isolated O157:H7 from patient’s stool cultures. The patients displayed symptoms of severe
cramps and abdominal pain, followed by watery diarrhea that eventually became grossly bloody. The illness was termed hemorrhagic colitis (HC). Subsequently, Karmali et al. in 1983 used culture filtrates from \textit{E. coli} serotypes including O157:H7 and demonstrated irreversible cytotoxicity on cultured African green monkey (Vero) cells (11), so the toxin was designated “Vero-cytotoxin”. The study established a link between sporadic cases of HUS and the \textit{E. coli} serotypes isolated from stool that express Vero-cytotoxin. O’Brien et al. had previously isolated a toxin that could be neutralized by an antitoxin against \textit{Shigella dysenteriae} serotype 1 (12). Eventually, it was determined that Verotoxin and Shiga-toxin (Stx), were the same, because toxin isolated from O157:H7 had the same subunit structure and range of biological activity as Stx, hence the terms Vero toxin and Shiga toxin began being used interchangeably. In this review the O157:H7 cytotoxin will be referred as Shiga toxin (Stx). The \textit{stx} gene is borne on lamboid bacteriophages in \textit{E. coli}. There are over a 100 different serotypes of \textit{E. coli} that produce Stx, and are termed Shiga-toxin producing \textit{E. coli} (STEC). STECs are different from EHEC’s as the latter possess a 60 MDa plasmid and express additional virulence factors, including the ability to produce attaching and effacing (A/E) lesions (13).

\textbf{Epidemiology:}

\textit{E. coli} O157:H7 is a notorious foodborne pathogen owing to its low infectious dose of 10-100 CFU and accounts for over 90\% of all the HUS cases in developed countries (14, 15). Study of the geographic distribution of O157:H7 showed that US and Canada have a high incidence. Furthermore, \textit{E. coli} O157:H7 is an important foodborne pathogen with outbreaks reported in Europe, Australia, Japan, South Africa and South America, especially Argentina, where HUS rate are five times higher than in North America (14). There is also a seasonal component to the occurrence, as infections tend to peak during the summer months in both the northern and
southern hemisphere. Infections have been reported, either as part of large outbreaks involving hundreds of individuals, or sporadic cases (13, 14). Population based studies done in the United States since 1985 to 1986 showed 8 cases of HUS for every 100,000 people. The estimated incidence was 21,000 infections annually; out of this, O157:H7 contributed to 50-80% of reported infections (16).

*E coli* O157:H7 colonizes the gastrointestinal tract of healthy cattle. Cattle are the major reservoir, as O157:H7 is asymptotically carried in the bovine gut and shed periodically (17). Moreover, O157:H7 is also isolated from the fecal flora of sheep, goat, pigs and deer (13, 14, 18–20). *E. coli* O157:H7 transmission mostly occurs through the consumption of contaminated food and water, or through person to person contact. The most common food vehicle for *E. coli* O157:H7 is ground beef, and most of the infections are known to be caused by consuming undercooked meat (21). Meat may becomes contaminated at the time of slaughter through fecal or hide contact, or during the grinding process, when the interior of the meat comes in contact with the pathogen (14). Moreover, food can also get contaminated post-slaughter, as demonstrated in a study done by Xia *et al.*(22), who investigated *stx* contamination on retail meats. Testing of 7,258 isolates recovered from 4 states, between the time periods of 2002 to 2007, was done. Almost all the STEC positive isolates originated from ground beef, but some isolates were also found on pork chops, possible due to cross contamination. Another feature of transmission is the resilience of O157:H7, as exemplified by the outbreak in commercial salami. This outbreak not only demonstrated the versatility of food vehicles, but also tolerance of *E. coli* O157:H7 to drying and fermentation (23). Furthermore, produce is also commonly implicated in outbreaks. Through cross contamination, either by fecal carryover or irrigation water, fruits and vegetables become sources of O157:H7 (18). The largest O157:H7 outbreak occurred in Sakai City, Japan, in 1996, in which more than 6,000 school children became sick from consuming contaminated radish sprouts (21). Processed foods, like unpasteurized juices and apple cider have
also been implicated, as exemplified by the apple cider outbreak that occurred in 1991 (24) in southeastern Massachusetts. A multistate produce related outbreak occurred in 2006 that lead to 183 people getting ill by consuming bagged spinach. This strain had a high hospitalization rate of 52%, and one casualty (25). Table 1 is a list of outbreaks that have occurred since 2006, showing the year and different food vehicles implicated.

Table 1. List of *E. coli* O157:H7 outbreaks in the United States since 2006.  
(Source [www.cdc.gov/ecoli/outbreaks.html](http://www.cdc.gov/ecoli/outbreaks.html))

<table>
<thead>
<tr>
<th>Year</th>
<th>Food vehicle</th>
</tr>
</thead>
<tbody>
<tr>
<td>2014</td>
<td>Multistate outbreak in ground-beef (Wolverine Packing Co.)</td>
</tr>
<tr>
<td>2013</td>
<td>Multistate outbreak in ready-to-eat salad</td>
</tr>
<tr>
<td>2012</td>
<td>Organic spinach and spring mix blend</td>
</tr>
<tr>
<td>2011</td>
<td>Romaine lettuce</td>
</tr>
<tr>
<td></td>
<td>Lebanon bologna</td>
</tr>
<tr>
<td></td>
<td>In-Shell hazelnut</td>
</tr>
<tr>
<td>2010</td>
<td>Bravo farms cheeses</td>
</tr>
<tr>
<td></td>
<td>Beef (National Steak and Poultry)</td>
</tr>
<tr>
<td>2009</td>
<td>Beef (Fairbank Farms)</td>
</tr>
<tr>
<td></td>
<td>Beef (JBS Swift Company)</td>
</tr>
<tr>
<td></td>
<td>Prepackaged Cookie Dough</td>
</tr>
<tr>
<td>2008</td>
<td>Beef (Kroger/Nebraska Ltd.)</td>
</tr>
<tr>
<td>2007</td>
<td>Totino’s Pizza</td>
</tr>
<tr>
<td></td>
<td>Ground beef patties (Topp’s Co.)</td>
</tr>
<tr>
<td>2006</td>
<td>Spinach</td>
</tr>
</tbody>
</table>
Detection:

The first symptom of O157:H7 is watery diarrhea, followed by abdominal cramping, and an absence of fever (26, 27). When an O157:H7 infection is suspected, the stool sample is procured and one or more of three different categories of tests is done. First category is culture confirmation of O157:H7 from stool sample, second is detection of fecal Stx, and third is the test for elevated antibody levels to O157 LPS or other EHEC antigens in the serum (13, 28, 29). Culture confirmation is done by plating on Sorbitol MacConkey (SMaC), which utilizes the inability of O157:H7 to ferment sorbitol, giving rise to colorless colonies. The putative O157:H7 colonies can be assayed with antiserum or latex agglutination kits (14, 25). Stool samples cultured within 2 days of onset of diarrheal symptoms show a 100% recovery of O157:H7; however, the rate of recovery is lowered to 33% after 6 days (13). Another biochemical test utilizes the β-glucoronidase-negative phenotype of E. coli O157:H7. It is unable to hydrolyze the substrate 4-methyl-umbelliferyl-D-glucuronide (MUG), allowing it to be distinguished from other bacteria. Apart from culture confirmation, serotyping is another way to ensure the isolates are O157:H7. Antisera for O157 lipopolysaccaride and H7 antigen are used in latex agglutination kits, enzyme linked immunosorbent assays (ELISA) or other methods to confirm the identity of isolates. Additionally, commercially available ELISA kits can detect fecal toxins and/or E. coli O157:H7 antigens. However, ELISA suffers from the limitation of high false positives, requiring a culture confirmation on SMaC (13). Other less commonly used methods include immunomagnetic separation (IMS), using magnetic beads coated with the specific antibody. This is a very sensitive technique that equaled PCR in detection rate, while surpassing direct fecal culture on SMaC. (28). Molecular methods are also widely used. Most common gene targets are stx genes, and other virulence factors specific to EHEC, including intimin (eae), β-glucoronidase (uidA), pO157
marker (*ehx*), H-specific (*fliC*) etc. (13, 30–33). PCR reaction or DNA probes are designed to target these genes, either in single or multiplex reactions.

The FDA Bacteriological Analytical Manual (BAM) protocol for detecting *E. coli* O157:H7 strains in food samples is as follows. The first step is overnight 37 °C incubation of the homogenized samples in EHEC-enrichment broth. The broth is supplemented with cefixime and novobiocin to limit growth of other microflora. After incubation samples are plated on Sorbitol MacConkey agar containing cefixime and tellurite (CT-SMaC), and incubated for 20 to 24 hours at 37°C. Samples positive for O157:H7 will show a white, colorless colony. A confirmatory test using PCR primers that target *stx1* and/or *stx2* and the *eae* genes is also done. Other variations of this method include using modified buffered peptone water (mBPW), which is supplemented with pyruvate. Antigenic tests to confirm the serotype of O157:H7, immunomagnetic separation and selective chromogenic media are also used for the confirmation of O157:H7 (34).

**Evolutionary model and subtyping of O157:H7:**

There is great diversity even within the O157:H7 serotype, where strains differ in their phenotype based on the source of isolation (35). Determining the genetic diversity is important component for studying strains during outbreak investigations. Most of this diversity has originated from loss or gain of genes through horizontal gene transfer or nucleotide polymorphisms (36–38). Multilocus enzyme electrophoresis (MLEE) was one of the first attempts at classifying *E. coli* strains and explaining the steps that lead to the emergence of the most recent ancestor of O157:H7 (36). Feng *et al.* looked at 163 *E. coli* isolates that included 78 O157:H7 strains implicated in clinical cases of HUS. Clusters were assigned based on a strain’s ability to ferment sorbitol (SOR), the nucleotide sequence of the β-glucoronidase (GUD/uidA)
gene, presence/absence of \textit{stx1} and \textit{stx2}, and the MLEE pattern using 20 enzyme loci in 46 representative strains. There were 15 electrophoresis types (ETs) identified, in which the ET1 encompassed all O157:H7 strains, as well as some non-motile O157. Based on these results, an evolutionary model was proposed, in which the serotype O55:H7, having phenotype GUD$^+$SOR$^+$, was designated as the earliest ancestor. The O55:H7 diverged by gaining \textit{stx2} through transduction of an Stx2-converting phage. The next event was lateral transfer of \textit{rfbE} region, which has similarity to perosamine synthetase of \textit{Vibrio cholera}, that lead to an antigenic shift from O55 to O157, in addition to the acquisition of the pO157 plasmid in an independent event.

The GUD$^+$SOR$^+$ and \textit{stx2}$^+$ O157:H7 strain further diverged into two separate lineages, in which one became non-motile through loss of the flagellar antigen, while the other lineage arose through loss of SOR and gain of the \textit{stxl} phage. This divergent lineage of O157:H7, which was GUD$^+$SOR$^+$ and \textit{stxl}$^+$ and \textit{stx2}$^+$, lost its GUD activity through a frameshift mutation in the \textit{uidA} gene, giving rise to the immediate ancestor of present-day O157:H7 clone (36, 39).

The O157:H7 clones have been further categorized by multiple molecular methods, including pulsed field gel electrophoresis (PFGE), multi-locus sequence typing (MLST), and single nucleotide polymorphism (SNP) typing. The earliest method looked at plasmid profile and Shiga toxin allele present (40) in 88 isolates from the Washington State Public Health Library. The information regarding clinical status of these strains was also known, thus permitting correlation of disease severity and \textit{stx} genotype. Among the 88 isolates, 13 plasmid patterns were identified, out of which 4 included 82% of isolates. The drawback, however, was that all isolates were restricted to one geographical area. The method was unable to distinguish outbreak strains from each other; however, they demonstrated the genetic diversity in O157:H7 by identifying existence of multiple ETs. Moreover, the study also recognized that isolates carrying \textit{stx2} genes were more likely associated with clinical cases of HUS.
PFGE is the gold standard for distinguishing strains implicated in outbreaks, and involves digestion of chromosomal DNA with restriction enzymes, followed by electrophoretic separation of the fragments (38). It was first used by Bohm and Karsh (41) to test clustering of epidemiologically-independent strains belonging to different serogroups and isolated from different geographic locations. The XbaI digested DNA from 36 isolates, resulted in identical banding patterns of O157:H7. The study concluded that although PFGE could separate the O157:H7 from other non-toxigenic strains, it was unable to distinguish epidemiologically distinct O157:H7 strains. More work was done by Barrett et al (42) by including a more expansive collection of 233 isolates and performing PFGE, as well as phage typing. The data showed that PFGE pattern could distinguish outbreak strains from sporadic cases, and was able to cluster isolates from a common source into the same group. A similar approach was used by Samadpour et al. to test 168 isolates by using bacteriophage lambda restriction fragment length polymorphism and cluster the outbreak and sporadic cases separately (43). Based on its high epidemiological concordance, PFGE gained acceptance and utility for its ability to separate the outbreak strains from non-outbreak, or sporadic cases. However, the drawback was its low discriminatory power (D) and ineffectiveness at deciphering genetic relatedness in E. coli O157:H7. In an attempt to increase the discriminatory power, Davis et al (44) used two restriction enzymes XbaI and BlnI to digest 62 isolates from cattle herds. The study showed that some DNA fragments were composed of non-homologous elements, despite migrating similarly in the gel. This lead to their conclusion that the banding patterns were poor measures of relatedness, and that PFGE could not be used to study the genetic diversity. Additional shortcomings of PFGE include long and labor intensive protocols, low-throughput and subjective results that have difficulty in comparison across laboratories. To counter such drawbacks, the next approach tested was multi locus sequence typing (MLST), in which specific internal fragments of housekeeping genes were analyzed to get a measure of genetic relatedness (45). Studies by Noller et al using 77 strains with
diverse PFGE patterns, showed no sequence variation. Thus, the efficacy of MLST to study genetic diversity was dismissed. Noller et al. targeted seven housekeeping genes and two outer membrane proteins (ompA and espA), which were expected to be under selective pressure. However, there was a surprising lack of sequence diversity among the tested strains (45). The same group used a different approach, by exploiting short tandem repeat regions that evolve rapidly and are associated with high diversity. Multilocus variable number of tandem repeat analysis (MLVA), identifies alleles based on the differences in number of repeats at a single loci (46). Fully sequenced O157:H7 genomes were used to identify tandem repeats in 7 loci that evolve rapidly and differed in sequence copies. PCR of the 7 loci resulted in 6 to 30 alleles, which gave rise to 64 MLVA types in the 80 isolates tested. This method was comparable to PFGE in sensitivity and ability to distinguish the outbreaks and sporadic cases. MLVA was also able to counter all the shortcomings of PFGE in being fast, repeatable, non-subjective and high throughput.

With the exception of MLVA, all attempts made to characterize genotypic diversity resulted in conclusions regarding the clonal nature of E. coli O157:H7, where strains from different geographic distribution would cluster separately (45–48). On the contrary, earlier studies that used plasmid profiling and bacteriophage typing had showed genetic diversity in O157:H7 (40). These conflicting results between the multilocus sequence typing and PFGE or plasmid profiling lead Kim et al. (48) to utilize the method targeting 23 of 150 octamer sequences in the E. coli chromosome. These sequences are over-represented and have a bias towards the lagging strand in the genome. Fluorescently-labeled forward primers and non-labeled reverse primers are used to amplify fragments that are then size-fractionated using automated sequencers, which can resolve up to one nucleotide. The patterns showed the O157:H7 isolates from geographically and temporally unrelated strains could be grouped into two lineages. Lineage I, which comprised of both human clinical isolates and some bovine isolates, was distinct from lineage II that had strains
exclusively of bovine origin. Kim et al. hypothesized that this non-random distribution observed between the two lineages could be due to physiological adaptations in lineage II strains, which makes the latter more successful inhabitants of the bovine gut. Alternatively, the lineage I isolates may have virulence factors that allow easier transmission to humans (48). A simpler method to segregate strains into lineages was developed by Yang et al. (47). The multi-locus PCR assay that targets 6-regions was termed Lineage Specific Polymorphism Assay (LSPA). Multiplex primer targets gave unique amplicon sizes that were genotyped by assigning a binary code. In the code proposed by Yang et al. 111111 corresponds to lineage I, while 211111, 212111 and 222222 were assigned lineage II. The study also confirmed, using over 1,500 isolates that the host source of O157:H7 was consistent with findings of Kim et al. The LSPA code, introduced by Yang et al. was rectified by Zhang et al (49), where lineage I/II was assigned to LSPA type(s) 211111 and lineage II strains were LSPA type(s) 222222, 222211, 222212 and 222221. The newly defined lineage I/II had equal distribution in human and clinical isolates using comparative genome hybridization (49). The phenotypic implications of these were also studied extensively. Using a gnotobiotic pig model, Baker et al (50, 51) demonstrated, lineage II isolates from healthy cattle were less virulent as compared to lineage I isolates from clinical patients. This was confirmed by Zhang et al. (35), where they showed that lineage I and lineage I/II were expressing significantly higher levels of Stx2 than lineage II. Moreover, they demonstrated that the Q gene is replaced by pphA in lineage II. The stx2 allele is also different, where the lineage I/II strains are more likely to carry both stx2 and stx2c while lineage I and lineage II are more likely to carry the stx2 and stx2c, respectively. Overall, the strains associated with human clinical illness produced significantly more Stx2, as compared to bovine strains (35). Their studies concluded that diversity in lineages could be attributable to differences in prophage content, and stx2 alleles carried.

Most of the previous molecular studies based data on the two fully sequenced prototypical strains, EDL933 and Sakai. Zhang et al. (52) employed comparative genome
sequencing microarrays to 11 clinical strains of O157:H7 strains and investigated the intergenic nucleotide polymorphism. A total of 1199 chromosomal genes and 92 kb of virulence plasmid pO157 were studied to identify informative SNPs. In addition to 906 SNPs in 523 genes, a high level of DNA polymorphism was observed in the pO157 plasmid. Apart from estimating the evolutionary time frame of O157:H7, the knowledge of SNPs allowed development of subtyping techniques that could be used for devising faster detection methods. Classification of O157:H7 strains into nine clades was subsequently done by Manning et al. (53) based upon 96 SNPs loci. The rationale for subtyping was to understand why some E. coli O157:H7, like the strain implicated in the 2006 spinach outbreak, was more virulent and associated with a higher incidence of HUS that the other strains. This approach lead to the grouping of strains by clades and identification of a subpopulation of O157:H7 with increased virulence.

Clawson et al. (38) noted that the drawback of Manning et al. clade classification was, the emphasis on clinical strains as well as low representation of O157:H7 isolated from bovine origins. The authors proposed another classification to understand the full spectrum of O157:H7 genetic diversity (38), in which 193 strains were sequenced by 454-sequencing platform and observed 178 SNP loci. Out of the identified SNPs, 42 genotypes were identified, which clustered strains according to host source. Another attempt at classifying and determining the phylogeny of subtypes of O157:H7 was done by Bono et al. (54) in which the authors used both, polymorphism-derived genotypes, and a phage insertion typing system to classify strains from outbreaks and also try and replace PFGE as the gold standard. Although the method was unable to surpass the epidemiological concordance shown by PFGE, it successfully categorized strains by genogroup and identified 8 lineages. The results from Bono et al. identified 175 genotypes tagged using 138 SNPs. Out of the 8 lineages, cattle were defined as the reservoir for 7. The most recent proposition for classification was put-forward by Jung et al. (55), who typed O157:H7 strains at 48 SNPs loci, identifying 11 genogroups within the 8 lineages proposed by Bono et al.
The researchers used 530 isolates and further classified the 8 lineages into subgroups based on isolated host.

Genomics:

Genome sequencing of non-pathogenic E. coli strain MG1655 by Blatter et al. 1997 (56) revealed a 4.6 Mb chromosome. Subsequently, two outbreak strains of E. coli O157:H7, Sakai and EDL933, were sequenced by Hayashi et al. (21), and Perna et al. (57), respectively. Comparative genomics showed that the O157:H7 strain Sakai was 5.5 Mb, which was 859 kB larger than the sequenced MG1655. The strain Sakai, contained a highly conserved 4.1 Mb sequence, which was designated as the backbone, or core genome of E. coli (21). This backbone had 98.3% sequence identity between the two strains and is free of any rearrangements, including translocation and inversion (21). However, the conserved sequence is interrupted by a 1.4 Mb unique sequence, termed strain-specific loops or S-loops. There are 296-loops distributed in an uneven manner throughout the chromosome. These S-loops may have been acquired through horizontal gene transfer by bacteriophage, as evidenced by the higher G+C content and lower codon usage. The S-loops vary in size, with each being at least over 19 bp, and were considered most likely spots for recombination and integration of foreign DNA to occur. Most of the large loops incorporated the prophage and prophage-like genes, and other horizontally acquired genes that housed major virulence factors. Sakai chromosome has 18 prophage and prophage-like elements, called Sakai prophage (Sp), and were numbered Sp1-Sp18. Thirteen out of 18 prophage are lambda-like, including Stx1- and Stx2-converting phage, present on Sp15 and Sp5, respectively. The other 5 phages include a Mu-phage, P4 as well as P2-like and P-22-like phage. The 6 Sakai prophage-like elements (SpLE), numbered SpLE1-SpLE6 were also horizontally acquired. SpLE1 or the S-loop 72 carries tellurite resistance genes and adherence conferring
islands SpLE4 carries genes for the locus of enterocyte effacement (LEE). The other virulence factor(s) are borne on the 92.7 kB plasmid pO157, which carries genes encoding the enterohemolysin operon, EspP protease, and catalase peroxidase genes. In the Sakai strain, there are 1632 protein coding genes, constituting 88.1% of the total chromosome. Among the protein coding genes, 131 have virulence-related functions (21). Comparison between two E. coli O157:H7 strains, EDL933 and Sakai, showed multiple differences in genome organization. A 420 kb inversion at the replication terminus, as well as duplication of 86 kb prophage-like element designated SpLE1 in Sakai, was observed in the EDL933 chromosome (57, 58).

**Virulence factors: Shiga Toxin**

The pathogenicity of E. coli O157:H7 was largely due to acquisition of multiple virulence factors through horizontal gene transfer (21, 39, 58, 59). The presence of virulence factors and ability to express them is not sufficient to make the bacteria highly pathogenic. The bacterial background, as well as combination of the virulence factors, confer notoriety to E. coli O157:H7 (60). This point is further illustrated by the higher incidence, hospitalization, and mortality rate associated with E. coli O157:H7, as compared to other Shiga-toxin producing E. coli (STEC). The non-O157 strains contribute to only 25% of total STEC incidence (61, 62). Moreover, pathotypes including ETEC, EPEC and EAEC are not implicated in outbreaks that parallel in magnitude to EHEC (14). The exception, however, was EAHEC, which was implicated in German outbreak in 2011 (5). Virulence factors, such as expression of Stx1 and/or Stx2, are either found on lamboid prophages, (21), or on the chromosome as integrative elements (IE), such as the genes found on SpLE4 that encode factors involved in production of attaching/effacing lesions (63, 64). In addition, there are genes borne on pO157 that increase pathogenicity of O157:H7.
The nomenclature of Shiga toxin (Stx) originated from identification of *E. coli* strains whose culture filtrates showed toxicity towards African Green monkey kidney cells or Vero cells (65). These toxins were neutralized when rabbit anti-toxin against *S. dysenteriae* Serotype-1 were applied, hence the name Vero toxin. The terms Vero cytotoxin and Shiga toxin have been used interchangeably, until it was clarified that the two are the same (12). Apart from EHEC, *Shigella dysenteriae* serotype-1 is also known to express Stx1 (12). Among the virulence factors found associated with O157:H7, Stx expression is the most prominent and important one (66). Stx is a potent cytotoxin that halts protein synthesis using the same mechanism of action as plant toxins such as ricin and viscum (67). The expression of Stx2 is triggered by lytic conversion of prophage, which in turn, is caused by DNA damage (68). This limits the ability to use antibiotics that target DNA replication, for treating O157:H7 infections.

**Structure:**

Shiga toxin(s) are approximately 70 kDa proteins that are comprised of an A and pentameric B subunit. Earliest reports purified Stx by chromatography and ammonium sulfate fractionation to reveal the 32,225 Da A subunit and multiple copies of the 7,691 Da B subunit (69–72). Subsequently, the translated nucleotide sequence also confirmed the observation of molecular weight (73). The B subunits have anti-parallel β-sheets and an α-helix, which together, constitute the binding site for Stx receptor. There are two types of antigenically distinct Stx, that are immunologically non-cross reactive, and named Stx1 and Stx2 (74). The sequences of *stx1* and *stx2*, have 56% amino acid identity (74, 75). The electrophoretic mobilities of Stx1 and toxin from *S. dysenteriae* serotype-1 are the same when run on sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE) (69). The *stx1* has a single reported variant designated *stx1c*, which was associated with O-groups other than O157:H7. The genes for *stx1c* shares 97.1% and
96.6% identity to the A and B subunit of stx1, respectively (76). On the other hand, there are multiple allelic variants of stx2 including stx2a, stx2b, stx2c, stx2d, stx2dact, stx2e. Both stx2a and stx2c have 100% amino acid identity in the A subunit, while differing in the B subunit. Similarly, stx2d and stx2c have the same B subunit, but dissimilar A subunit.

**Mechanism of action:**

Mechanistically, the prototypical Stx starts acting when the B subunit attaches to the receptor on mammalian cells. Toxin-adherence assays using radiolabelled Stx, showed binding to microvillus membrane in the brush border epithelium of rabbit ileum (77), and also identified a carbohydrate motif to be the receptor for the B subunit (77, 78). Analysis revealed that the receptor for Stx including stx1, stx2a, stx2c and stx2d was a glycolipid globotriasoyl-ceramide (Gb3), while globotetrasoyl-ceramide (Gb4) bound stx2e. The X-ray crystallography structure of B subunit indicated three carbohydrate binding sites on each B-subunit, which, when mutated with site-directed mutagenesis, and showed reduced toxicity (79). Non-receptor mediated entry may also exist and play a role in the infections becoming systemic, without contacting the epithelial cells. Disease distribution within organism is also known to be more common in organs that express the Gb3 on its surface (72). Upon attachment of the pentameric B subunit to the receptor(s), the AB₅ is endocytosed and follows one of two possible paths. Stx either moves to the lysosomes for degradation, or is transported to the Golgi apparatus, and subsequently to the endoplasmic reticulum (67, 72). The A subunit in the cytoplasm is proteolytically cleaved to yield two fragments; one being a 28 kDa peptide which has the enzymatic activity, while the other is a 4 kDa peptide, that links to the B subunits. Before being cleaved, the two A-units are linked by a disulphide bond. The larger cleaved portion of A subunit acts on the 60S ribosomal subunit,
which has N-glycosidase function, that removes the adenine residue from the 28S rRNA. This action abrogates protein synthesis in the eukaryotic cell, ultimately causing cell death (80).

**Toxicity studies using different models:**

Earliest observations regarding the role of Stx in pathogenicity of *E. coli* O157:H7 was done using gnotobiotic pig models, in which researchers observed attaching and effacing lesions, as well as renal damage, when the animals were orally fed with O157:H7 (81). Another study using the same model also showed that the strains isolated from healthy cattle were less pathogenic to gnotobiotic pigs, compared to clinical isolates. There appears to be a correlation with the amount of Stx2 expressed by the strains and pathogenicity (50). Mouse studies also demonstrated that *E. coli* O157:H7 strains expressing Stx2 were more likely to develop acute renal necrosis and die, as compared to the strains that express Stx1 (82, 83). To test the hypothesis, regarding increased potency of Stx2 as compared to Stx1, pure Stx(s) were injected into mice, and their histopathology was studied. Results showed that intravenous (IV) and intraperitoneal (IP) injections of Stx2 had 400 times lower LD<sub>50</sub> than Stx1. Mechanistically, this difference in potency was not mediated by receptor binding to Gb3, as demonstrated by solid-phase binding assay, which revealed that Stx1 had a higher affinity. Their study investigated stability of the two Stxs to reveal that Stx2 was more heat and pH stable macromolecule, as compared to Stx1 (83). Another animal study included a primate model that used baboons, and IV injected with 25 ng/kg of Stx1 and Stx2, every 12 hours. The baboons receiving Stx2 started developing symptoms after 60 hours, and became moribund by hour 72, while the Stx1 and control groups did not show any symptoms (84). Studies on cell lines also showed that the Stx1 bound with higher affinity to the human renal microvascular endothelial cells (HRMEC), as compared to Stx2, however the latter was 1000 times more cytotoxic (85). The other possible
mechanistic explanation of lowered hemolytic uremic syndrome (HUS) incidence with Stx1 could be due to the difference in tissue targets. Radioactive iodine-labeled Stx1 and Stx2 were injected in mice and whole body autoradiography, with emphasis on specific targets was done. The results showed that lungs and nasal turbinates were major targets and showed most accumulation of Stx1, while Stx2 was found in the renal cells at a higher incidence (86). It was also observed, through epidemiological studies, that the allelic variants within Stx2 show differences in potency and HUS incidence. Studies looking at 201 STEC strains from clinical and environmental sources showed that stx2a and stx2c alleles were most frequently associated with high virulence and clinical samples. The milder and asymptomatic strains carried stx2d, stx2e, stxl and stx1c. Phenotypically, the amount of Stx expression in vitro, between strains were statistically indistinguishable between the HUS and non-HUS associated strains. The study concluded severity of HUS symptoms is determined by the stx2 allele of strains, along with some unknown virulence factor, instead of the Stx expression. (87). Studies on the structural and biochemical differences between the stx2 alleles stx2a, stx2b, stx2c, stx2d and elastase-cleaved stx2d (stx2dact), using Vero-cells and renal cells, showed that Stx2a, Stx2d and Stx2dact were 25 times more potent than Stx2b and Stx2c (88).

**Clinical manifestations and course of infection**

After ingestion of STEC, the clinical course of HUS infections commences with an onset of loose and watery diarrhea. The interval may range between 2 to 12 days, depending upon factors including dose, STEC serotype and gender, age or immune status of the host (10, 27, 89, 90). *E. coli* O157:H7 infections are distinguished from other possible gastrointestinal infections by the absence of fever during diarrhea. After approximately 3 days, the diarrhea becomes bloody, in about 90% of the cases (90). This is generally the point when patients or their family
members consult clinicians, who would then check their leucocyte counts. Onset of subsequent symptoms, including increased abdominal pain and painful cramps while defecating are observed (26, 91). Once diagnosed, the patients are secluded, in order to prevent secondary infections, and given intravenous rehydration as well as hypotonic solutions to maintain fluid balance. Giving patients antibiotics is strictly avoided because of their strong association to development of HUS (92, 93). This observation is because stx are borne on lamboid prophages, which become lytic and release Stx. Moreover, loss of competitive microflora in the gut could also attribute to increased susceptibility of the host. Furthermore, antimotility agents and narcotics are also avoided for patients showing symptoms of bloody diarrhea, as they are known to aggravate symptoms (93). Usually, after 5 to 13 days of diarrhea and abdominal cramps, the infections progress to HUS. Approximately, 38 to 61% of individuals develop hemorrhagic colitis (HC), while 13-9% of individuals infected will develop HUS (90, 92). This statistic varies between outbreaks and strains implicated, as exemplified by the spinach outbreak in 2006 that showed development of HUS in 16% of the cases which was comparatively higher than numbers reported in other outbreaks (25). Incidence of HUS is estimated to be 2.1 cases per 100,000 people/year. Children younger than 5 years are considered to be a high risk population (94). HUS is clinically defined by thrombocytopenia, or low blood platelet count, followed by hemolysis, and characterized by ruptured erythrocytes. Biomarkers for HUS onset are increased creatinine concentrations and anemia. Most of the infected patients recover naturally (26, 27); however, 55 to 70% cases may further progress to acute renal dysfunction that may either resolve gradually, or result in kidney failure (16, 91). Treatments are supportive, and involve administering saline to maintain the electrolyte balance, except in case of renal failure, where dialysis becomes necessary. In cases showing severe anemia, blood transfusions are done to restore the blood counts (14, 16, 91).

Detection strategies primarily involve the identification of E. coli O157:H7 using SMaC, or other culture based methods, details of which are in the section on “detection of E. coli
O157:H7. Laboratories studying Stx expression use methods including Vero cell assay, Western blot, and ELISA. Vero cell assays were the first to be used to detect Stx (65, 74) by incubating cell cultures with titers of bacterial lysates and profiling the cytotoxicity. Western blot analysis use antibodies that target the Stx, which is first bound to membranes like polyvinylidene fluoride (PVDF). Fluorescent secondary antibody binds to the antigen-specific primary and the strength of signals correlate with toxin quantity (95). Finally, enzyme linked immune sorbent assay (ELISA), is another colorimetric assay that also uses antibodies, to bind to the Stx antigen captured on wells of the ELISA plate. It is a very sensitive and specific method. Multiple commercial kits are available that can detect Stx including Premier® EHEC, Biopharm Ridascreen and ProSpecT (96). The methods mentioned can be used to quantify the Stx by generating a standard curve using pure Stx, and plotting unknowns on the curve.

**Virulence factors: Others**

Other virulence factors include genes on the chromosome and virulence plasmid whose products express adhesins, hemolysins and other factors that boost colonization and increase pathogenicity of O157:H7. Although Stx is an important determinant for causing disease in humans (66), studies have shown that it is not required for colonization in cattle (97). Mutant strains that were deficient in expression of virulence factors, including intimin (eae), translocated intimin receptor (Tir) and pO157, were unable to colonize or persist in the cattle rectal mucosa. These findings further supports the hypothesis that all the virulence factors work in tandem to confer high pathogenicity to
the O157:H7 serotype, which is why the latter is highly pathogenic, as compared to other STECs.

Locus of enterocyte effacement (LEE) is an important virulence factor expressed by *E. coli* O157:H7, and is found on the 33.2 kb pathogenicity island (98). Genes encoding the outer membrane protein intimin (*eae*), type three secretion system (T3SS), secreted protein (*espA, espB, espC*), and the translocated intimin receptor (*tir*) are found on this locus. The pathogenicity island, absent in benign *E. coli* strain K-12, confers the pathogenic organism an ability to attach to the intestinal mucosa (98, 99). The entire LEE island is under regulation from the LEE-encoded regulator (*ler*) operon that regulates function in EHEC (100). The multiple genes found on the loci are arranged into polycistronic operons designated LEE1 through LEE5. LEE4 houses *sepL* and *ecsF* that are essential for T3SS apparatus, as well as *espA, espB, espD* and *espF* that are effector proteins (100–103). The T3SS acts like a molecular syringe, where effector proteins are injected into the host cell. Mechanistically, the effector proteins, including Tir, EspG, EspH and Map, enter the host through the filamentous extension formed by EspA, EspB and the T3SS apparatus. The central domain of Tir interacts with the 94 kDa protein intimin, expressed on the surface of the EHEC (103, 104). Next, a polymerized actin-complex is formed directly beneath the adhered cell which is composed of actin, talin, ezrin and and α-actin, and resembles a pedestal (105, 106). This follows the localized degeneration or effacement of the brush border microvilli. These formations are also seen in enteropathogenic *E. coli* (98, 100, 105)

The other virulence factor is the 60 MDa plasmid pO157, which is also carried by uropathogenic (UPEC) *E. coli*, considered an extraintestinal pathogen implicated in
A study on the hemolytic property of the pO157 was done by transforming non-pathogenic strain (C600) with the pO157, and hemolytic properties were observed in the benign *E. coli* strain (107). Strains expressing hemolysin (*hly*) genes have the ability to lyse erythrocytes. The hemolytic toxins expressed by UPEC strains are termed α-hemolysin, while EHECs including O157:H7, express enterohemolysin (*ehx*). Ehx forms turbid zones on blood-agar plates after 18 hours of incubation. Alternatively, hemolysin shows clear zones within 4 hours of incubation. This phenotype is used to detect and differentiate O157:H7 containing the pO157 (60). The plasmid, that ranges from 93.6 to 104 kb (107) carries genes that share 60% identity with *hlyA* from uropathogenic *E. coli* (108, 109). Ehx secretion was regarded as defective, as compared to Hly from UPEC, as seen in toxicity studies in erythrocytes that showed lack of hemolytic activity when supernatants of *E. coli* O157:H7 were used. This observation was due to a defect in the genes related to transport, as the expression of Ehx increased when *hly* transport system from UPEC was transferred to *E. coli* O157:H7 (107, 110). The *ehx* genes are commonly associated with the O157:H7 serotype, and were found on 99% of the 107 strains tested by Levine *et al.* (111). The exact role of Ehx in human infection is not fully understood, however, the *ehx* genes show high level of conservation among O157:H7 strains. It is possible that the operon is maintained due to some putative role it plays in the survival of bacterium (66). Additionally, pO157 encodes some adherence factors, that mediate attachment to intestinal derived epithelial cells (112) and a catalase peroxidase *katP* (113).
Bacteriophage:

Prophage is the term for bacteriophages that, through horizontal gene transfer, integrate themselves into the bacterial genomes and replicate within the bacterial host. The phages become a source of variation between different bacterial strains (114, 115). The incoming phage has one of two pathways that it may follow. In the lysogenic life cycle, the phage parasitizes the host and multiplies with the bacteria. Alternatively, in the lytic life cycle, phage can use host machinery to assemble and package its DNA into viral particles, as well as express proteins, which would lyse the host and release infectious virions. These virions can infect other bacterial hosts, and follow either of the life cycles (115, 116). Temperate phages are maintained in a lysogenic state by repressors, but switch into lytic phase when phage encoded repressors are proteolytically cleaved. This conversion of temperate phages from lysogenic to lytic state is called “induction” (117–119). Whole genome sequencing of the prototypical strain Sakai revealed 18 prophage and prophage like elements, designated Sakai prophage 1 (SP1) through SP18. (21). Out of the 18 SPs 11 are lambda-like and carry virulence genes. Most of the 18 Sp are predicted to be defective, due to gene deletions or disruptions, which make them unable to induce and propagate (59). The stx genes are borne on lamboid temperate phages (120, 121). The Sp5 carries stx2 genes, while Sp15 encodes stxl in Sakai (21). The other types of phages characterized in Sakai include P2 like (Sp13), P4 like (SP1), Mu-like (SP18) and four untypable phages, Sp2, Sp7, Sp16 and Sp17. The latter category is designated untypeable due to the disrupted and chimeric nature of the phage backbone. (59). The lamboid phage encoding Stx2 genes is discussed in the subsequent section, as it was the
primary focus of our experiments. Morphologically, the Stx2 phage have a 70 nm wide hexagonal head and short contractile tails (122).

**Genetic structure:**

The complete genome of Stx2-converting phage was sequenced as part of the whole genome sequencing project of EDL933 strain (122). The genome organization of \textit{stx2} is represented in Fig 3-1. Overall, the phage has high level of similarity in genome organization with lambda (31, 115, 122). In lambda, site-specific recombination between the core-phage sequence, \textit{attP} and bacterial chromosomal sequence \textit{attB} allows the integration of the phage into the host. The \textit{attL} and \textit{attR} flank the integrated phage and when the phage excises, the two genes circularize by a similar site-specific recombination event and exit the bacterial chromosome. The integration site in prototypical strains Sakai and EDL933 is \textit{wrbA}, which encodes a TrpR binding protein (123–125). The sequencing of \textit{stx2} phage genome 933W, from the strain EDL933 revealed a 61,670 bp sequence, with 81 open reading frames (ORFs), out of which 45 were annotated and showed sequence similarity to known lambda or P22-like phage genes (122).

The entire genome could broadly be divided into four functional regions; namely recombination, early genes, replication and late genes. The recombination genes start from phage-encoded recombinase or Integrase (\textit{int}) that works with excisionase (\textit{xis}) to allow strand cleavage, rejoining and excision. Other genes found annotated in the recombination region include \textit{exo}, \textit{bet}, \textit{gam}, and \textit{kil}. The next regions are the “early genes” that include \textit{cIII}, N-antiterminator protein (\textit{N}), serine/threonine protein kinase
(stk), cI repressor (cI), cro, cII and the O and P genes. cI repressors are important in regulation and maintenance of lysogeny. Structurally, the cI consists of 238 amino acids (aa) that fold into two monomeric carboxyl and amino domains. The cI monomers associate to form dimers that are bound to the operator site. Cro and cII are also functionally similar as they promote maintenance of lysogenic state. Replication proteins expressed by genes in the loci were designated as O and P, based on 98% identity with genes in phage lambda and H19-B phage (122). The “late genes” include Q antiterminator, Shiga toxin genes (stx2A and stx2b), lysis genes including the S and R that encodes holins and endolysins, respectively. The function of late genes is described in-depth in the subsequent section termed “regulation”.

Figure 3-1. Stx2 phage: A schematic showing organization and transcription patterns of the lamboid Stx2-converting phage (genes not drawn to scale). The organization of the early genes (a) and late genes (b) shows the order. Beige arrow indicates genes that are under the regulation of N-antiterminator protein, while green arrow shows genes under the regulation of Q antiterminator.
Regulation

The prophages, under favorable conditions, divide along with the bacteria and are maintained in their lysogenic state. Under normal growth conditions, the dimeric cI represses transcription mediated by phage promoters P_L and P_R, by binding to operators O_L and O_R. The switch from the lysogenic state to lytic, a phenomenon termed “induction”, occurs when a DNA damaging agents, including UV light, hydrogen peroxide and antibiotics, obstruct bacterial DNA-replication (126–128). By sensing single stranded DNA, the SOS response is triggered in the bacteria, which leads to the cleavage of cI repressor by RecA. When the cI is cleaved, the P_R and the P_L derepressors become active. The N antiterminator protein is transcribed, permitting RNA-polymerase read-through. This step allows the expression of the “replication” and “late genes”, downstream of the N-antiterminator. The Q antiterminator is transcribed next, that acts by binding to the Q utilization site (qut) within phage late promoter P_{R'}; after which RNA-polymerase can process through the downstream terminators like t_{R'}. As a result, transcription of the late genes, including stx2A and stx2B, lysis and structural genes is achieved (129). Subsequently, the holin-protein S is expressed, which forms pores in the host bacterial cytoplasm (130, 131). Downstream of the S are R-proteins that are endolysins (31, 115, 132). After lysis, the mature phage virions are released and are free to infect other bacteria in their vicinity (31, 133). Upon infection of other bacteria, the phage can either choose a lysogenic or lytic life cycle. Due to the expression of the late genes, phage induction in O157:H7 strains that carry Stx2 leads to increased expression of Stx2 and phage (134).
**Diversity in phage sequences:**

Another phage sequence derived from the Sakai strain is designated VT2-phi. Comparison with the 933W showed similarity, but not complete identity in the genome. The VT2-phi had 90 ORFs and 8 blocks of genes that showed very low identity with the 933W. The regions included the regulatory genes in the “early gene” region, replication and initiation genes, some promoters and the position of the IS629 element (21, 64). It was inferred that multiple genetic rearrangements may have occurred leading to differences in the phage genomes between the O157:H7 strains (135). By nature, phage genomes are highly mosaic, modular and repetitive, indicating high levels of recombination, which is why there is a great deal of diversity in their genomes (59).

When analysis of 30 stx2 phage sequences derived from cattle isolates was done by Muniesa et al. (136), 9 showed 100% identity with 933W phage, while the others were similar to a Nil12 phage, previously isolated from cattle (137). The stx allelic type stx2a was the most abundant stx2 phage, followed by stx2c (136). Allelic variation in the sequence of Q genes has been previously reported, but the studies investigating whole phage genomes have been limited (138). A study of the Stx2-converting phage from 22 different O157:H7 strains was done by Yin et al. (123), where phage genomes were sequenced, assembled de novo and annotated after being induced from the bacteria. The study identified 9 phage sequence types (PST). These PST clustered into three groups, out of which PST1-cluster encompassed 6 PST, where the phage genomes shared high level of similarity with the Sakai-like phage. The second PST2-cluster grouped two clade 8 strains, PA2 and PA8, both of which have been used in our present study, while the
PST3-cluster had one strain. Yin et al. described the most comprehensive look at phage genome diversity, as phage was obtained from a diverse collection of 22 O157:H7.

**Bovine mucus, colonization and nutrient utilization by E. coli O157:H7**

Mucus glycoproteins are viscous, gel-like constituents of the gastrointestinal tract, and can be divided into three phases based on their *in vivo* location. The first phase is the pre-secreted mucus found associated within the epithelial cells, while the second phase is the gel-like substance that is firmly attached to the mucosal layer of the lumen. The latter forms a protective layer that coats the mucosal epithelial cells and prevents damage by food, digestive enzymes and microorganisms (139, 140). The third layer is the outermost dynamic layer that moves along the colon slowly and is comprised of partially proteolysed and degraded luminal mucus, mixed with other contents of the mucus layer. Functionally, mucus acts as a lubricant in the large intestine (140). The secreted mucus is approximately 10 MDa in molecular weight and is composed of carbohydrate side chains, which are packed around a protein core. The primary glycoprotein is composed of 47.5% carbohydrate and 52.6% protein (w/w) (139). Galactose (42.1%) is the most abundant oligosaccaride, followed by N-acetylglucosamine (24.1%), N-acetylgalactosamine (23.6%), fucose (4.7%) and mannose (3.1%). The amino acids present include glutamate (13.2%), aspartate (11.2%), threonine (9.6%) and serine (9.2%) (139, 141).

The gastrointestinal tract of cattle is colonized with *E. coli* O157:H7 asymptomatically, with particular prevalence in the hindgut. The primary site is the mucosal epithelium in the terminal rectum, which is the site of tropism for *E. coli* O157:H7 (141–144). The successful colonization of bovine mucus in rectum is determined by various factors that include, but are not limited to, penetration of the mucus layer, adhesion to epithelial surfaces and mucus derived oligosaccharides, and the ability to catabolize mucin-derived nutrients (145). *In vitro* studies that
investigated growth of O157:H7 in mucus using streptomycin treated mice as a model showed that EDL933 peaks to $10^7$ CFU/mL in the colonic mucus (82). Several *ex vivo* studies using bovine mucus have also demonstrated that *E. coli* O157:H7 strains grow well in the mucus (146–149). In addition to adhesins expressed by pO157, and *eae* being a requisite that allows colonization (150), ability to catabolize mucin derived nutrients also gives an advantage to O157:H7. Aperce *et al.* demonstrated that growth of *E. coli* O157:H7 was proportional to the mucus concentration added as a medium. A more comprehensive study done by Bertin *et al.* investigated the carbohydrate utilization patterns in EDL933 strain. Minimal media supplemented with six carbohydrates, namely, galactose, N-acetylglucosamine (NAcGlu), N-acetylgalactosamine (NAcGal), N-acetylgalactosamine (NAcGal), N-acetylgalactosamine (NAcGal), N-acetylneuramic acid (NAcNeu), fucose and mannose, were used to grow EDL933. All the sugars were able to support the O157:H7 strain, however the most robust growth curve was seen with NAcGlu and NAcNeu. Enzymes involved in degradation of the specific sugars were highly expressed during exponential phase. EDL933 also showed faster catabolism of the sugars as compared to the commensal flora, which lead to the inference that differential nutrient utilization may give a competitive advantage to *E. coli* O157:H7 (151). This observation was further supported by findings of Snider *et al.*, in which mutants of *E. coli* O157:H7 lacking the ability to catabolize L-fucose or NAcGlu could not colonize cattle mucus, while the wild type could (152). Another study showed that the O157 uses fucose to modulate pathogenicity and metabolism in the recto-anal junction (153). Apart from carbohydrate utilization, another mechanism that confers a competitive advantage to *E. coli* O157:H7 is the presence of the ethanolamine utilization (*eut*) gene cluster, that allows EDL933 to break down free ethanolamine (EA) present in the bovine gut and use it as a nitrogen source (146). The *eut* genes are either absent or poorly expressed in other commensal strains thus allowing the *E. coli* O157:H7 to compete better. Carbohydrate utilization was compared between a commensal adapted *E. coli* strain MG1655, and pathogenic EDL933, by feeding streptomycin-treated mice
with mutants deficient in sugar utilization for a particular carbohydrate. The colonization of the mutants was profiled by measuring fecal counts. The results demonstrated that, with the exception of fucose and NAcGlu, both strains break down different sugars. EDL933 catabolizes galactose, hexuronates, mannose and ribose, while MG1655 utilizes gluconate (154).

These aforementioned findings, coupled with previous studies showing the differences in the rate of utilization, lead to the inference that the pathogenic strain O157:H7 may be gaining an advantage by either simultaneously consuming several sugars, such as fucose, mannose, and NAcGlu; or using sugars that are not utilized by the commensal strains, like mannose and galactose. The difference in nutrient composition and their utilization by the O157:H7 helps understand why O157:H7 is able to colonize the mucus better than commensal and non-pathogenic strains in vivo.
Chapter 3:

Review of factors that alter Shiga toxin expression

Introduction:

Ingestion of *E. coli* O157:H7 causes symptoms starting from watery diarrhea, that progresses to become bloody, and in some cases, may lead to hemorrhagic colitis and HUS (10, 16, 81). HUS development is due to expression of Shiga toxin (Stx) which is also an important determinant of pathogenicity in *E. coli* O157:H7 (66). Release of Stx is solely through the lysogenic to lytic conversion of temperate lamboid prophages, which house the stx genes. There are no dedicated secretion systems. Thus, phage induction is always associated with cell lysis of the host bacteria, also termed lysogen (155, 156). Structurally, Stx are a 70-kDa protein that is comprised of an A and pentameric B subunit. The A subunit of the AB₅ toxin has enzymatic activity, while the B subunit is responsible for binding to the receptor (69–72). There are two types of immunologically distinct Stx, named Stx1 and Stx2 (74). The subtypes of Stx1 and Stx2, have 56% amino acid identity (74, 75). The Stx1 has high level of identity with *S. dysenteriae* serotype-1 (69), and has a variant designated stx1c. On the other hand, there are multiple allelic variants of stx2 including stx2a, stx2b, stx2c, stx2d, stx2dact, and stx2e. Both stx2a and stx2c have 100% amino acid identity in the A subunit, while differing in the B subunit. (88). The B subunit of stx1, stx2a, stx2c and stx2d attaches to the glycolipid receptor on mammalian cells, termed globotriasoyl-ceramide (Gb3). Upon attachment of the pentameric B subunit to the receptor, the AB₅ is endocytosed and follows one of two possible paths. Stx either moves to the lysosomes for degradation, or is transported to the Golgi apparatus and subsequently, to the endoplasmic reticulum (67, 72). The A subunit in the cytoplasm is proteolytically cleaved to yield two
fragments, one being a 28 kDa peptide which contains the enzymatic activity, while the other is a 4 kDa peptide, that is the site for connection with the B subunits. The larger cleaved portion of A subunit has N-glycosidase function that acts on the 60S ribosomal subunit, removing the adenine residue from the 28S rRNA. This step abrogates protein synthesis in the eukaryotic cell, ultimately causing cell death (80).

A correlation between the amount of Stx2 expressed by the strains and pathogenicity has been suggested. Mouse studies demonstrated that *E. coli* O157:H7 strains expressing Stx2 were more likely to develop acute renal necrosis and die, as compared to the strains that express Stx1 (51, 82, 83). In a mouse model, purified Stx(s) had had 400 times lower LD<sub>50</sub> than Stx1. Another animal study used baboons, and administered 25 ng/kg of Stx1 and Stx2, every 12 hours. The baboons receiving Stx2 started developing symptoms after 60 hours, and became moribund by hour 72, while the Stx1 and control groups did not show any symptoms (84). Studies on cell lines also showed that the Stx1 bound with higher affinity to the human renal microvascular endothelial cells (HRMEC), as compared to Stx2. However, the latter was 1000 times more cytotoxic (85). The following discussion will review the known and characterized factors that alter Stx2 expression in O157:H7. Characterization factors enhancing Stx2 help understand the conditions that enhance pathogenicity of O157:H7; whereas, reviewing causes for decreased Stx2 levels help identify agents that will have a protective effect.
Factors Enhancing Stx2 Expression

DNA damaging agents:

Genes encoding the Stx expressed are present on lamboid prophages that are in a lysogenic state and replicate with O157:H7. When stress signals are sensed, the lysogenic phage switches into the lytic phase and excises itself from the bacterial chromosome, exiting *E. coli* O157:H7. In case of Stx1-converting phage, the induction is triggered by low iron concentrations (157–159). On the other hand, the Stx2 phage is induced by the RecA mediated cleavage of the cI repressor (115, 131). The SOS response is triggered when single-stranded DNA, generated due to replication errors accumulating in bacterial cell. This action activates RecA, which has proteolytic activity. RecA cleaves the cI dimers and N protein is expressed. Expression of N protein promotes the Q anti-terminator expression, which in turn allows late- gene expression including Stx2, phage morphogenesis and lysis genes (31, 115, 126, 160). DNA damage in *E. coli* O157:H7 lysogenized with Stx2-converting phage leads to induction as well as lysis of the host bacteria. Examples of DNA damaging agents, including antibiotics, UV light and hydrogen peroxide, are discussed in the subsequent section.

*Antibiotics:*

Among the various categories of antibiotics, the class that cause errors in DNA replication is particularly potent in inducing Stx2 expression (68). Rocha and Piazza *et al.* demonstrated that addition of quinolone antibiotics causes significantly higher levels of Stx2 and phage induction as compared to Stx1 (161). Stx1 is induced by low iron concentration (157, 159), while Stx2 expression is induced following DNA damage. Therefore it is understandable why more Stx2 is detected, as compared to Stx1. DNA damaging antibiotics, added to an early log
phase culture, engenders a rapid cell lysis. This is visualized by a drop in optical density in the antibiotic supplemented culture, otherwise absent in uninduced cultures (161). Different antibiotics have different effects on the Stx2 expression, and the antibiotics tested in literature are summarized in Table 2.

A study on phage induction by different classes of antibiotics was done by McGannon et al. where three classes of antibiotics having different mechanisms of action were tested for phage induction and Stx2 overexpression, as compared to uninduced O157:H7 (68). As expected the DNA-damaging antibiotics, including ciprofloxacin and trimethoprim sulfamethoxazole caused enhanced Stx2 expression, while the cell wall inhibitors and ribosome or RNA polymerase targeting antibiotics caused little to no increase in Stx2 expression (68). This observation was previously shown in gnotobiotic piglets, where administering antibiotics that inhibit protein synthesis did not cause any development of HUS. In contrast, quinolones, including ciprofloxacin and norfloxacin, increased Stx2 expression (162).

McGannon et al. focused on Stx2 expression from a single O157:H7 strain. However, Grif et al did a comprehensive study on the effects of administering subinhibitory concentrations of 13 antibiotics on three strains of O157:H7. The three E. coli O15:H7 strains tested carried either stxl, stx2, or both genes (134). The results demonstrated that the nature of the strain and which stx genes carried by them determined the Stx2 levels. Certain antibiotics, which were previously reported to show no effect or decrease in Stx2, now showed increased Stx2 expression. For example, this study reported increased Stx2 with azithromycin and gentamicin, which conflicted with McGannon et al. who demonstrated decreased Stx2 levels. Another study that conflicted with the findings of McGannon et al. had shown increased Stx2 expression with azithromycin. The increase was also coupled by high recA and Q expression (163). Subinhibitory concentrations of cotrimoxazole, a combination of trimethoprim and sulfamethoxazole added to growing E. coli O157:H7 strains increased Stx2. The results of Stx2 levels reported for growing
O157:H7 with antibiotics that disrupt cell wall synthesis have been ambiguous. This class includes penicillin, ampicillin and ceftriaxone. Ampicillin was reported to increase Stx2 expression to 10 fold (164), while other studies showed no effects (68). In Grif et al study, media containing cefixime, ceftriaxone, or erythromycin caused an increased expression of Stx2 from strains carrying stx1, 2 and stx2 only. Moreover, penicillin, streptomycin, ciprofloxacin, fosfomycin and sulfamethoxazole caused increased Stx2 expression in the stx2 only strains but not in strains carrying both stx1, 2 or stx1 alone (134). The disagreement and conflicting results between these studies may be due to strain specific differences between E. coli O157:H7.

Table 2. List of antibiotics with their mechanism of action and the effect on Stx2 expression.

<table>
<thead>
<tr>
<th>Mechanism of action</th>
<th>Antibiotics tested</th>
<th>Mechanism of action</th>
<th>Effect on Stx2 expression</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell wall inhibitors</td>
<td>Fosfomycin</td>
<td>Inhibits cell wall biogenesis by stalling enzyme MurA</td>
<td>Decrease</td>
<td>(68, 165)</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>β-lactam inhibitor</td>
<td>Moderate increase</td>
<td>(68)</td>
<td></td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>Blocks peptidoglycan synthesis</td>
<td>No change</td>
<td>(134)</td>
<td></td>
</tr>
<tr>
<td>Penicillin</td>
<td>β-lactam inhibitor</td>
<td>Increase</td>
<td>(134)</td>
<td></td>
</tr>
<tr>
<td>Stalled DNA replication</td>
<td>Ciprofloxacin</td>
<td>Inhibits DNA-gyrase, thus causing strain when replicating DNA is unwound</td>
<td>Increase</td>
<td>(68, 134, 161)</td>
</tr>
<tr>
<td>Trimethoprim-sulfamethoxazole</td>
<td>Blocks/ nucleotide synthesis</td>
<td>Increase</td>
<td>(134, 166)</td>
<td></td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>Introduces DNA cross-links</td>
<td>Increase</td>
<td>(167)</td>
<td></td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>DNA gyrase (same as ciprofloxacin)</td>
<td>Increase</td>
<td>(168)</td>
<td></td>
</tr>
<tr>
<td>Blocking protein</td>
<td>Azithromycin</td>
<td>Binds to 50S ribosome</td>
<td>Increase</td>
<td>(134)</td>
</tr>
<tr>
<td>antibiotic</td>
<td>action</td>
<td>effect</td>
<td></td>
<td></td>
</tr>
<tr>
<td>------------</td>
<td>--------</td>
<td>--------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Doxycycline</td>
<td>Binding to aminoacyl tRNA (like tetracycline)</td>
<td>Decrease</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gentamicin</td>
<td>Binding to the 30S RNA</td>
<td>Increase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rifampin</td>
<td>Inhibits RNA polymerase</td>
<td>No change</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Identification of antibiotics that increase Stx2 expression is pertinent. Some clinical studies have reported exacerbated HUS symptoms when antibiotic therapy was used during O157:H7 infection. Prescribing antibiotics for O157:H7 infections has always been controversial due to conflicting literature. Some studies have shown use of fosfomycin, which was given to patients during the 1996 EHEC outbreak in Japan, is unsafe, as it triggers Stx1 expression (170). However, in vitro Vero cell assays have showed decreased Stx1 and Stx2 levels with fosfomycin. Wong et al, did a prospective cohort study to investigate the effects of administering antibiotics and development of HUS in children under the age of 10 years. The study included 71 children whose caregivers filled out questionnaires answering whether antibiotics or anti-motility agents, that inhibit peristalsis, were prescribed to patients. Out of the 71 included, antibiotics were given to 9 children between day 1 and 3 of illness. Development HUS was observed in 10 patients. 5 of who had received antibiotics. This lead to the conclusion that patients receiving antibiotics showed a higher chance of developing HUS when antibiotics were prescribed (92). Similarly, the diagnosis of patients with O157:H7 infections during an outbreak in central Scotland was investigated. Out of 120 patients, 34 showed symptoms of HUS. Antibiotics were prescribed to 14, out of whom 8 developed HUS symptoms (171). Another meta-analysis searched reports on cases of EHEC infections and antibiotic use between Jan 1983 to 2001. Safdar et al. measured the odds ratio for 9 studies, which includes 3 prospective and 6 retrospective studies. Four of the retrospective studies showed no association between HUS development and antibiotic, while the remaining two either showed a positive correlation in one, and a negative in the other. Among the
three prospective studies, one study showed increased HUS with antibiotics, and one revealed no association. The last prospective study showed protective effect with fosfomycin. Overall, the results of the meta-analysis demonstrated that antibiotics were not associated with higher risk of HUS development, and a more randomized trial should be conducted to better characterize the association between HUS and O157:H7 infections (172).

Other SOS dependent factors:

Apart from antibiotics, other factors that trigger the SOS response also enhance Stx2 expression. These triggers include UV light, hydrogen peroxide and neutrophils. Previous studies have demonstrated that oxidative stress, following colonization of the intestine by enteric bacteria, results in production of H$_2$O$_2$ (173). Phage induction by H$_2$O$_2$ is due to oxidative damage of the bases by the reactive oxygen species (ROS). A dose lower than 2.5mM thwarts actively growing cells by induction of the SOS response (174–176). A study done by Los et al. compared the efficiency of phage titers in 5 STEC lamboid phages, including 933W, when exposed to H$_2$O$_2$ and two DNA damaging antibiotics, mitomycin C and norfloxacin. Firstly, it was observed that frequency of phage induction varied between different O157:H7. Secondly, the results showed that H$_2$O$_2$ induced phage and Stx2 in some of the phages; however the efficiency was much lower than that observed with antibiotics. The kinetics of phage induction showed low or absence of lysis, measured by a drop in OD$_{600}$. This lead to the inference that H$_2$O$_2$ mediated phage induction was occurring in only a fraction of the population (177). Another study demonstrated phage and Stx2 expression was up regulated when a clinical O157:H7 strain was cultured with H$_2$O$_2$ and neutrophils (178). Understanding the induction by H$_2$O$_2$ has more physiological relevance. The agents known to enhance Stx2 expression, including UV light and antibiotics, may show higher phage induction efficiency; owerver, they are not associated with the gut (173, 174, 178).
Additional factors which may have an additive effect to the SOS-mediated phage induction include temperature (177), culture media (161), and high pressure (179). Studies on the effect of temperature and osmotic stress were performed by Los et al. This finding was based on previous observations with lambda phage, which showed increased rate of induction in a cI repressor regulated phage when the salt concentration was high (180). When H₂O₂, UV light and mitomycin C induction was done at a temperature of 43 °C, a higher efficiency of phage induction was observed. Salt concentration had no effect on the phage induction in 933W phage, which was contrary to behavior of other lamboid phages (177).

**Bacterial factors:**

**Lineage, source of isolation and clade**

The O157:H7 bacterium has diverged into genetically distinct lineages. The strains were classified based on banding patterns obtained, using octamer based genome scanning (48) (reviewed in the section “classification and subtyping of E. coli). Lineage I strains are more commonly associated with human clinical isolates, while lineage II strains show over-representation of bovine isolates. Lineage I/II, on the other hand, has strains represented from both the sources (49). Phenotypic analysis of Stx2 expression has shown that lineage I and I/II strains express significantly more Stx2 than lineage II strains (181–184). Moreover, it was also shown that cattle derived isolates express significantly lower Stx2 than human isolates (181, 182, 184). In a gnotobiotic pig model, strains derived from cattle produced significantly lesser Stx2 than human clinical isolates. (50). Based on the difference in Stx2 expression, it was hypothesized that lineage I strains are more pathogenic and adapted to human transmission, while lineage II are more frequently associated with bovine gut (185). This could also explain the
difference in Stx2 expression between bovine and human clinical isolates. Another classification system was devised to group O157:H7 based on their virulence. Out of the 9 clades identified by SNP-typing, O157:H7 belonging to clade 8 encompassed the hypervirulent strains. The enhanced virulence is exemplified by strains implicated in the spinach outbreak in 2006. The latter was associated with higher rate of HUS development and over 50% hospitalization rate (53). Analysis of Stx2 expression from the clade 8 strains showed higher levels of Stx2 expression, as compared to clade 1 through 3 (95). In addition to the lineage, host source and clade, the subtype of stx associated with O157:H7 is also a determinant of Stx2 expression. Strains that carry stx2 only are known to produce higher levels of Stx2, as compared to strains that carry both stx1 and stx2 (17, 186–188). Thus, human clinical isolates, strains clustered into lineage I and I/II or clade 8, or the ones carrying stx2 only are more likely to overexpress Stx2.

Another factor that relies on the bacteria are the number of phages carried by it, also known as the prophage burden. Multiple phages can infect a host bacterium and establish stable lysogens (189). In this case, prophage burden is known to influence the Stx expression. When isogenic double lysogens were compared to single lysogens, the former were associated with approximately 2.5 fold higher Stx2 and phage induction as well as faster lysis time. Induction in the absence of antibiotics, or spontaneous induction, was also higher for the double lysogens (190). Moreover, the host harboring the phage is also known to influence the induction and phage production.

**Co-culture with commensal bacteria:**

Increase in the amount of Stx2 can also be observed when an E. coli O157:H7 is co-cultured with non-pathogenic susceptible bacteria (191). Even in the absence of DNA damaging agents, O157:H7 produces basal levels of phage and Stx2 through spontaneous induction (fig 3-
2). When co-cultured with non-pathogenic *E. coli*, this induced phage can infect susceptible bacteria, shown in fig 3-2 (represented as infection 1). The infectious phage goes directly into the lytic cycle and uses the benign *E. coli* to produce more infectious phage and Stx2. The phage released from lysed non-pathogenic bacteria can now infect other susceptible *E. coli*, (illustrated as infection 2) (Fig 3-2). This release results in increased Stx2 accumulation when *E. coli* O157:H7 is co-cultured with susceptible bacteria. The susceptible *E. coli* constitutes about 10% of the commensals associated in our gut. (192).

Figure 3-1. Model proposed by Gamage *et al.* showing how phage mediated lysis causes increased Stx2 expression when coincubated with a susceptible *E. coli*.

To test coincubation model, a lysogen containing the phage 933W was engineered (C600::933W) and co cultured with non-pathogenic strain C600. An *E. coli* strain resistant to superinfection was generated as a negative control. This strain (C600::Δtox) was lysogenized with a phage having an inactivated toxin (Δtox). Gamage *et al* reported 40 fold higher Stx2 levels when C600::933W was coincubated with the C600, as compared to the C600::933W alone. In contrast, co-culture of C600:933W with C600::Δtox resulted in reduced Stx2. A pool of 37
commensal *E. coli* strains were derived from the feces of healthy individuals, and infected with the 933W phage. Out of the 37 commensal strains, 3 significantly increased, while 1 lowered the Stx2 levels. The coincubation was also performed in a mouse model. The Stx2 was quantified from the feces of the mice inoculated with the C600::933W and C600::Δtox showed significant reduction in Stx2 levels. The mice where C600::933W was cocultured with susceptible C600 showed variation and was not statistically distinguishable from the monoculture controls (191). Another study by Gamage *et al.* investigated the diversity and host range of 933W and 6 other phage from clinical isolates. It was shown that despite the clinical strains having similar Stx2 expression, and PFGE patterns, the phage induced were diverse. Coinfection of 72 commensal strains from the ECOR collection with the phage showed only two of the 6 phages amplified Stx2. There were differences in the fold amplification and susceptibility to phage between different commensals. For example, 933W phage amplified Stx2 by 256, 8 and 512 fold in ECOR-4, ECOR-6 and ECOR-13 respectively. On the other hand, phage PT32 amplified Stx2 in the ECOR-6, ECOR-13 and ECOR-51 by 32, 1034 and 4 fold. As the next step, three classes of commensal *E. coli* from the ECOR collection were characterized. The ability of susceptible, resistant and toxin-neutralizing commensal strains to colonize mouse intestine and amplify Stx2 was investigated in a streptomycin-treated mouse model (192). The presence of any strain reduced colonization of *E. coli* O157:H7. Stx2 quantified from the three experimental setups had lower detectable Stx2 as compared to the O157:H7 alone. The mouse study was unable to replicate the *in vitro* results of Stx2 amplification, which was understandable due to the inadequate colonization of the O157:H7. The study used pre-colonized mice and started with low concentration of O157:H7, which failed to colonize. Nevertheless, the work of Gamage *et al.* on characterization of Stx2 amplification by phage mediated lysis was a seminal study that recognized a phenotype which takes into account the role of commensal, as well as *E. coli* O157:H7.
An additional mechanism by which commensal flora can enhance Stx2 expression by expressing DNase colicins. This finding was reported by Toshima et al. where commensals carrying plasmid that express different colicins were co-cultured with the O157:H7. The DNase-colicin enhanced Stx2 expression by 8 to 64 fold, while RNase colicin reduced the Stx2 levels (193).

**EDTA**

In addition to phage mediated lysis following co-culture of O157:H7 with susceptible commensals, another factor enhancing Stx2 expression, without directly triggering SOS response is ethylenediaminetetraacetic acid (EDTA). Lysogens were generated in RecA mutants, which were unable to cleave cI dimers, and initiate the SOS response. These strains would show increased phage, compared to the uninduced *E. coli* O157:H7 when 20 mM of EDTA was added. The chelating nature of EDTA affects Stx2 phage induction, by binding to Mg$^{2+}$ and disrupting the outer membrane and triggering a stress response. This was validated by using another chelating agent, citrate, that had a similar impact on phage induction. Two genes involved in the response, were identified as *rcsA* and *dsrA*. RcsA is responsible for colonic acid synthesis, while *dsrA*, is a small regulatory RNA, whose function includes preventing the degradation of DsrA. Gene mutants lacking stress response to EDTA disrupting the bacterial envelope, did not show increased phage levels. (194).

**Factors Decreasing Stx2 Expression**

Apart from the antibiotics mentioned in the previous section that reduce Stx2 expression, other factors are also known to decrease Stx2 levels. Zinc is also known to reduce the Stx2
expression both at the transcription and translation level. *In vitro* studies showed zinc acetate at concentrations of above 0.4 mM reduced Stx2 expression from EDL933. Moreover, an *in vivo* rabbit model demonstrated reduced adherence to the mucosa as well as Stx induced histological damage with zinc acetate (195). Nitric oxide, expressed during innate immune responses also reduces the Stx2 levels by binding to RecA, stopping cI dimers from getting cleaved (196).

When co-cultured with commensal *E. coli* only 10% of the strains were reported to be susceptible. The rest of them either have a neutral interaction, or be resistant to the O157:H7 through generalized phage-resistance mechanisms (197). A specific strategy that reduces Stx2 levels upon co-culture with commensal is through binding of Stx2 to the commensal LPS. The O-group O117 and O107 bind the Stx2 and neutralize it (198). A non-*E coli* commensal *Bacteroides thetaiotaomicron* can also partake in reduced Stx2 expression. A study of prokaryotic molecules, released by human microbiota grown in the cecal contents of gnotobiotic rats, was done by de-Sablet *et al.* A protein with molecular weight of <3 kDA caused repression of spontaneous induction, and reduced Stx2 levels (199).
Chapter 4:

Subset of *E. coli* O157:H7 strains changes from low to high Stx2 producer when coincubated with susceptible commensal strains
**Introduction:**

The Enterobacter *Escherichia coli* can either be non-pathogenic, as exemplified by the bacteria associated in our normal gut flora, or pathogenic. Intestinal pathogenic *E. coli* are further classified into six pathotypes, among which enterohemorrhagic *E. coli* (EHEC) is a causative agent of bloody diarrhea, hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS) through the production of Shiga toxins (2, 13, 14). Since its first characterization in 1983 (10), the serotype O157:H7 rapidly established itself as a notorious foodborne pathogen, owing to a low infectious does of less than 100 CFU/mL (200). A recent study estimated that O157:H7 causes over 96,534 cases in the U.S. annually (201). *E. coli* O157:H7 transmission occurs through the consumption of contaminated food and water, or through person to person contact (18, 20). Cattle are the main reservoir, where it is asymptotically associated and shed seasonally by the animals (14). There have been multi-state outbreaks that have been linked to consuming either raw-undercooked beef, or produce (2, 14, 20, 202, 203). Cross contamination through fecal carryover to produce and water has become common, as witnessed by the outbreaks linked to spinach (18), apple cider (24), lettuce (204) and most recently in ready-to-eat salad (205). Two strains, Sakai and EDL933, are considered as prototypical O157:H7 as they were the first to be fully sequenced (21, 57). Sakai was implicated in an outbreak associated with consuming radish sprouts caused illness in over 9,000 children in Sakai city, Japan (206). On the other hand, EDL933 was linked to consumption of tainted hamburgers in Michigan (90).

Shiga toxins are one of the most important virulence factors associated with STEC O157:H7 (66, 91). The *stx* genes are encoded within the functional prophages of the lambda family. Shiga-toxins are found along with the late genes of bacteriophages, and the toxin is released after the phage induced lysis of the bacterial cell (31). Shiga toxin(s) are approximately 70-kDa proteins that comprises of an A and pentameric B subunit. Upon attachment of the
pentameric B subunit to the receptor(s), the AB₅ is endocytosed and follows one of two possible paths. Stx either moves to the lysosomes for degradation, or is transported to the Golgi apparatus and subsequently to the endoplasmic reticulum (67, 72). The A subunit in the cytoplasm is proteolytically cleaved to yield two fragments, one being a 28 kDa peptide which has the enzymatic activity, while the other is a 4 kDa peptide, that links to the B subunits. The larger cleaved portion of A subunit acts on the 60S ribosomal subunit, which has N-glycosidase function, that removes the adenine residue from the 28S rRNA. This abrogates protein synthesis in the eukaryotic cell, ultimately causing cell death (80). There are two types of antigenically distinct Stx, named Stx1 and Stx2 (74). Despite having an amino acid identity of 57%, Stx1 and Stx2 possess dramatic differences in potency (74, 75). Stx2 is more severe, as seen by studies on primates where administration of Stx2 in caused symptoms to appear after 60 hours, while the Stx1 and control groups did not show any symptoms (84). Mouse studies also demonstrated that purified Stx2 were more likely to develop acute renal necrosis and die, as compared to the toxin Stx1 (82, 83). Furthermore, Stx2 also has allelic variants that are characterized based on the amino acid differences in the A and B subunits. The variants include stx2a, stx2b, stx2c, stx2d, stx2dₐₑₙ, and stx2e. Both stx2a and stx2c have 100% amino acid identity in the A subunit, while differing in the B subunit (88).

Stx2 production is enhanced by DNA damaging agents including antibiotics, UV light and H₂O₂ (68, 127, 128), because the Stx-converting phage switches from a lysogenic to lytic life cycle (31). Increased phage and Stx2 accumulation is also observed when O157:H7 is co-cultured with an E. coli strain susceptible to the phage (191). O157:H7 spontaneously induces phage virions, which may infect other susceptible E. coli hosts, leading to the production of additional infectious phage particles as well as Stx2. Gamage et al. also demonstrated that this phenomenon occurs in the mouse intestine (192), and 10% of E. coli commensals are susceptible to phage mediated lysis, thereby increasing Stx2 levels.
There is great diversity even within the O157:H7 serotype, where strains differ in their biological behavior, based on the source of isolation (35). DNA based methods including octamer based genome scanning (OBGS) showed the O157:H7 isolates from geographically and temporally unrelated strains could be grouped into two lineages. Lineage I, which comprised of both human clinical isolates and some bovine isolates, was distinct from lineage II that had strains exclusively of bovine origin (48). Further studies identified lineage I/II, that had equal distribution in human and clinical isolates using comparative genome hybridization (47, 49). The phenotypic implications of the lineages were demonstrated in a gnotobiotic pig model, where lineage II isolates were less virulent, compared to lineage I (50, 51). Moreover, *in vitro* studies have shown that lineage I and lineage I/II express significantly higher levels of Stx2 than lineage II (35). Subsequently, to understand why some O157:H7 strains, like the strain implicated in 2006 spinach outbreak, were more virulent and associated with a higher incidence of HUS that the other strains (25, 207), a SNP-based classification of O157:H7 strains was developed. Using 96 SNPs loci, nine clades were identified out of which clade 8 was contained a subpopulation of O157:H7 with increased virulence.(53).

Yin *et al*. grouped phage genomes of 22 O157:H7 strains into 9 phage sequence types (PST), that formed 3 distinct clusters; PST1, PST2 and PST3. Of the three, PST2 was the most notable, since it clustered hypervirulent non-O157:H7 strains including the German outbreak strain O104:H4 (5) and Norwegian outbreak strain O103:H25 (208). Moreover, this cluster comprised two strains, PA2 and PA8 that belonged to clade 8. The aim of our study was to characterize the phenotype of PA2, a lineage I/II and compare the phage and Stx2 production to two prototypical lineage I strains, Sakai (clade 1), and EDL933 (clade 3). We also studied whether co-culturing strains with a susceptible commensal, C600, would alter Stx2 production. We tested a collection of commensals belonging to different O-groups to test how many would be
susceptible to Stx2 amplification (192). The aim was also to compare the cumulative Stx2 levels when PA2, EDL933 and Sakai were co-cultured with the commensals.

**Material and Methods:**

**Strains and culture conditions:**

Strains and plasmids used in the study are reported in Table 3. The bacteria were routinely propagated in lysogeny broth (LB) (VWR, Ranor, PA) at 37°C, while culture stocks were maintained at -80°C in 10% glycerol. For coincubation and coinfection assays, calcium chloride (CaCl$_2$) (Sigma Aldrich, St. Louis, MO) at a final concentration of 10mM/mL was used to supplement LB agar media.

**Induction and supernatant collection:**

*E. coli* O157:H7 strains were grown overnight in LB and diluted to an OD$_{600}$ of 0.05. LB was added to dilute uninduced controls, while LB supplemented with ciprofloxacin (45ng/mL) was used for inducing the phage. The growth curve for both the induced and uninduced O157:H7 was profiled for 8 hours, with samples collected every two hours. OD$_{600}$, phage and Stx2 levels were measured for each collected sample. The peak OD$_{600}$, phage and Stx2 production through the time course is reported. For Stx2 quantification, samples were treated with 6 mg/mL of Polymyxin B (PMB) (Enzo Life Sciences, Farmingdale, NY) and incubated at 37 °C for 5 mins to lyse cells and release internal Stx2. The PMB treated samples were centrifuged 4,000 xg for 10 min, and supernatant was collected and either used immediately or stored at -80°C.
Table 3. List of strains used in the study

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<th>STRAIN</th>
<th>RELEVANT CHARACTERISTIC</th>
<th>REFERENCE</th>
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<td>(57)</td>
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**Phage preparation for sequencing, assembly and annotation of O157:H7 strains:**

Phage preparation protocol was adapted from Asadulghani et al. (59) with the following modifications. Briefly, 100 ml of O157:H7 culture at an OD_{600} of 0.05 was induced with 45 ng/ml of antibiotic, ciprofloxacin (Sigma-Aldrich, St. Louis, MO). The induction curves were profiled
until the strains lysed after which the cultures were centrifuged at 4,000xg for 10 min, and filter sterilized using 0.2 μm cellulose acetate filters (VWR, Radnor, PA). DNase and RNase treatment was done to remove the DNA from bacterial debris and minimize the background signals from the host bacteria. Solid NaCl was added to a final concentration of 1 M and incubated in ice for 1 hour. Polyethylene glycol (PEG MW 800) (Promega, Madison WI) was added to the tubes to a final concentration of 10% and incubated at 4°C overnight. Centrifugation was done for 2 hours at 4,000 xg 4°C. SM buffer, was added to soak the pellet and wash the walls followed by addition of proteinase K to digest the capsid. Equilibrated phenol:chloroform (1:1) was added and the aqueous phase containing the DNA was recovered. Sodium acetate was added along with 100% isopropanol to precipitate the DNA followed by a 70% ethanol wash. Concentration and purification of DNA sample was done using Genomic DNA Clean and Concentrator™ kit (Zymo Research, Irvine CA). The DNA was visualized in a 0.8% gel, followed by spectrophotometric quantification using Nanodrop (Thermo Scientific, Hudson, NH) and Qubit (Invitrogen, Grand Island, NY). The extracted total DNA was submitted to the Penn State Genome Core Facility for sequencing using a Roche GS 454 FLX_system. The raw reads were assembled using NGen (DNASTAR, Madison, WI) and analyzed on SeqMan Pro (DNASTAR, Madison, WI).

Sequences were assembled using the same protocol as described by Yin et al (123). Briefly, both phage DNA reads (454 technology) and bacteria DNA reads (Illumina) were employed for phage genome assembly. Phage DNA reads were assembled de novo using SeqMan NGen (DNASTAR, Madison, WI). For strain PA2, VT2Phi (HQ424691.1) was used as reference for stx2a-converting phage assembly. Templated assembly with the phage DNA reads validated the draft phage genomes. Next, the draft genomes were used as a template for templated assembly with bacteria genome reads (Illumina) to resolve homopolymeric errors and check for structural variants. For IS elements, primers were designed and Sanger sequencing was used to confirm the
assembly. Phage genomes were auto-annotated using RAST and PHAST. The GenBank files generated by RAST were then modified in Artemis.

**Stx2 quantification using RELISA:**

Receptor-ELISA uses ceramide trihexoside (CTH), a Gb3 analogue, as a capture for the Stx. The 96 well polystyrene microtiter strip plates (Thermo Scientific, Waltham, MA) were coated with 2.5μg of CTH (Matreya Biosciences, Pleasant Gap, PA). The plates were incubated at 4°C overnight with blocking buffer (4% bovine serum albumin in 0.01 M phosphate buffer saline with 0.05% Tween20). Diluted or undiluted samples were dispensed in triplicate and placed on a shaking incubator at room temperature for 1 hour. Mouse anti-Stx2 (Santa Cruz Biotech, Santa Cruz CA) was added to a final concentration of 0.1μg/mL and incubated at room temperature for 1 hour. After 5 washes with PBST, goat anti–mouse IgG peroxidase conjugate was added to a final concentration of 0.1µg/mL, and incubated at RT for 1 hour. Subsequently, 100 µl of 1-Step Ultra TMB (3,3’,5,5’ tetramethylbenzidine) (Thermo-Fischer, Waltham, MA) was added to each well, and the plates were incubated for 10 min at room temperature. Finally, 100 µL of stop-solution (2M H₂SO₄) was added and the OD₄₅₀ was measured. Wells containing Stx1 served as a negative control, and any signal that was over its OD₄₅₀ was considered positive for Stx2. With each run, a two-fold serially diluted Stx2 of known concentration was run. Plotting the absorbance for serially diluted sample gave the standard curve, which could be used to calculate the quantity of Stx2 in our unknown samples.
Plaque assay:

Double overlay agar method for measuring plaques was adapted from Islam et al. (210) to measure the infectious phage. Phage preparation in induced and uninduced cultures was done by as mentioned above. The supernatant was centrifuged at 4000 xg for 10 min, and serial dilutions were made in SM buffer (0.1 M NaCl, 50mM Tris-Cl, 8mM MgSO₄ and 0.01% gelatin). Indicator strain JM109 and phage were added at a 2:1 ratio and mixed in LB-soft agar (0.75% agar) supplemented with CaCl₂. The soft-agar mixture was overlaid on modified LB agar and incubated at 42°C, to allow overexpression of the phage receptor bamA gene (211). Plaques were counted after 16 hours of incubation.

Coincubation experiments:

The co-incubation assays were developed based on experiments by Gamage et al (191). The overnight cultures of E. coli O157:H7 and C600 were diluted in LB to a final concentration of OD₆₀₀ 0.03. A “monoculture setup” was the O157:H7 or C600 present alone, while a “coincubation setup” contained both O157:H7 and C600 in a 1:1 ratio. Modified-LB with either the monoculture or coincubation setup was overlaid on 6 well plates (BD Biosciences Inc., Franklin Lakes, NJ) and incubated at 37 °C. Samples for enumerating cell density and Stx2 levels were harvested every three hours after the 6-hour time point. Cell density was measured by plating on Sorbitol MacConkey agar (SMaC), a differential media where O157:H7 appears as white colonies and C600 or other non-O157:H7 appears as red. Counting the red or white colonies on SMaC plate was done for monoculture controls. For the coincubation setup, plates having 30-300 total colonies were enumerated and the numbers of red or white colonies were reported, giving the counts of either O157:H7 or C600 for that time point. For Stx2
quantification, the harvested samples were treated with 6 mg/mL of PMB, prior to using RELISA to quantify the Stx2.

**Mucus assays:**

Three Angus cows, and one Jersey/Holstein mix were slaughtered at the Penn State Meat Laboratory or Rising Spring Mills Meat Co. (Rising Spring Mills, PA) and three feet from the rectum was sequestered to collect mucus. The mucus was scraped and an aliquot was saved for performing phenol-sulfuric acid test for total carbohydrate using the protocol by Masuko et al. 2005 (212). The total plate count, density and pH were also measured to assess batch to batch differences in the mucus. The remaining mucus was autoclaved and approx. 2.5 mL was added to each well in a 6-well polystyrene plate (BD Biosciences Inc., Franklin Lakes, NJ). Inoculum was prepared by re-suspending overnight cultures in PBS, to wash off residual media from the overnight culture. Dilution was done in PBS to a final concentration of 0.03. The monoculture and coincubation of PA2, EDL933 and C600 were set up. A negative control with mucus in PBS was also included. Growth curve was profiled on SMaC for every 3 hours, until 16 hours. After 16 hours, the mucus was collected in Eppendorf tubes and vortexed at maximum speed for 1 min. to homogenize the mucus. Subsequently, centrifugation was done for 15 min at 4,000 x g to collect the supernatant. ELISA was done using the Premier® EHEC ELISA kit (Meridian Biosciences Inc., Cincinnati, OH), which has a polyclonal primary antibody that cannot distinguish Stx1 and Stx2. The Premier EHEC kit was used instead of the R-ELISA, as the former is approximately 10 times more sensitive (data not shown). The inability to distinguish the Stx would be an issue in case of EDL933, but phage sequencing of EDL933 has shown that Stx2 is the major phage induced.
**Coincubation with non-pathogenic *E. coli* and clade 8 strains:**

To test whether other non-pathogenic *E. coli* strains amplify Stx2 when cocultured with the three O157:H7, a random collection of non-pathogenic *E. coli* strains was acquired. Six different O-groups with two representative strains were chosen to do the coincubation with PA2, EDL933 and Sakai; and C600 was the positive control for increased Stx2 accumulation after coincubation. Stx2 from monoculture controls of the three O157:H7 were compared to the coincubation setups. A non-pathogenic *E. coli* strain was regarded as positive for Stx2 amplification if there was statistically significant increase in Stx2 in the coincubated sample as compared to the monoculture. The cell counts were enumerated by calculating the $10^7$ dilution for first biological repeat, and $10^8$ for the second and third biological repeat. For testing Stx2 accumulation when other clade 8 strains are co-cultured with C600, 10 *E. coli* O157:H7 from the collection previously characterized by Hartzell *et al.* (209) were chosen. Clade 8 strains carrying the *stx2a* only were PA2, PA8, PA9, PA19, PA25 and PA24, while 3 strains PA3, PA13 and PA28 carried both the *stx2a* and *stx2c* alleles. The coincubation was done with C600 and Stx2 levels were compared between monoculture of O157:H7 and the coincubation setup. The cell counts from three biological repeats were enumerated from $10^8$ dilutions. In both the commensal, and clade 8 coincubation setups, the cell densities for a particular biological repeat were statistically indistinguishable. The results of competitive index was reported as percentage commensal, calculated using the equation:

$$\text{Percentage commensal: } \frac{\text{Red colonies on SMaC}}{\text{Red colonies + White colonies on SMaC}} \times 100$$

*E. coli* O157:H7 grown in spent supernatant

To test any possibility that the non-pathogenic *E. coli* expresses a factor that up regulates Stx2 expression in O157:H7, the spent supernatant experiment was done. The five non-pathogenic *E. coli* strains showing increased Stx2 expression, namely 0.1229, 0.1282, 0.1231,
1.1954 and C600 were grown in a shaking incubator at 37°C incubator. After 16 hours, the cultures were centrifuged at 4,000 x g for 15 min and filter sterilized using a 0.2 µm cellulose acetate filter (VWR, Radnor PA). The pH was measured and adjusted to 7. An overnight culture of PA2, EDL933 and Sakai was diluted to an OD_{600} and inoculated into the spent supernatant as well as fresh LB control. Static incubation at 37°C in a 6-well plate setup was performed. OD_{600} was recorded every two hours to profile the growth curve. ELISA was done to the PMB treated samples and results are reported as fold difference between fresh LB grown O157:H7.

**Data analysis:**

MS Excel was used to calculate the mean, standard deviation and standard error, while GraphPad Prism 8 software was used to calculate p value and generate figures.

**Results:**

**Differences in the phage genomes of PA2, EDL933 and Sakai.**

Yin *et al.* 2014, previously sequenced and characterized Stx2a-converting phages from 22 different *E. coli* O157:H7 strains and identified nine phage sequence types (PST) that formed three clusters designated as PST1, PST2 and PST3. PST2 was a notable cluster, containing two clade 8 strains PA2 and PA8, and hypervirulent non-O157:H7 strains including 2011 German outbreak strain O104:H4 (5), a 2006 Norwegian outbreak strain O103:H25 (208, 213), and O103:H2 (214). Our aim was to investigate the phenotypic manifestations of the observed genotypic differences, compared to the prototypical strains Sakai and EDL933. Sequence comparison of the Stx2 converting genome between PA2 and the two prototypical O157:H7
strains, EDL933 and Sakai, revealed distinct differences between them. The position of insertion sequence IS629 and the predicted phage early regulatory and replication regions varied between the O157:H7 (Fig 4-1). Moreover, the PA2 phage genome was unique in possessing a restriction/modification system bsuBL, which had previously been reported in O104:H4 str. 2011C-3493 (123). The cI and N genes were absent in PA2 Stx2-converting phage and were replaced by the bsuBL genes.

Figure 4-1. **Genome alignment of PA2, EDL933 and Sakai** Phage genomes from PA2 (A), EDL933 (B) and Sakai (C) were sequenced. Genome alignment shows differences in the regulatory and early replication genes.

**Differences in the induction profile, phage and Stx2 production between PA2, EDL933 and Sakai**

To begin, the kinetics of phage induction, and Stx2 and phage production were compared between PA2, EDL933 and Sakai. The three strains did not show any significant difference in their growth in LB media (data not shown). However, the growth profiles were different when
grown in subinhibitory concentrations of ciprofloxacin induced the lytic cycle. Both PA2 and EDL933 grew to their maximum OD$_{600}$ at hour 2, but PA2 showed a lower numeric peak as compared to EDL933. A drop in turbidity was observed at hour 4 for both PA2 and EDL933 indicating cell lysis (Fig 4-2a). Sakai, on the other hand, showed its highest OD$_{600}$ at hour 4, followed by a steady decline in optical density (Fig 4-2a). Among PA2, EDL933 and Sakai, the highest numeric value of OD$_{600}$ was observed for Sakai. The latter also took the longest time to show a drop in turbidity, indicating the latest lysis time. Next, RELISA was used to quantify Stx2 in the induced samples. An increase in toxin production over the time-course was demonstrated, with the induced samples producing significantly higher (p<0.0001) Stx2 than their uninduced counterparts (Fig 4-2b). Among the induced samples, Sakai produced significantly lower (p<0.005) Stx2 than EDL933 at hour 4; however, the levels became comparable thereafter. PA2 consistently produced lower Stx2 than both EDL933 and Sakai from hour 4 until hour 8 (p<0.005). Lastly, phage production, quantified by a plaque assay, showed that EDL933 produced significantly more (p<0.0001) plaques than PA2 and Sakai at hour 4 through 8 (Fig 4-2c).

Sequencing of the inducible phage demonstrated that Stx2 is the predominantly induced phage in both EDL933 and Sakai, while PA2 releases both Stx2 and a phage related to SP7, which was previously characterized in Sakai. Thus, in the cases of EDL933 and Sakai, most of the plaques enumerated were likely Stx2 converting phage, while PA2 plaques were likely a combination of Stx2 converting and SP7-like phag
Figure 4-2. **Phenotypic characterization of the O157:H7 strains**: Induction curve of O157:H7 strain PA2, EDL933 and Sakai induced with 45 ng/mL of ciprofloxacin (a). The OD₆₀₀ was measured every two hours and the error bars represent SD from three biological repeats. The Stx2 levels (b) were normalized to total protein in PA2, EDL933 and Sakai. Strains were either induced with ciprofloxacin or uninduced. Samples were collected every two hours and treated with PMB. Error bars represent SEM from three biological repeats. Total phage quantified using plaque assay from PA2, EDL933 and Sakai induced by ciprofloxacin (c). Error bars represent SEM from three biological repeats.
Coincubation with the commensal strain C600 amplifies Stx2 in PA2 and EDL933 in vitro

Gamage et al. (191) showed that coincubation of O157:H7 strains with non-pathogenic E. coli C600 leads to increased toxin levels, as compared to monoculture controls. This finding was due to the spontaneous induction of Stx2-converting phage, followed by lytic infection of C600, resulting in amplified phage and Stx2 production. We investigated whether there are any strain-specific differences in Stx2 levels when the three O157:H7 were individually cocultured with C600. Cell counts of PA2, EDL933, Sakai and C600, were enumerated in monoculture and coculture, to profile the population dynamics during the course of coincubation (Fig 4-4). When PA2 and C600 were coincubated, a sharp reduction in cell concentrations of C600 was observed between hours 9 and 16 (Fig 4-4a). Similarly, the cell counts in the setup where C600 was coincubated with EDL933 exhibited consistently lower concentrations than when C600 was present alone (Fig 4-4b). In contrast, co-culture with Sakai did not significantly reduce the colony counts of C600, at any time point during the coincubation time course (Fig 4-4c). The RELISA assay showed no difference in Stx2 levels between the monoculture and coincubated samples of all three strains between hours 6 and 9 (Fig 4-3); however, from hour 12 onwards, a significant increase in Stx2 levels in the PA2 coincubation setup was noted (Fig 4-4). At hour 16, both PA2 and EDL933 had significantly higher Stx2 levels in the coincubated sample as compared to O157:H7 monoculture controls. On the other hand, Stx2 levels in Sakai monoculture and coculture with C600 were statistically indistinguishable. A significantly higher (p<0.05) amplification was reported for PA2, with the fold difference being 20.3 (± 1.2), as compared to EDL933 and Sakai, which showed 2.7 (± 0.4) and 1.6 (± 0.4) fold difference respectively.
Figure 4-3. **Stx2 levels after coincubation with C600**: O157:H7 strains were statically incubated at 37°C, either alone (solid bars) or with C600 (dashed). Samples were harvested every 3 hours from 6 hour onwards and treated to PMB. Error bars are SEM from three biological repeats.
Figure 4-4. **Population dynamics in co-cultured samples.** O157:H7 strains were statically incubated at 37°C, either alone (solid lines) or with C600 (dashed lines). Samples were harvested every 3 hours, from 6 hour onwards and plated on sorbitol macConkey. In the total plate counts, the red colonies were counted for C600, while colorless were O157:H7. Error bars are SEM from three biological repeats.
Stx2 amplification occurs when strains are grown in *ex vivo* cattle mucus.

We developed an *ex vivo* model to investigate whether toxin amplification described above was repeatable in environments other than laboratory media. Cultures of C600 and O157:H7 in cattle mucus showed a similar trend of increased Stx in the coincubation samples with respect to the monoculture controls (Fig 4-5a). There was repeatable increase in the coincubated sample, as compared to the monoculture controls. The fold increase ranged from 1.7 to 7.4 in case of PA2, while EDL933 ranged from 1.1 to 3.1 (Fig 4-5a). The growth kinetics of pure culture showed that C600 concentration was lower than the observed counts for O157:H7 strains; however, this difference was statistically insignificant. In the co-culture setup C600 concentration is significantly reduced (p<0.01) compared to the O157:H7 it is coincubated with, however, the concentration of C600 in the co-culture and monoculture was statistically indistinguishable (Fig 4-5b-c).
Figure 4-5. **Shiga toxin and cell counts in the ex vivo mucus model.** (a) Fold increase in the Stx levels in the monoculture as compared to the co-culture with C600. A positive fold change indicates increased Stx2 levels in the coincubated samples. Time course of coincubated PA2 with C600 (b) and EDL933 with C600 (c) as coincubated (dashed lines) and monoculture (solid lines). Cultures were grown for 16 hours and plated on sorbitol MacConkey (SmaC). O157:H7 showed as white, while C600 was a red colony.
Shiga toxin amplification is also seen in other non-pathogenic *E. coli* belonging to O-18, O-6 and O-11 group.

Next, we investigated whether Stx2 accumulation occurs when non-pathogenic *E. coli*, other than C600 were co-cultured with PA2, EDL933 and Sakai. Twelve *E. coli* strains that belonged to six O-groups were cocultured with the three O157:H7. Four strains, namely, 0.1229, 0.1282, 0.1231 and 1.1954 showed increased Stx2 production as compared to monoculture controls of PA2, EDL933 and Sakai (Fig 4-6a-c). Average Stx2 level quantified from Sakai coincubated with non-pathogenic *E. coli* strains(s) (Fig 4-6c) were significantly lower than EDL933 (p<0.05) (Fig 4-6b) and PA2 (p<0.0001) (Fig 4-6a). The cell densities of non-pathogenic *E. coli* and *E. coli* O157:H7 were enumerated and expressed as a percentage (Fig 4-7c-d). Based on our observations with C600, we expected to see a reduction in cell density for non-pathogenic *E. coli* that amplified Stx2, however, none of the 12 *E. coli* strains were less than 50% of the total population.

Figure 4-6. Stx2 production when PA2, EDL933 and Sakai are coincubated with non-pathogenic *E. coli* strains. Stx2 levels from 13 different commensal strains (12 belonging to 6 O-groups and C600) coincubated with PA2 (a), EDL933 (b) and Sakai (c). The error bars represent SEM from three biological repeats. The asterix shows that the difference in means has a p <0.0001 for samples that had higher Stx2 than the monoculture.
Figure 4.7. **Percentage of non-pathogenic *E. coli* in the total coincubated sample when PA2, EDL933 and Sakai are coincubated with commensal strains** Figure shows the relative cell counts expressed as a percentage of red colonies growing on SMaC plates from (either the $10^7$ or $10^8$ dilution fold) from 13 different commensal strains (12 belonging to 6 O-groups and C600) coincubated with PA2 (d), EDL933 (e) and Sakai (f). The error bars represent SEM from three biological repeats.

**Spent supernatant from *E. coli* strain 0.1229 enhances Stx2 expression**

To examine whether the *E. coli* strains are expressing a factor that up regulates Stx2 expression in the O157:H7, the spent supernatant experiment was done. Sakai grown in the spent supernatant of 0.1229 showed 3 folds higher Stx2 levels as compared to when the strain was grown in fresh LB (Table 4). PA2 and EDL933, on the other hand, gave a saturated signal when grown in 0.1229 spent supernatant. The Stx2 expression from PA2, EDL933 and Sakai, grown in LB was statistically indistinguishable from the Stx2 levels quantified when the three O157:H7 were grown in spent supernatants of 0.1282, 0.1231, 1.1954 and C600 (Table 4).
Table 4. Fold difference in the Stx2 levels when O157:H7 strains were grown in the spent supernatant of non-pathogenic *E. coli* strains

<table>
<thead>
<tr>
<th>O157:H7 Growth Media</th>
<th>PA2</th>
<th>EDL933</th>
<th>Sakai</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spent supernatant (0.1229)</td>
<td>Saturated</td>
<td>Saturated</td>
<td>3.41 (±1.4)</td>
</tr>
<tr>
<td>Spent supernatant (0.1282)</td>
<td>1.6 (±0.3)</td>
<td>1.0 (±0.1)</td>
<td>1.8 (±0.3)</td>
</tr>
<tr>
<td>Spent supernatant (1.1954)</td>
<td>1.4 (±0.3)</td>
<td>0.9 (±0.1)</td>
<td>1.6 (±0.7)</td>
</tr>
<tr>
<td>Spent supernatant (0.1231)</td>
<td>1.2 (±0.3)</td>
<td>0.8 (±0.0)</td>
<td>1.2 (±0.5)</td>
</tr>
<tr>
<td>Spent supernatant (C600)</td>
<td>1.1 (±0.1)</td>
<td>0.8 (±0.1)</td>
<td>1.3 (±0.3)</td>
</tr>
<tr>
<td>Fresh LB</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

**Coincubation with other clade 8 strains causes toxin amplification.**

Based on observations that cocultures of PA2 and C600 show a drastic increase in Stx2 levels compared to PA2 alone, we hypothesized that other clade 8 strains would amplify Stx2. We chose 10 clade 8 strains from the previously characterized PA Health Isolate collection (209). PA2 carries only the *stx*2a gene; therefore, we selected 5 other clade 8 strains that carry *stx*2a, namely PA8, PA9, PA19, PA25 and PA34. Three other strains, PA3, PA13 and PA28, which carry both *stx*2 and *stx*2c gene were chosen to represent more typical *stx* genes for clade 8 strains (53). The Stx2 quantification after coincubation with clade 8 strains (Fig 4-8a) demonstrated that PA8 co-cultured with C600 also increased Stx2 levels by 24.1 (±2.5) fold as compared to monoculture controls. Other strains including PA3, PA19 and PA25 showed increased Stx2 levels by 2.3 (±0.1), 2.7 (±1.1) and 1.9 (±0.5) fold, albeit the numbers were not statistically significant. The cell counts enumerated from PA2, PA8 and PA34 showed that less than 50% of total colonies were C600 (Fig 4-8b). The co-cultured samples of PA8 and PA34 were the only ones to show reduced cell densities that were comparable to C600 counts recorded when coincubated with PA2.
Figure 4-8. Stx2 levels and percentage of commensals after coincubation of clade 8 strains with C600: clade 8 strains with stx2 (PA2, PA8, PA9, PA19, PA25 and PA34) or stx2, 2c (PA3, PA13 and PA28) were coincubated with C600. (a) Stx2 level quantified from the coincubation samples demonstrated that PA2 and PA8 significantly amplified Shiga-toxin2 in comparison to its monoculture control (b) relative cell densities of C600 showed that PA8 and PA34 significantly lowered the number of C600 to the same extent as PA2.
Discussion:

Previous studies have characterized an increase in Stx2 levels when O157:H7 is co-cultured with susceptible commensal *E. coli*, due to spontaneous induction of phage (191, 192). Our work augmented the understanding of this phenotype by demonstrating strain-specific differences in extent of amplification. Moreover, we revealed a set of O157:H7 strains that belong to a unique and notable genomic cluster, PST2 (123), that produces low Stx2 when induced with ciprofloxacin (Fig 4-2b), but show drastic increase in Stx2 accumulation, due to phage mediated lysis of susceptible *E. coli* (Fig 4-4 and 4-8). By using a broader collection of *E. coli* strains, we identified other non-pathogenic *E. coli* that employs different mechanisms to amplify Stx2 (Fig 4-6). We recognized four categories of commensals (Fig 4-9, table 4) that alter Stx2 production when co-cultured with O157:H7, by either increasing or reducing levels through defined mechanisms.

We used prototypical strains Sakai and EDL933 to characterize the phenotype of PA2. The former strains were the first *E. coli* O157:H7s to be fully sequenced (21, 57). Sakai is a lineage I, clade 1 strain carrying both *stx1* and *stx2* and was part of one of the largest O157:H7 outbreak that affected over 9,000 children in Sakai city, Japan. On the other hand, EDL933 is a clade 3, lineage I strain, also carrying *stx1* and *stx2*. It was implicated in the multistate hamburger outbreak in 1982 that caused 43 people getting sick (215). Studies on the Stx2 expression showed that human clinical isolates belonging to lineage I or I/II produce higher Stx2 as compared to cattle derived lineage II isolates (35, 48). Further classifying the strains based on SNP typing revealed a subset known as clade 8, which encompasses more virulent O157:H7 strains that are associated with higher incidence of HUS as compared to any other clade (53). An example of a large outbreak caused by clade 8 strains, is the multi-state Spinach outbreak in 2006, which reported 205 cases and 3 casualties (18, 207). It resulted in hospitalizations in over 60% cases,
while recording over 13% HUS rates (25, 53). The studied isolate PA2 belongs to clade 8, lineage I/II strain, and carries \textit{stx2}. Additionally, it is notable that it is not a great producer of Stx2 when induced with ciprofloxacin (Fig 4-2b). Clustering of the phage genome from this strain shows how it is in the same group as phage from the German outbreak strain O104:H4 and the Norwegian outbreak strain O103:H25 (213).

Sequencing of the inducible phage pool showed that the prototypical strains primarily produce Stx2 phage, while PA2 produces a combination of Stx2 and an untypeable phage showing sequence similarity to SP7-like phage (21, 59, 123). The Stx2 phage genomes were different from Sakai and EDL933 in the early regulatory and replication genes (Fig 4-1). The differences in the lysis time, peak OD$_{600}$, phage and Stx2 production (Fig 4-2a-c) confirmed that differences in genome content would translate to phenotypic differences. Sakai showed the highest peak OD$_{600}$ and completed its lysis at hour 8, while EDL933 showed the highest phage and Stx2 expression. This was consistent with previous findings, as EDL933 and Sakai are lineage I strains that produce more Stx2 (35). On the other hand, PA2 expressing low levels of Stx2 was contrary to studies by Neupane et al, that looked at five clade 8 strains, and showed a range of 0.88 to 5 fold increase in Stx2 expression with ciprofloxacin induction (95). Moreover, clade 8 strains have also known to up regulate the production of locus of enterocyte effacement (LEE) genes, which is another important virulence factor in O157:H7 (63, 183). Besides lineage, clade and host source, other factors also contribute to the Stx2 expression (35), including host background (123) inducing agent and antibiotics (127, 128, 216) and other phages carried by the bacteria (190). It is possible that PA2, despite being clade 8, could be a low producer of Stx2 owing to the aforementioned factors, or due to the difference in the phage genome or a combination of both.

According to the model proposed by Gamage et al. spontaneously inducing phage from O157:H7 infects a susceptible host, leading to lysis and Stx2 production, thus amplifying the
basal levels of Stx2. (191, 192). We hypothesized that the Stx2 accumulation trends we observed
when O157:H7 was grown in presence of ciprofloxacin would repeat when the susceptible host
C600 was co-cultured with three O157:H7. This hypothesis was based on the work of Gamage et
al who demonstrated a correlation between phage titers and Stx2 concentration. However, we
were surprised to see that the lowest Stx2 producer showed highest Stx2 levels and even
surpassed the levels quantified from C600 coincubated with EDL933 (Fig 4-3). Kinetics study of
cell counts showed reduced numbers of the susceptible host when present with PA2; strongly
suggesting that phage-mediated lysis of the C600 was occurring (Fig 4-4a). Moreover, addition of
induced supernatants, containing phage sans the O157:H7 background, to C600 showed increased
Stx2 levels (data not shown). This creates a compelling argument to reconsider strategies that
assess virulence potential of O157:H7 strains by solely reporting the Stx2 production in presence
of antibiotics, as the patterns observed were opposite of monoculture levels. Additionally, the
strain specific variations in Stx2 production illustrate how the prototypical strains may not be
representative of O157:H7 phenotypic diversity.

We also tested the validity of Stx2-amplification phenotype in non-laboratory setup by
developing an ex vivo model that uses mucus from slaughtered cattle. Mucin, the primary
component of colon-mucus, is a viscous, heterogenous and nutrient-rich glycoprotein. The
objective was also to observe the population dynamics in an environment that could represent the
intestine and give an indication about how the two would interact in the gut. The mucus
replicates, three of which were derived from Angus and one from a Jersey-Holstein mixed cattle,
consistently showed higher signals when C600 and O157:H7 was co-cultured, as compared to the
monoculture controls (Fig 4-5a). Despite variation in observed fold amplification, possibly due to
differences in the chemical composition of mucus, and/or biological differences caused by breed,
age or gender or resident flora of the cattle, we can conclusively say that the monoculture controls
would show lower Stx2 levels as compared to the C600 coincubated samples even in the mucus
model. Additionally, the suppression of C600 in the setup further demonstrates how O157:H7 reduces the cell densities coincubated (Fig 4-5 b-c). Compositionally, the mucus glycoproteins contain oligosaccharides including galactose (42.1%) N-acetylgalactosamine (24.1%), N-acetylgalactosamine (23.6%), fucose (4.7%) and mannose (3.1%) (139, 141). Ability to utilize fucose lets O157:H7 establish itself in the mucus, and modulating its pathogenicity (153). This may have helped O157:H7 to better colonize the mucus, as compared to the C600.

Isolating E. coli strains from 37 healthy individuals previously characterized commensals that amplify Stx2. The study showed that only 10% amplified Stx2 and phage (191). We procured a collection of 12 commensals, isolated from humans, to test whether amplification with other commensals is also observed. This collection had two representatives from six O-groups. Four out of the 12 (33.33%) amplified the Stx2 with all three O157:H7 (Fig 4-6 a-c), thus demonstrating that this phenomenon can be seen with different commensals. The cell densities did not show a decimation that paralleled C600 (Fig 4-7a-c), which could be attributed to reasons including, but not limited to, Stx2 induction by mechanisms other than phage mediated lysis.

Based on the possible mechanisms that the commensals employed to alter Stx2, we recognized 4 categories (Fig 9). The first category is strains that amplify Stx2 through phage-mediated lysis. These strains, exemplified by C600, amplify as well as show reduced cell densities (Fig 4-4a-c and Fig 4-5). The categories 2 and 3 include strains that amplify Stx2 but do not drop to lower than 50% of the total cell counts (Fig 4-7a-c, 4-8a-c). Category 2, represented by 0.1229, are commensals that possibly produce DNA damaging agents, like colicins (217) which induce Stx2 production in O157:H7. In this case, O157:H7 strains grown in spent supernatants of these commensals tend to produce more Stx2, as compared to the strains grown in fresh LB (Table 4). The third category of commensals, typified by 0.1282 and 1.1954 are Stx2 amplifying strains that employ mechanisms other than category 1 and 2, to enhance Stx2 production. These could include lysogenic conversion of commensals, such that they express Stx2, contributing toxin
accumulation. A high rate of lysogeny was previously reported when 41% of the 72 strains belonging to the ECOR collection incorporated Φ933W:Δtox phage upon infection (218). The fourth and final category of non-pathogenic *E. coli* includes strains that do not amplify Stx2. The mechanisms could either be non-specific competitive exclusion of O157:H7, or others that involve phage resistance reviewed by Labrie *et al.* (197). Some phage resistance mechanisms tested for O157:H7 include shielding by the O-group (219, 220) or binding of Stx2 to the O-antigen of the LPS, thereby reducing total Stx2 carriage (198).

Another noteworthy finding in our study was the phage cluster PST2. We hypothesized that the amplification phenotype shown by PA2 could be generalized to the clade 8 strains that carry *stx2a* genes. We also included three clade 8 strains that carry both *stx2* and *stx2c*, as most clade 8 strains carry the two alleles. We observed that PA8 was the only O157:H7 that showed increased Stx2 (Fig 4-8a). This strain also clusters in PST2, and the phage genome is identical to PA2 except for the position of an insertion sequence *IS629*. PA8 also induces both Stx2 as well as SP7 like phage, and is characterized as a low Stx2 producer as a monoculture (123). Knowing the phage genomes of both these strains can drive future studies in identifying candidate genes that can predict whether Stx2 amplification will occur or not.
Figure 4-9. **Categories of non-pathogenic* E. coli *based on their phenotype when co-incubated with O157:H7 strains.** Based on the altered Stx2 expression and competitive index, the *E. coli* strains are assigned one of four categories.

In conclusion, our study showed strain specific differences in Stx2 accumulation, when co-cultured with a non-pathogenic *E. coli*. This study expands the model proposed by Gamage *et al.* (191) where it was inferred that fold amplification was proportional to the amount of Stx2 expressed by a monoculture of O157:H7. In our study, a low Stx2 producing PA2 strain increased Stx2 levels 20 fold, as compared to a high Stx2 producer EDL933 that only amplified Stx2 by 3 folds. This demonstrates that there could be reasons, in addition to the phage and Stx2 produced by O157:H7 alone, which causes enhanced Stx2 production. The increased Stx2 production when O157:H7 is co-cultured was also seen in an *ex vivo* model that used cattle mucus as a growth media for the O157:H7. Additionally, we showed that phage-mediated lysis is not the sole mechanisms that increase Stx2 levels. There can be factors expressed by the non-pathogenic *E. coli*, as seen in case of strain 0.1229, which can induce higher Stx2 expression by the O157:H7.
Chapter 5

Conclusions and Future Directions

Conclusions

The overarching aim of our study was to look at the differences between the strains from the PST2 cluster, especially *E. coli* O157:H7 strain PA2, and prototypical strains, Sakai and EDL933. The first objective was to investigate the kinetics of phage induction, phage production and Stx2 expression from PA2, EDL933 and Sakai. The growth curve of the three O157:H7 strains, in the absence of antibiotics was identical; however there were strain specific differences when ciprofloxacin was added. The PA2 and EDL933 both lysed completely by hour 4, while Sakai phage continued to induce till 8 hours. Moreover, the lowest numeric OD\textsubscript{600} was observed for PA2, whose value was significantly lesser than what was recorded for EDL933 and Sakai. The phage production was highest for EDL933, while PA2 and Sakai were statistically indistinguishable. The Stx2 expression was significantly lower for PA2, as compared to EDL933 and Sakai, which expressed similar levels. The results showed that PA2 expressed the lowest levels of Stx2 when induced with ciprofloxacin.

For our second objective, the Stx2 and population dynamics of PA2, EDL933 and Sakai alone, as well as when co-cultured with C600 were investigated. In the co-culture samples, PA2 coincubated with C600 showed a 20 fold higher Stx2 expression as compared to PA2 alone. The Stx2 levels from the former were the highest among all the strains coincubated with C600. This was noteworthy because PA2 was the lowest Stx2 producer when induced with antibiotics. EDL933 co-cultured with C600 increased Stx2 by 3 fold, while Sakai alone and co-cultured Stx2 levels were statistically indistinguishable. The cell counts of C600, when co-cultured with
EDL933 and PA2 showed significantly lower numbers, as compared to C600 present alone. This was indicative of phage mediated lysis that decimated the C600 population in the coincubation sample. The studies on Stx2 in the coculture demonstrated how a low Stx2 producing O157:H7 strain PA2, dramatically increases the Stx2 levels when co-cultured. Moreover, the results illustrate a strain specific difference in the Stx2 fold amplification, which has not been reported previously. Increased Stx2 expression when PA2 and EDL933 were co-cultured with C600 was also demonstrated in an *ex vivo* model. This model used cattle colonic mucus as a growth media to highlight how Stx2 expression occurs, even in a non-laboratory setup. In the mucus model, the Stx quantified from the coincubated samples was higher than the monoculture levels, indicating that Stx amplification is repeatable when we grew the strains in a non-laboratory media setup.

The third objective was to assess the host range by co-culturing 12 different non-pathogenic *E. coli* strains with the three O157:H7 and testing whether there was an increase in Stx2. Four out of the 12 *E. coli* strains, namely 0.1229, 0.1282, 0.1231 and 1.1954, increased Stx2 levels with respect to the O157:H7 monoculture. Comparing the fold amplification with the three O157:H7, PA2 showed the highest cumulative fold increase as compared to EDL933 and Sakai. The competitive index was measured by enumerating the percentage of non-pathogenic *E. coli* in the total co-culture. More than 50% of the total cell counts were commensal, showing that all four strains outcompeted O157:H7. In contrast the C600 co-cultured with O157:H7 showed higher cell density of the latter. It was hypothesized that the non-pathogenic *E. coli* may be expressing a factor that can putatively induce Stx2 expression in the O157:H7, therefore the O157:H7 was grown in the spent supernatant of the non-pathogenic *E. coli*. The spent supernatant from the strain 0.1229 caused increased Stx2 expression as compared to the O157:H7 grown in fresh LB, supporting this hypothesis. This lead to the identification of four categories based on altered Stx2 levels and competitive index. If strains do not alter Stx2 expression upon co-culturing with O157:H7, they are assigned category 4. If there is a reported increase, the
competitive index is measured. For strains, like C600, where Stx2 amplification is accompanied by a competitive index favoring O157:H7 strain, the putative mechanism is phage-mediated lysis. In case that the competitive index favors the non-pathogenic *E. coli*, it is either assigned category 2, as exemplified from strain 0.1229, or category 3, where Stx2 is increased by some unknown mechanism.

The final objective was to test the Stx2 levels when O157:H7 strains that belong to clade 8 were co-cultured with C600. Strains PA2 and PA8 belonged to the PST2 cluster, while PA28 clustered within PST3 cluster. Six other clade 8 strains, namely PA9, PA19, PA25, PA34, all carrying *stx2* only and PA3, PA13 that carry the *stx2,2c* allele were included. Out of the 9 tested, only PA2 and PA8 from the PST2 cluster amplified Stx2 with respect to their monoculture control. Therefore, both the strains that cluster within the PST2 cluster enhance Stx2 expression.

Overall, our study showed strain specific differences in Stx2 accumulation when co-cultured with a non-pathogenic *E. coli*. This expands the previous model where it was inferred that fold amplification was proportional to the amount of Stx2 expressed by a monoculture of O157:H7. In our study, a low Stx2 producing PA2 strain increased Stx2 levels 20 fold, as compared to a high Stx2 producer EDL933 that only amplified Stx2 by 3 folds. This demonstrates that there could be reasons, in addition to the phage and Stx2 produced by O157:H7 alone, which causes enhanced Stx2 production. The increased Stx2 production when O157:H7 is co-cultured was also seen in an *ex vivo* model that used cattle mucus as a growth media for the O157:H7. Additionally, we showed that phage mediated lysis is not the sole mechanisms that increases Stx2 levels. There can be factors expressed by the non-pathogenic *E. coli*, as seen in case of strain 0.1229, which can induce higher Stx2 expression by the O157:H7.
Future Directions:

The results from the aforementioned studies generate some questions that could lead to further studies. The possible areas that could be investigated further are listed below.

1. Stx2 amplification with C600 in vivo

   Increased Stx2 levels were reported when PA2 was co-cultured with C600 in LB (in vitro) and in the cattle mucus (ex vivo). By using a germ-free mice model, we can test the hypothesis of whether Stx2 amplification would occur in vivo. The germ free mice will be a suitable model; as we can investigate the direct interaction between C600 and O157:H7 without any background flora present. Apart from measuring the Stx2 and the cell densities over course of infection, there are additional data points that can highlight disease severity including assessing the renal damage, change in body weight and urine density.

2. Identification of candidate genes in the PST2 cluster

   Our data demonstrated how both PA2 and PA8, both belonging to the PSt2 cluster showed high Stx2 fold amplification when co-cultured with C600. Since we know the phage genome, we can identify candidate genes that might confer this phenotype to the strains. Comparative genomics with the strains EDL933 and Sakai has shown PA2 to be unique in possessing the bsuBI and the higA genes. Molecular manipulations can be done to knock the genes out and measure its effect on the Stx2 amplification phenotype.
3. Role of Sp7-like phage

The sequencing data revealed both PA2 and PA8 release Sp7-like phage in the same levels as the Sp5 or Stx2-converting phage. The role of this phage in the Stx2 amplification phenotype is unknown. It can be hypothesized that the Sp7 acts as a helper phage. Molecular manipulations to cure the O157:H7 of the Sp7 phage can be undertaken. The Stx2 amplification between the Sp7-cured and wild type can be measured to test the hypothesis.

4. Characterization of factor up-regulating Stx2 expression in category 2 of non-pathogenic E. coli

The non-pathogenic E. coli strain 0.1229 expresses a factor that induces Stx2 production in O157:H7 strains. Studies can be performed to characterize the chemical nature of the molecule that prompts O157:H7 to switch from the lysogenic to lytic cycle. After understanding the nature of the compound, we can test its specificity and host range with other O157:H7 strains, to verify whether induction with this molecule is can be generalized to other O157:H7.

5. Mechanism of Stx2 amplification in category 3

Category 3 non-pathogenic E. coli strains amplify the Stx2 when co-cultured with O157:H7, but do not get outcompeted, thus negating phage mediated lysis as the principal mechanism. Growing O157:H7 in their spent supernatant did not enhance Stx2 expression, thus the cause for enhanced Stx2 expression with co-cultute is not exclusively one or the other. There can be multiple hypotheses to
test that explain increased Stx2 levels, but no reduction in counts of the non-pathogenic *E. coli* strains. It is possible that the *E. coli* integrate the Stx2 phage into their chromosome, which is maintained in the lysogenic cycle and continues to express basal levels of Stx2 themselves. Alternatively, it is possible that the non-pathogenic *E. coli* strains have robust growth rates, due to which the rate of phage mediated lysis is compensated by newer cells, therefore the non-pathogenic *E. coli* are category 1 after all. Furthermore, the non-pathogenic *E. coli* could also be category 2, where the factor expressed by them is unstable, showing transient induction leading to Stx2 accumulation. Nevertheless, understanding the mechanism of Stx2 amplification when coincubated with these strains will allow a better understanding of the phenotype.

6. Mechanism of Stx2 reduction in category 4

The fourth category assigned to non-pathogenic *E. coli* comprised of strains that do not alter Stx2 expression when co-cultured with O157:H7. This also includes strains that reduce the Stx2 levels with respect to the monoculture controls. Previously characterized mechanism of lowering Stx2 levels includes binding of the Stx to lipo-polysaccaride (LPS) membrane. Other mechanisms range from specialized to generalized bacteriophage resistance, including prevention of adsorption, expression of restriction modification proteins to digest incoming phage DNA or abortive infection of the infected cells. By characterizing the mechanisms of phage resistance and delving into benign non-pathogenic *E. coli*
that reduce Stx2 expression as well as have competitive indices that favor themselves, we can select probiotic strains that protect from O157:H7 infections.

7. Interaction between categories

All the co-culture experiments focused on interactions between the o157:H7 and a single non-pathogenic *E. coli* strain. It would be worthy to test how two or more categories would interact when they are co-cultured. For example, would there be Stx2 amplification when 0.1229, which expresses factors to enhance Stx2 expression, is co cultured with O157 and C600. Similarly, measuring be the Stx2 levels when category 4 strains are co-cultured with O157:H7 and 0.1229, would demonstrate which non-pathogenic *E. coli* overpowers the mixed culture.
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Appendix:

Qualitative data of Stx2 and phage production

Hartzell et al. previously characterized 52 isolates to identify their lineage, stx subtype and whether they belonged to clade 8 or not. These isolates were from the Pennsylvania department of health. This was a diverse collection of strains, as there were representatives of all three lineage, different stx alleles. Moreover the PFGE patterns, date and place of isolation from these strains was also known.

Our aim was to sequence the phage DNA, after they have induced from the O157:H7. This way, the inducible and defective phage can be distinguished. Moreover, due to modular and mosaic nature of phage, assembly of the phage genomes during whole genome sequencing becomes problematic. By performing de-novo assembly, one can obtain better sequence data. However, due to strain specific differences in phage and Stx2 levels expressed by different O157:H7, a screen to distinguish high from low phage and Stx2 producers had to be done. The relative expression of phage and Stx2 was evaluated for the 52 isolates, by comparing it to the prototypical strain Sakai, which is a high phage and Stx2 producer.

Methods:

Phage induction for Stx2 and phage estimation:

The phage and Stx2 levels were measured for all the 52 strains of O157 that were sequenced. The strains were induced with 45 ng/ml of ciprofloxacin, and the supernatant was collected 8 hours post induction. Aliquots of the supernatant were made such that one portion was used for the phage estimation, while the other was used to quantify the amount of Stx2 using slot blot.
Phage estimation using qPCR assay:

For the qPCR, the supernatant was diluted 1:1 (v/v) and 5 units/ml of DNase (Promega, Madison WI) was added. This was incubated at 37°C for 2 hours followed 90°C for 15 minutes to inactivate the DNase. Then proteinase K (EMD, Darmtadt, Germany) was added to a final concentration of 20 μg/ml was added and incubated at 65°C for 1 hour to digest the capsid, followed by a 95°C for 15 minutes to inactivate the enzymes. Each reaction contained 10μl of PerfeCta™ SYBR Green FastMix™ for iQ™ (Quanta Biosciences, Gaithersburg, MD), 0.1 μM of forward and reverse primers that amplify stx2, and sterile DNase/RNase free water (Promega, Madison WI) was added to make up the volume to 25 μl. DNase and proteinase K treated sample was added (25μl) as the template for qPCR. Each reaction was run in triplicates and 50 ng of Sakai DNA served as positive control while E. coli C600 was the negative control. The Ct values obtained were compared to the prototypical strain Sakai.

Stx2 estimation using Slot-Blot:

To quantify the amount of Stx2, slot blot was done in the 48-well Manifold ITM System (Whatman, Piscataway, NJ). Supernatant (500 μl) was added to the wells of the slot blot, and was bound to the 0.45 μm to polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA). Following an hour long incubation in blocking buffer, mouse anti-Stx2 (Santa Cruz Biotech.,Santa Cruz CA) diluted to a concentration of 1:200 was added. After washing five times with PBS (with 0.1% Tween-20), secondary antibody, IRDye 800CW goat anti-mouse IgG (LI-COR, Lincoln, NE) was diluted to 1:15,000 (v/v) and was incubated for 1 hour. Odyssey Infrared Imaging System (LI-COR, Lincoln, NE) was used for fluorescence detection. Signal intensity from each sample was analyzed using Odyssey Application Software Version 3.0 and the result was normalized against Sakai, which was our positive control.

Results:
The 52 isolates were induced with 45 ng/mL of ciprofloxacin. Out of the 52, two strains, PA24 and PA39 did not induce. Isolates showing more phage and Stx2 expression than Sakai were designated as “high”, while isolated that were comparable or lower than the latter were designated as “moderate” or “low”, respectively. The results showed a higher percentage of strains express equivalent, or higher levels of phage and Stx2 than Sakai. Phage DNA isolation was done from the isolates characterized as high or moderate.

![Phage production (qualitative) and Stx2 production (qualitative)](image)

a. Phage production (qualitative)

b. Stx2 production (qualitative)

Figure A1- Phage production (a) and Stx2 expression (b) in 52 isolates from the Pennsylvania department of health, previously characterized by Hartzell *et al.*
Vita

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Poster and Oral Presentations:
▪ “Growth Dynamics and Toxin Production in *E. coli* O157:H7 co-incubated with non-pathogenic strain in an *ex-vivo* cattle mucus model” Goswami K. and Dudley E.G. Sensing and Signaling across the mucosa: from homeostasis to pathogenesis. 2013. University Park, PA
▪ “Identification of Variable Regions within Genomes of Shiga Toxin Prophage from *Escherichia coli* O157:H7” Goswami K., Chen C., Dudley E.G. - International Association of Food Protection, 2012, Providence RI
▪ “Multiplex, quantitative real time PCR for detection, identification and quantification of mycotoxigenic Fusarium species” Goswami K. and Hall C.E. at NC 213 Grain Quality Consortium meeting (2010) at Kansas City
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