THE DEVELOPMENT OF A SHIGA TOXIN 2A QUANTIFICATION METHOD AND APPLICATION TO STUDY THE ENHANCED TOXIN PRODUCTION OF ESCHERICHIA COLI O157:H7 BY NONPATHOGENIC E. COLI

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ABSTRACT

The foodborne pathogen, *Escherichia coli* O157:H7, is a causal agent for gastrointestinal diseases and life-threatening hemolytic uremic syndrome (HUS) and hemorrhagic colitis (HC). Shiga toxin 2 (Stx2), the prominent virulence factor expressed by *E. coli* O157:H7 strains, is encoded in prophages. Its release is due to induction of the phage lytic cycle, and essential for fatal HUS development. Epidemiological studies have revealed that two subtypes of Stx2, namely Stx2a and Stx2c, are more likely to be associated with severe diseases. The quantitative measurement of Stx is important to evaluate the virulence of *E. coli* O157:H7. Immunoassays are the widely used method for quantifying Stx2 production by Shiga toxin (Stx)-producing *Escherichia coli* (STEC). The application of available immunoassay in routine usage is constrained by either high cost or requiring two sets of anti-Stx2 antibodies from different species, but most commercially available anti-Stx2 antibodies are derived from the same species. A sensitive and specific receptor-based ELISA (R-ELISA) was developed using all commercially available reagents (Chapter 3). Hydroxyl acyl ceramide trihexoside, an analogue of Stx2 receptor globotriaosylceramide (Gb3), was used for antigen capture, and several critical steps were identified that must be adhered to ensure repeatability. No cross-reactivity was observed in this assay with Stx1, and linear curves could be constructed using bacterial lysate from a high Stx2a-producing O157:H7 strain. Evaluation of Stx2 levels in a collection of *E. coli* O157:H7 strains showed that toxin production did not necessarily correlate with strain phylogeny using this assay.

Enhanced Stx2a expression was reported when specific nonpathogenic *E. coli* strains were co-cultured with *E. coli* O157:H7. Therefore, it was hypothesized that this phenotype required the former to be sensitive to infection by the stx-converting phages. In this study, 33%
of nonpathogenic *E. coli* strains were shown to be able to enhance the Stx2a production of an *E. coli* O157:H7 strain PA2, which belongs to the hypervirulent clade 8 cluster. Different competitive indexes for nonpathogenic *E. coli* strains versus PA2 were observed after co-incubation. *E. coli* C600 was outcompeted by PA2 in co-culture, which led to our hypothesis that *stx2a*-converting phages produced by PA2 caused the cell lysis of phage susceptible strain *E. coli* C600 and resulted in Stx2a amplification. By replacing the essential gene *bamA* (receptor for *stx*-converting phages) in *E. coli* C600 with a heterologous *bamA*, we were able to generate phage resistant strains and they did not amplify Stx2a in co-culture with PA2, supporting our hypothesis. Moreover, the extracellular loops (loop 4, 6, 7) BamA *E. coli* were demonstrated to be important for phage lytic infection. Unlike *E. coli* C600, commensal *E. coli* strains showed growth advantages over PA2 in co-cultures. *E. coli* strain 1.1954 was selected as the representative strain, and its chromosomal *bamA* knockout mutant did not eliminate the ability to amplify Stx2a. Additionally, the phage susceptible strain *E. coli* 1.1954 was not lysogenized by *stx2a*-converting phages after co-culturing with PA2. The data collectively indicated *E. coli* 1.1954 had an additional mechanism of Stx2a amplification. Additionally, the commensal *E. coli* strain 0.1229 was shown to secrete an unknown molecule in its supernatant which amplified Stx2a production. Therefore, we proposed three mechanisms for explaining the Stx2 amplification phenotype observed for nonpathogenic *E. coli* strains when co-cultured with *E. coli* O157:H7, which may explain the variations in the severity of diseases observed for patients infected with the same pathogen O157:H7 strain.
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LIST OF ABBREVIATION

AE  attaching and effacing
ANOVA  one-way analysis of variance
BSA  bovine serum albumin
CTH  ceramide trihexoside
DAEC  diffusely adherent E. coli
E. coli  *Escherichia coli*
EAEC  enteroaggregative E. coli
EHEC  enterohemorrhagic *E. coli*
EIEC  enteroinvasive *E. coli*
ELISA  enzyme-linked immunosorbent assay
EORC  *E. coli* Reference Center
EPEC  enteropathogenic *E. coli*
ETEC  enterotoxigenic *E. coli*
Gb3  globotriaosylceramide
Gb4  globotetraosylceramide
HC  hemorrhagic colitis
HRP  horseradish peroxidase
HUS  hemolytic uremic syndrome
IS  insertion element
LB  Luria-Bertani
LSPA  lineage-specific polymorphism assay
lyso-Gb3  deacylated Gb3
MLVA  multiple-locus variable number tandem repeat analysis
mRNA  messenger RNA
OBGS  octamer-based genome scanning
OD  optical density
PBS  phosphate buffer saline
PST  phage sequence types
qPCR  real time polymerase chain reaction
R-ELISA  receptor based ELISA
rRNA  ribosomal RNA
SNP  single nucleotide polymorphism
STEC  Shiga toxin producing *E. coli*
Stx  Shiga toxin
T3SS  type III secretion system
Tir  translocated intimin receptor
TMB  3,3’,5,5’ tetramethylbenzidine
tRNA  transfer RNA
USDA  United States Department of Agriculture
WGS  whole genome sequencing
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Chapter 1 Statement of the Problem

The foodborne pathogen, *Escherichia coli* O157:H7, is the causal agent for gastrointestinal diseases and life-threatening hemolytic uremic syndrome (HUS) and hemorrhagic colitis (HC). Upon ingestion of fewer than 100 cells of *E. coli* O157:H7, people may develop symptoms ranging from the asymptomatic carriage, bloody diarrhea, to fatal HUS. It has been estimated to cause 73,000 illnesses and 60 deaths annually in the United States, with an estimated economic loss of about 400 million dollars. Understanding its pathogenesis will help to better provide effective treatments for patients and prevent economic loss.

Stx2, one of two immunologically distinct isoforms, is more potent than the other isoform, Stx1. Moreover, the Stx2-producing strains are correlated with the development of HUS, leading to the hypothesis that high toxin-producing strains are more likely to cause severe disease symptoms. Thus, accurate quantification of Stx2 is important for studying the virulence of *E. coli* O157:H7. However, the application of current immunoassay in routine usage is constrained by either high cost or requirement of two sets of anti-Stx2 antibody for both antigen capture and detection steps. Therefore, one of the objectives of this study (Chapter 3) was to develop a quantitative receptor based in enzyme-linked immunosorbent assay (ELISA) by using all commercially available reagents, using a chemical analogue of Gb3 as the receptor for antigen capture.

The *stx*2 is encoded in prophages. Like the lambdoid phage, *stx*2-converting phages possess a lysogenic and a lytic life cycle. Phage induction, switching from lysogenic to lytic state, can be triggered by DNA damaging agents such as certain antibiotics, leading to a release
of Stx2 and host cell lysis. Significant enhancement of Stx2 production was shown when *E. coli* O157:H7 was co-cultured with certain nonpathogenic *E. coli*. A model was previously proposed where stx2-converting phages produced by *E. coli* O157:H7 increased Stx2 production through infection of susceptible commensal *E. coli* strains. Previous studies corroborated this model, and additionally demonstrated that toxin amplification was accompanied by a reduction in cell density of laboratory strain *E. coli* C600 when grown in the presence of *E. coli* O157:H7. However, the amount of Stx2a produced in co-cultures varied among three tested *E. coli* O157:H7 strains, PA2, EDL933 and Sakai. The highest level of Stx2a production was observed when *E. coli* C600 was co-cultured with PA2. Although the current model suggests toxin amplification requires phage infection of *E. coli* C600, no genetic evidence exists to support this assumption. It is also unknown whether toxin amplification by other commensal *E. coli* occurs by a similar mechanism. Thus, the hypothesis was that multiple mechanisms accounted for the Stx2 amplification when *E. coli* O157:H7 was co-cultured with commensal *E. coli* was addressed in Chapter 4.
Chapter 2 Literature Review

2.1. Introduction

2.1.1 Escherichia coli

*Escherichia coli* (*E. coli*) is a rod shaped, Gram-negative, facultative anaerobic bacterium. It was first isolated from the feces of a healthy infant and described by Theodore Escherichia in 1885 (Wasteson, 2001). Most *E. coli* strains are harmless and normal flora in the gastrointestinal track of human and animals. They benefit the host by providing vitamin K (Wilson, 2004), or preventing colonization of pathogens by producing bacteriocins (Callaway *et al*., 2004; Jordi *et al*., 2001; Murinda *et al*., 1996; Patton *et al*., 2008; Schamberger and Phillips, 2004; Schamberger and Diez-Gonzalez, 2004; H Toshima *et al*., 2007). However, some *E. coli* strains have evolved into pathogens after acquiring virulence factors through horizontal gene transfer (Feng *et al*., 1998; Zhao *et al*., 2015). Diverse *E. coli* population are classified into six main phylogroups: A, B1, B2, D, E and F, as well as several cryptic lineages (Gordon *et al*., 2008; Jaureguy *et al*., 2008; Walk *et al*., 2009). Although virulence determinants vary, a relationship between strain phylogeny and virulence has been reported. Phylogroup A and B1 are usually linked to commensal *E. coli* strains, while B2 and D are frequently associated with strains causing extraintestinal diseases (Picard *et al*., 1999).

2.1.2 E. coli O157:H7

Six pathotypes were identified for the pathogenic *E. coli* based on their virulence features: enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC). Based on the antibodies that bind to three distinct antigens (O, lipopolysaccharide;
H, flagella; K, capsule) on the bacterial surface, the strains can be divided into different serogroups (Nataro and Kaper, 1998). \textit{E. coli} O157:H7 is a well-recognized serotype of EHEC, which has a high outbreak rate (Rangel \textit{et al}., 2005) and pathogenicity, causing foodborne diseases (Gould \textit{et al}., 2009) and leading to a spectrum of symptoms ranging from the asymptomatic carriage, bloody diarrhea, to fatal hemolytic uremic syndrome (HUS) (Tarr \textit{et al}., 2005).

Epidemiological investigations have shown that healthy cattle are the major reservoir for \textit{E. coli} O157:H7 (Borczyk \textit{et al}., 1987). The ability of this organism to survive in water, soil, and manure has important implications for its persistence in cattle herds and contamination of water supplies and crops (Kudva \textit{et al}., 1998; Maule, 2000). Its transmission routes to human can be through contaminated water or food such as ground beef (Elder \textit{et al}., 2000), cheese (Honish \textit{et al}., 1983) and fresh produce (Ackers \textit{et al}., 1998). Due to its low infectious dose of fewer than 100 cells (Strachan \textit{et al}., 2005), the United States Department of Agriculture (USDA) has enforced a zero tolerance policy to regulate its prevalence in foods. Annually, 73,000 illnesses, 1800-3600 hospitalizations, and 60 to 541 deaths have been estimated to occur in the United States, which cost close to 400 million dollars (Frenzen \textit{et al}., 2005).

The \textit{E. coli} O157:H7 implements two main virulence infecting strategies: the formation of attaching and effacing (AE) lesions on enterocytes for colonization and the expression of Shiga toxins (Stx) for potency. The pathogenicity island called locus of enterocyte effacement (LEE) (Deng \textit{et al}., 2004) is responsible for AE lesion. LEE encodes a type III secretion system (T3SS) (Jarvis \textit{et al}., 1995) that exports effector molecules; several secreted proteins (Esp) as part of T3SS (Spears \textit{et al}., 2006), an adhesion named intimin (Jerse \textit{et al}., 1990) and its own translocated intimin receptor (Tir) (Kenny \textit{et al}., 1997). Tir is injected into the host cell through the syringe-like structure made of T3SS apparatus. The intimin expressed on the surface of \textit{E. coli} O157:H7
interact with Tir, resulting in striking cytoskeletal changes of the intestinal epithelial cells which
develop to the effacement of intestinal microvilli and formation of a pedestal-like structure. This
characteristic intestinal histopathology is known as AE lesions (Knutton et al., 1989). Other
virulence factors such as plasmid pO157 (Schmidt et al., 1994) also contribute to the pathogenicity
of O157, however, they are not the main focus of this dissertation. More details about EHEC
pathogenicity can be found in other reviews (Kaper et al., 2005; Nataro and Kaper, 1998).

The key virulence factor of *E. coli* O157:H7 is Stx, which is essential for HUS development
(Karmali et al., 1983; O’Brien and LaVeck, 1983). As revealed by X-ray crystallography, the Stxs
have an AB₅ molecular structure consisted of an A subunit with a pentamer of identical B subunits
(Fraser et al., 1994; Stein et al., 1992). The pentamer B subunits of Stxs selectively bind to
globotriaosylceramide (Gb3) (Jacewicz et al., 2006) or globotetraosylceramide (Gb4) (Tyrrell et
al., 1992) on the surface of host cell membrane, and act as the receptor for subsequent
internalization of toxin into the cytoplasm. In the cytoplasm, the RNA N-glycosidase active A
subunit cleaves an adenine nucleotide from 28S ribosomal RNA (rRNA) of eukaryotic ribosome,
preventing transfer RNA (tRNA) binding and protein synthesis, resulting in necrosis and cell death
(Sandvig and van Deurs, 1992; Saxena et al., 1989). **This review will discuss Stx, the prominent
virulence factor of foodborne pathogen *E. coli* O157:H7, and Stx receptor as this serves as
the basis for the development of a toxin quantification method. Moreover, it will cover the
diversity of *E. coli* O157:H7 strains, particularly the segment contributed by the stx2a-
converting phages, to understand the strain level virulence difference of *E. coli* O157:H7 as
well as to identify the limits of our understanding.**

2.2 Overall review of Stx and its receptor
2.2.1 Stx subtypes

Stxs from *E. coli* O157:H7 have two immunologically distinct alleles, designated as Stx1 and Stx2. Stx1 is identical to Stx from *S. dysenteriae* type 1, differing by a single amino acid in the catalytic A subunit. However, Stx1 shares only 56% identity with Stx2 at the amino acid sequence level (Jackson *et al*., 1987). Within each Stx allele, minor DNA sequence differences are used to classify Stx1 into Stx1a, Stx1c and Stx1d; and Stx2 into seven variants, from Stx2a to Stx2g (Scheutz *et al*., 2012). Stx1a, Stx2a, Stx2c and Stx2d are often linked to human diseases (Persson *et al*., 2007; Tinetti *et al*., 1988), while the Stx2e, Stx2f and Stx2g are mostly found in animal infections. For example, Stx2e is associated with edema disease in swine, while Stx2g has been associated with feral pigeons (Leung *et al*., 2003; Schmidt *et al*., 2000; Weinstein *et al*., 1988). Epidemiological investigation showed that Stx2a and Stx2c are found more frequent in strains causing HUS (Friedrich *et al*., 2002; Persson *et al*., 2007).

2.2.2 Toxicity of Stx subtypes

The toxicity of Stx1 and Stx2 was compared in different animal models. Studies in mouse models have shown that Stx2 is 400 times more potent than Stx1 (Tesh *et al*., 1993). Administration of Stx2 alone in primates can lead to HUS, while the same dose of Stx1 does not cause HUS (Siegler *et al*., 2003). Thus, Stx1 is less toxic than Stx2, though the former demonstrated a greater affinity to receptor Gb3 *in vitro* (Function *et al*., 1991). A possible explanation for this may be due to their different target tissues. After tracking radioactive iodine labeled Stx1 and Stx2 in mice, it was observed that Stx1 accumulated mainly in the lungs and nasal turbinate, while renal cells were the major target for Stx2 (Rutjes *et al*., 2002). The primary sequences of the Stx2 A subunit are relatively conserved, however, sequence variations in the Stx2
B subunit result in alterations of toxicities and receptor affinities. The potencies of Stx2 subtypes were characterized in vitro by using renal proximal tubule cells and in vivo using a mouse model. The subtype Stx2a and Stx2d were more potent than Stx2b and Stx2c (Fuller et al., 2011). Different binding affinity to receptor was hypothesized as a reason for the potency differences within Stx2 subtypes, as the initial structure modeling showed the amino acid polymorphisms existed within the receptor binding face of the B subunit in Stx2 (Rutjes et al., 2002). The toxin-receptor binding was further assessed in vitro by receptor based enzyme-linked immunosorbent assay (R-ELISA), and the holotoxin Stx2a and Stx2d showed comparable glycolipid binding profile, followed by Stx2c and Stx2b (Gallegos et al., 2012). The lower toxicity of Stx2c when compared to Stx2d was ascribed to the instability of Stx2c holotoxin (Bunger et al., 2015).

2.2.3 Stx and Gb3 binding

2.2.3.1 Binding sites in Stx

As mentioned previously, the Stx B subunit specifically binds to the Gb3 receptor. Even without the A subunit, it still functioned equivalently to holotoxin in terms of receptor binding (Donohue-Rolfe et al., 1989). Previous crystal structure and modelling studies of the B subunit of Stxs with or without Gb3 trisaccharide analogue has revealed that there are two or three Gb3 binding sites per B subunit (Bast et al., 1999; Nyholm et al., 1996; Soltyk et al., 2002). Thus, for a single Stx B pentamer will have a maximum of 15 binding sites for Gb3. The primary importance of binding site 2 in the B pentamer with respect to Gb3 binding was confirmed by multiple mutational analyses. Although binding site 1 and 3 were shown to be involved in binding to Gb3 (Bast et al., 1999; Ling et al., 2000; Soltyk et al., 2002), they are less frequently occupied by the trisaccharide analogue of Gb3 than binding site 2 (Kitova et al., 2007).
2.2.3.2 Gb3 as Stx receptor

That Gb3 plays an important role in Stx-receptor recognition. The incorporation of exogenous Gb3 into Gb3-deficient cells sensitized them to Stx1 (Waddell et al., 1990), and purified Stx2 demonstrated a specific binding ability to extracted Gb3 from human kidney (Waddell et al., 1988). Therefore, Gb3 expression is crucial for the toxic effects of Stx and its relation with the severity of HUS. The Gb3 expression level varies in bio-distribution, tissue types and age groups. It primarily locates in kidney as well as the brain endothelium. Moreover, the endothelial cells of renal microvascular with high Gb3 expression are considered as the principal targets for Stx. HUS often occurs in the young who express high levels of Gb3 in renal glomeruli. In addition, the elderly’s inappropriate responses of aging immune system in their renal glomeruli leads to induction of Gb3 (Obrig et al., 1993).

Gb3 (galactose α1-4galactose β1-4glucosyl β1-ceramide) is formed by a polar trisaccharide group and a nonpolar ceramide component. The ceramide is a sphingosine base linked to a fatty acid chain via an amide bond (Lindberg et al., 1987). The Gb3 structure is showed in Figure 2.1. Although the variant Stx2e specifically recognizes Gb4 as its receptor, it still binds to Gb3. The only difference between Gb4 and Gb3 is that the former has an additional N-acetylated galactose residue (DeGrandis et al., 1989). The terminal Gal α1-4Gal carbohydrate residue of Gb3 is the essential binding site for Stx, as the absence or modification of it abolishes the toxin-receptor interaction (Waddell et al., 1988). Moreover, digalactosyl diglyceride has a similar terminal galactose residue as Gb3, but does not bind to the toxins (Obrig et al., 1993), indicating that the ceramide component is necessary for binding Stx. Although the trisaccharide residue of Gb3 is invariable, ceramide is heterogeneous, affecting the lateral mobility of lipid in the plasma membrane and conformation of the trisaccharide moiety. In addition, the fatty acid component
within the ceramide moiety has a profound effect on Stx binding to receptor, as the deacylated Gb3 (lyso-Gb3) which lacked the fatty acid chain showed significantly decreased affinity to Stx1 when compared to Gb3 (Pellizzari et al., 1992). Thus, factors such as the fatty acid isoforms, chain length, degree of unsaturation and hydroxylation contribute to the heterogeneity of toxin-receptor binding.

**Figure 2.1** Chemical structure of Stx receptor, Gb3 [adapted from (Engedal et al., 2011)]. The sphingosine backbone often has a relatively invariable chain length of 18 carbons, whereas fatty acyl chain varies both in length and saturation (here shown as C16:0).

The Gb3 extracted from human kidney is a complex mixture. It contained fatty acid isoforms with chain length ranging from carbon 14 to 24 carbons, and varied in levels of unsaturation and hydroxylation. Different fatty acid containing Gb3 were hypothesized to cooperatively increase binding affinity to Stx, as the mixture of C16:0 with C24:0 Gb3 was showed to augment binding to Stx1 when compared to either one of them (Pellizzari et al., 1992). In addition, the binding of Gb3 to Stx1 increased with the increase in fatty acid chain length. With respect to the unsaturation state of Gb3, C18:1 and C22:1 fatty acid isoform showed the highest binding capacity for Stx2c and Stx1 respectively (Fatiy et al., 1994). Moreover, both Stx1 and Stx2 demonstrated increased affinity to C22 hydroxylated Gb3 in R-ELISA (Binnington et al., 2002). The Gb3 locates in lipid rafts (detergent-insoluble glycolipid-enriched domains), so the membrane environment including
the cholesterol levels is crucial for toxin-receptor recognition. With addition of cholesterol into Gb3, the affinities of both Stx1 and Stx2 to receptor significantly increased in vitro (Gallegos et al., 2012). A clinical study suggested that patients who did not develop HUS had less hydroxylated fatty acid content in their red cell Gb3 than those with HUS (Newburg et al., 1993).

In summary, the pentamer B subunit of Stx specifically binds to the trisaccharide moiety of Gb3, and heterogeneity within Gb3 fatty acid chain affects Stx-Gb3 affinity. Thus, understanding the complexity of the Stx-Gb3 interaction and the diversity within Gb3 is crucial for studying Stx toxicity as well as serving the basis for development of effective toxin quantification methods.

2.2.4. Current Stx2 quantification methods

Stx2 producing strains are correlated with the development of HUS (Ethelberg et al., 2004), leading to the hypothesis that high toxin-producing strains are more likely to cause severe disease symptoms. Most studies infer Stx2 concentration using Vero cell cytotoxic assay (Noda et al., 1987), semi-quantitative commercial kits (Amigo et al., 2015), qPCR/microarray (Abu-Ali et al., 2010) or semi-quantitative western blots (Neupane et al., 2011). Although the cytotoxic assay is the most sensitive method for detecting active Stxs, it is expensive, labor-intensive and time consuming. Moreover, several studies have reported that Stx2 transcript levels in messenger RNA (mRNA) quantification do not always correlate with Stx2 toxin production in terms of mRNA quantification (Amigo et al., 2015). The disadvantage of commercial kits is the expense of testing large sample sets and the inability to differentiate Stx1 from Stx2.

Several other methods were previously described that use either immobilized antibodies specific for Stx (He et al., 2013; Ball et al., 1996) or a receptor mimic (Ashkenazi and Cleary,
1989; Acheson et al., 1990; Basta et al., 1989) as the antigen capture in sandwich ELISA. However, none of the previously reported immunoassays were optimized to be quantitative. Therefore, it would be useful to develop a simple, cheap but specific and sensitive immunoassay for measuring a large set of samples containing Stx2a. Chapter 3 will discuss the development of a quantitative R-ELISA by using commercially available receptor mimic, hydroxylated C18 ceramide trihexoside.

2.3 stx2-converting phages and its diversity

2.3.1 stx2-converting phages

2.3.1.1 stx-converting phages belong to lambdoid family

The gene encoding Stx is in prophage. The complete sequencing of two prototypical O157 strains has highlighted the importance of the phages’ contributions to the bacterial genome. Eighteen phage-like sequences were identified when sequencing the genome of EDL933, which was isolated in 1982 from Michigan ground beef, and the gene encoding for Stx2a was found in the intact prophage 933W (Perna et al., 2001). Another O157 strain, Sakai, which was isolated in 1996 from an outbreak affecting more than 6000 primary schoolchildren in Sakai City Japan, has been fully sequenced and eighteen phage-like elements were identified. Thirteen of them, including stxl- and stx2a-converting phage named Sp15 and Sp5, respectively, were related to lambdoid phages. They resembled each other, and were reported to be defective due to gene disruption by an insertion element (IS) (Hayashi et al., 2001). These facts have enormous implications on both Stx expression and pathogenesis of O157 strain (Ortegren, 2014). Subsequent studies have revealed stx-converting phages belong to the lambdoid family and share great similarity in terms of DNA sequence, genome organization and gene function (Huang et al., 1987;
Miyamoto *et al.*, 1999). In *stx*-converting phages, the genes for encoding Stx locate within phage late genes, downstream of phage late promoter P$_R$' and upstream of the lysis cassette (Iii *et al.*, 1999), suggesting that Stx expression is determined by phage cycle.

### 2.3.1.2 Regulation of phage life cycles

Like lambdoid phages, the *stx*-converting phages have two life cycles: lysogenic and lytic. The switch between these two cycles is controlled by early regulatory genes (Figure 2.2). In the lysogenic state, the *stx*-converting phages remain as prophages which replicate and divide along with O157 host chromosome. The CI repressor forms dimer, and binds to the left and right operator sites (O$_L$, O$_R$), inhibiting the activity of early promoter P$_R$ and P$_L$. Therefore, the downstream genes are not expressed. In the lytic cycle, the prophage excises from the O157 chromosome, replicates by using host machinery, assembles new phage particles and escapes the host through cell lysis. The switch from lysogenic into the lytic cycle is called “induction”, and is triggered by stressors such as DNA damaging agents. The host genome sustains DNA damage and reacts by activating the LexA regulon, leading to a cellular change in gene expression called the SOS response (Sutton *et al.*, 2000). One component of the regulon, RecA, is produced and activated by interacting with single-stranded DNA resulting from the DNA damage, leading to the autocleavage of CI repressor through its co-protease activity. The release of the CI repressor results in the initiation of transcription from P$_R$ and the expression of the anti-terminator N and Q. Protein Q activates the late promoter, P$_R$ ', which controls the late phage genes including *stx*. During this process, the O157 strain undergoes cell lysis and Stxs are released concurrently (Mühldorfer *et al.*, 1996; Waldor and Friedman, 2005)
The importance of CI repressor in phage incorporation and toxin production were previously investigated. A study showed deletion of both \textit{cI} and \textit{cro} led to more than four log fold decrease in the lysogenized rate when compared with wild type Lys933W, but the complementation of \textit{cI} restored the mutant’s lysogenized rate (Serra-Moreno \textit{et al.}, 2008). In addition, CI repressor is necessary for Stx production, because a point mutation in \textit{cI} making it resistant to RecA-mediated autocleavage, abrogated Stx2 production by \textit{E. coli} K37. This demonstrated that autocleavage of CI is critical for Stx2 production (Tyler \textit{et al.}, 2004).

\textbf{Figure 2.2} Simplified composite genetic map of \textit{stx}-converting phages showing the regulatory region, associated genes including \textit{stx} and transcription pattern (not drawn to scale) (a) Repressed prophage: repressor CI forms dimer and binds at the operators \textit{O.L} and \textit{O.R}, halting the transcriptions that initiate the regulatory cascade (shown in [b]). The transcription from \textit{P.RM} directs synthesis of CI in the lysogenic state. In the absence of \textit{Q}, transcription initiating at \textit{P.R}\textsuperscript{'}, terminates at immediate downstream terminator. (b) Induced prophage: cleavage of CI repressor CI results in release of repression. The regulatory cascade begins with transcription initiating at \textit{P.L} and \textit{P.R} and lead to the expression of \textit{N} and \textit{Q}. In the presence of \textit{Q}, the transcription initiating at \textit{P.R}\textsuperscript{'}, and downstream genes including \textit{stx} and \textit{lys} as well as most of the genes required for forming viable phage particles are transcribed. Genes and function of their products as follow: Rec, recombination; Rep, replication; O, operators; P, promoters; \textit{cI}, repressor; \textit{cro}, inhibitor of
CI synthesis; cII, activator of CI synthesis; N and Q, transcription anti-termination; stx: Shiga toxin; lys, lysis; T: terminators; arrow represents the initiation of transcription.

2.3.1.3 External phage inducing agents and their mechanisms

The spontaneous induction of stx-converting phages from lysogenic to lytic cycles occurs at an extremely low rate (Livny and Friedman, 2004) in the absence of an external inducing agent, referred to as the baseline Stx production level (Zhang et al., 2010). External inducing agents were shown to trigger prophage induction, for example, nitric oxide, hydrogen peroxide, iron and antibiotics (Calderwood and Mekalanos, 1987; McGannon et al., 2010; Mühldorfer et al., 1996; O’Brien et al., 1982; Vareille et al., 2007; Wagner et al., 2001a; Zhang et al., 2000).

Treatment with nitric oxide of O157 strains was shown to interfere with their bacterial SOS responses by causing drastic reduction of RecA, leading to less phage and Stx2 production (Vareille et al., 2007). Incubating either hydrogen peroxide or neutrophils with EHEC, Stx production increased. This was most likely due to activation of SOS response by H$_2$O$_2$ and NO (Wagner et al., 2001a). In addition, iron was showed to have a dose-dependent effect on Stx1 overexpression: at a low concentration, it stimulated Stx1 production; yet at high levels, it inhibited Stx1 expression. This is because the promoters associated with the stx1 and stx2 genes differ significantly in sequence and regulation. The promoter for stx1 exclusively contains a ferric uptake regulator (Fur) box, which is a binding site for the Fur protein, coupling with iron to repress transcription (O’Brien et al., 1982; Calderwood and Mekalanos, 1987). However, there is no Fur box in promoter for Stx2. Thus, Stx2 expression is not regulated by iron (Sung et al., 1990). Many studies have shown that some antibiotics such as ciprofloxacin (McGannon et al., 2010; Zhang et al., 2000); trimethoprim-sulfamethoxazole (McGannon et al., 2010) and mitomycin C (Mühldorfer et al., 1996), which cause DNA damage or inhibition of DNA replication, provoke the bacterial
SOS responses, leading to phage induction and Stx production. However, other antibiotics targeting protein synthesis or cell wall synthesis would not cause phage induction (McGannon et al., 2010). Because of the mode of action, ciprofloxacin and mitomycin C are commonly used in research on phage induction and toxin production. For instance, exposed to mitomycin C, O157 cultures produced significantly more Stx than non-induced cultures (Ritchie et al., 2003).

Overall, the stx2-converting phage shares the same life cycle regulation as lambdoid phage does, and the inducing agents like antibiotics, which trigger the SOS responses, will promote prophage induction and resulted in Stx2 release.

2.3.2 stx2-converting phage diversity

2.3.2.1 Phage morphology, sequence and genome size

An extensive study of thirty infectious stx2-converting phages revealed that most of them were 933W-like (Muniesa et al., 2004), possessing an isometric capsid and a short tail, the latter of which had a great impact on the phage adsorption to host membrane (Smith et al., 2007). However, at least three morphological differences within the phage tails were identified, along with varying genome sizes, ranging from 50.7 kb to 66.4 kb (Muniesa et al., 2004). With development of sequencing technologies, more in-depth genomic analyses and comparisons of the stx2-converting phages have been done. Diversifications within early regulation and replication regions of stx2-converting phage backbone were initially observed when comparing the sequences of two stx2-converting phages from the prototypical E. coli O157:H7 strains, EDL933 and Sakai. The predicted amino acid sequences for regulatory region such as N, CI, Cro, O and P did not show significant similarity (Miyamoto et al., 1999). This was further verified in a later study on comparing ø24_B, which is capable of frequently infecting a single bacterial host, along with other
ten sequenced stx2-converting phage genomes (Smith et al., 2012). Two more recent comprehensive studies on stx2-converting phages genomes from different Stx producing E. coli (STEC) strains or different Stx2 allelic types added more evidence to show the diversity within the regulatory regions of stx2-converting phages (Ogura et al., 2015; Yin et al., 2015). Additionally, the phage backbone, the insertion element (IS) and single nucleotide polymorphisms (SNPs) contributed to the diversity, as the mobile element was able to disrupt gene normal function and possibly affect toxin production (Eppinger et al., 2011; Yin et al., 2015).

2.3.2.2 Phage insertion sites

The stx-converting phages are known to have preferred integration sites in the host bacteria. Five common insertion sites related with O157 strains are described including wrbA which codes a tryptophan repressor-binding protein, yehV which is a transcriptional regulator encoding gene, argW encoding tRNA-Arg, sbcB which produces an exonuclease and yecE with unknown function (Muniesa et al., 2004; De Greve et al., 2002; Mellor et al., 2013; Serra-Moreno et al., 2007; Shringi et al., 2012). The stx2a-converting phage usually prefers argW and wrbA, while stx2c-converting phage incline to sbcB (Yin et al., 2015; Ogura et al., 2015). Such variations in phage insertion sites have a biased distribution in human and bovine as well as in different geographic locations. An extensive study on stx-converting phage insertion sites in a collection of 606 E. coli O157:H7 strains from different geographic or animal origin revealed that genotype stx1 stx2c argW sbcB yehV was more frequent in Australia, while stx1 stx2 wrbA yehV was more prevalent in the United States, indicating a divergent evolution of O157 strains. In both countries, stx2c occurred in a greater proportion of cattle than human isolates (Mellor et al., 2013). Moreover, the availability of the insertion sites in the host genome is a determinant for phage integration. A study has shown
that if the primary insertion site in the host chromosome is already occupied, the stx2-converting phages will integrate at the second site (Serra-Moreno et al., 2007).

### 2.3.2.3 Phage receptors

BamA (also called YaeT), which is essential for outer membrane protein biogenesis (Wu et al., 2005), has been reported to be the receptor for short-tailed stx2-converting phages (Islam et al., 2012; Smith et al., 2007). The bamA gene is highly conserved among Enterobacteriaceae (Smith et al., 2007). Within species, the extracellular loops of bamA have heterogeneity (Ruhe et al., 2013). Deletion of bamA is lethal, so the characteristic of serving as a phage receptor was investigated biochemically using anti-BamA serum, or the genetically overexpressing BamA in a target organism to see if there was any change in phage adsorption rate (Islam et al., 2012; Smith et al., 2007). Thus, direct evidence through gene knockout is still lacking to prove bamA’s phage receptor function.

Other outer membrane proteins such as FadL and LamB were hypothesized to be stx2-converting phage receptors (Islam et al., 2012; Watarai et al., 1998). However, contradictory results were reported. In one study, two stx2-converting phages, designated Stx2 ø-I and Stx2 ø-II, were identified from two clinical strains of STEC strains associated with the outbreaks in Japan in 1996, and Stx2ø-I resembled 933W. By using outer membrane protein fractions in the phage neutralization experiment, FadL was shown to be receptor for 933W and Stx2ø-I, while LamB was for Stx2ø-II (Watarai et al., 1998). In contrast, deletions of fadL and lamB in E. coli K-12 MG1655 had no effect on stx2a-converting phages from E. coli O157:H7 Sakai (Islam et al., 2012). One potential explanation for it is that diversity within stx2-converting phages such as the tail protein
may influence its preference to phage receptor, or perhaps BamA is the primary stx2-converting phage receptor while the other two are less preferred.

2.3.3 stx2-converting phage, pathogenesis and toxin production

The stx2-converting phage plays a major role in the pathogenesis of O157 and Stx production. First, prophage induction was shown to be required for renal disease and lethality in vivo. By using an EDL933 derivative with a single mutation in the 933W prophage, the resulted mutant, EDL933clind1, had a noncleavable CI repressor and its prophage was uninducible (Tyler et al., 2013). When tested in vivo, the mouse group administrated with EDL933clind1 were healthy and produced negligible Stx2 while those ingested with wild type died. Second, the O157 strain colonization, acid tolerance, and mobility are greatly affected by stx2-converting phages. Xu et al. showed that the deletion of stx2-converting prophage resulted in a significant increase in T3SS and LEE1 expression by comparison with isogenic wild type EDL933. This indicated that stx2-converting phage regulated T3SS, and then coordinated O157 colonization (Xu et al., 2012). Moreover, the integration of stx2-converting phage into a nonpathogenic E. coli MG1655 host led to changes in host gene expression and enhanced its acid resistance (Su et al., 2010). Third, the total number of prophage in the O157 genome has a profound influence on its toxin production. It was shown that the existence of more than one stx2-converting phage in the same host background resulted in a significant change (either increase or decrease) in Stx2 production when comparing the scenario with only one prophage (Fogg et al., 2012; Serra-Moreno et al., 2008). However, apart from stx2-converting phage, the host background also modulates its Stx2 production. Significant differences were observed regarding induced phages and corresponding Stx2 production, when the same phage were lysogenized into two different nonpathogenic E. coli background (Iversen et al., 2015).
2.3.4 stx2a-converting phage subtyping

Subtyping methods based on phage diversity have been established. Ogura et al. studied a collection of ten O157 strains with single stx2-converting phage (either stx2a or stx2c) at different insertion sites. This study revealed that stx2c-converting phages were highly divergent from stx2a-converting phages, but homogeneous within the group. However, stx2a-converting phages possessed obvious intra-group variations in the replication protein. This characteristic was used for developing a two-step PCR-based subtyping method to differentiate them. Four subtypes (ø2a_α, β, γ and δ) were identified, with Sp5 from E. coli O157:H7 Sakai in group ø2a_α and 933W from EDL933 in ø2a_γ. However, some phages were untypeable, which shared the same replication protein but differed in the 5’-half of the O gene. This trait was utilized to classify them into two minor subtypes (φ Stx2a_ε, φ Stx2a_ζ). Furthermore, phage types were correlated with Stx2 production level: strains carrying ø2a_γ produced the highest amount of Stx2, and strains with ø2a_α was the second highest than other two types after antibiotic induction. Although more strains need to be included for a reliable analysis, strains with Stx2a_ζ seemed to produce more Stx2 than the other minor subtype. Twelve clade 8 strains sharing the same stx2a-converting phage insertion sites were investigated and consequently classified into two subclades: one was sub-clade 8a, including the spinach outbreak strain TW14359 carrying ø2a_γ; the other was sub-clade 8b with only ø2a_δ. In addition, strains in sub-clade 8a contained stx2c converting phage (Ogura et al., 2015).

Another phage subtyping method was proposed. Analysis of stx2a-converting phages from a Pennsylvania collection, including several clade 8 strains, successfully categorized 22 different O157:H7 strains into 9 phage sequence types (PST) based on phage insertion site, SNPs, copy number and orientation of IS 629. These nine PST were further classified into three clusters named
as PST1, PST2 and PST3. PST1, including Sp5 from Sakai, can be further divided into six minor clusters. PST2 was a notable cluster as it harbored two strains from clade 8 (PA2 and PA8). Moreover, strains in PST2 were grouped with hypervirulent non-O157 outbreak strains possessing a high HUS rate, including the 2011 German outbreak strain O104:H4 and the 2006 Norwegian outbreak strain O103:H2. This study also provided strong evidence to demonstrate the role of the host genetic background in toxin production by making use of naturally existing O157 strains. Within the cluster PST1-1, five O157 strains which had 100% identical phage DNA sequence and same phage insertion site were tested for their Stx2 levels after antibiotic induction. Significant differences of Stx2 productions were observed. However, the analysis of their genomes revealed that these five strains were phylogenetically separated (Yin. *et al.*, 2015). In conclusion, these phage subtyping studies shed light on using phage subtype for future prediction of O157 potential virulence. It also suggests that other factors such as host genome contribute to variations in Stx2 production in different O157 strains.

The diversity within stx2-converting phages has been revealed in multiple aspects, including morphology, genome size, sequence, insertion sites and phage receptors. The established phage subtyping methods, therefore, provides a new perspective to study phage diversity and its relation to Stx production.

2.4 *E. coli* O157:H7 phylogeny and its implication

Both epidemiological and genetic studies have revealed noteworthy chromosomal heterogeneity and diversity in expression of virulence factors among different O157 strains (Boerlin *et al.*, 1999; Stanton *et al.*, 2014). Multiple subtyping methods have been developed to help understand the phylogeny and pathogenesis of O157 strains.
2.4.1 Lineage classification

Octamer-based genome scanning (OBGS) and lineage-specific polymorphism assay (LSPA) were able to divide O157 strains into epidemiologically relevant groups. OBGS used the over-represented, strain-based octamer as primers and compared the size differences of genomic segments lying between them, while LSPA was a simplified alternative of OBGS by looking at only six loci and comparing the PCR patterns. The OBGS divided O157 strains into two lineages, I and II; however, the LSPA with a relatively better resolution further identified the third lineage, I/II (Kim et al., 1999; Yang et al., 2004). These three lineages varied in isolate origin, association with human disease as well as toxin production. Lineage I has been commonly associated with O157 human clinical isolates, lineage II are predominately bovine origin and rarely cause human disease. Lineage I/II which share the characteristics of the two previous identified lineages, are frequently related with human infection and contained a hyper-virulent cluster composing of a 2006 multi-state spinach outbreak strain (Kim et al., 1999; Laing et al., 2009; Yang et al., 2004; Zhang et al., 2007). Additionally, strains within lineage I and I/II produced significantly more Stx2 than lineage II strains (Zhang et al., 2010).

2.4.2 Clade subtyping

Later, a new typing method built on 96 SNPs within O157 loci was proposed and gave a refined classification. It divided over five hundred clinical O157 strains into nine clades, with Sakai in clade 1, EDL933 in clade 3, and the spinach outbreak strain TW14369 in clade 8. Moreover, the subpopulation of clade 8 has raised a great attention for its strong association with a high hospitalization and HUS rate, and more frequently carried only stx2a or both stx2a and stx2c rather than stxl and stx2a gene. The parallel genomic comparisons among Sakai (clade 1), EDL933
(clade 3) and TW14369 (clade 8) revealed the Sakai genome was relatively similar to EDL933’s, but quite different from TW14369’s (Manning et al., 2008). The strain TW14369 seemed to possess unique genetic features such as two putative T3SS effector protein which might be crucial for the disease (Kulasekara et al., 2009). Three major virulence elements of O157 including the LEE, stx and pO157 were up-regulated in clade 8 strains relative to clade 2 strains (Abu-Ali et al., 2010). Strains in clade 8 were reported to have higher Stx2 expression than other clades (Abu-Ali et al., 2010; Neupane et al., 2011). However, the number of clade 8 strains was limited in those studies, besides one of them showed a very low level of Stx2 production. Thus, it is not exclusive for clade 8 strains to have high Stx2 production. A recent study which focused on the Stx2 level after ciprofloxacin induction of a collection of clade 8 strains corroborated this (Goswami et al., 2015).

### 2.4.3 Whole-genome sequencing typing

The recent application of whole-genome sequencing (WGS) has provided a high-resolution phylogenomic subtyping method for studying O157 strains, providing concordant results with the epidemiological data. Eppinger et al. have established an SNP panel after studying the core genes of 16 E. coli O157:H7 genomes derived from three 2006 outbreaks related with fresh produce. Based on this panel which consisted of 1,225 SNPs, it was possible to cluster these outbreak strains into different branches, matching with metadata analyzed by either the lineage classification or clade typing. This method was further validated by testing 229 O157 strains after screening selected 19 canonical SNPs, showing its power to group closely related strains with high phylogenetic accuracy and resolution. The information obtained from WGS also unveiled the plasticity of the O157 genomes, which were attributed to lateral acquired genomic islands and prophages, especially stx-converting phage. Moreover, the IS within stx-converting phages were
speculated to be the drive for its prophage microevolution. Some of the SNPs were found to account for distinct phenotypes observed in the Phenotypic Biolog characterization. This study predicted a combination of the genomic and metabolic information would be useful if further differentiation of the closely related strains was needed (Eppinger et al., 2011).

The promising potential for WGS used for O157 outbreak detection and epidemiological surveillance is also endorsed by another research group. Holmes et al. used WGS to investigate a collection of 105 O157 strains isolated over a five-year period from human fecal samples in Scotland. A total of 8,721 SNPs were characterized in the core genomes, with 40% of them ascribed to six atypical strains. Based on their SNP profile, 81 genotypes were identified and further classified into different phylogenetic groups, which were consistent with epidemiological data obtained after using multiple-locus variable number tandem repeat analysis (MLVA). Three or fewer SNP differences were observed within the same epidemiological group. The authors pointed out that the WGS data added more to reveal the prevalence of genes responsible for virulence and antibiotic resistance, providing an alternative to current routine laboratory testing (Holmes et al., 2015).

The phylogenetic relationships among _E. coli_ O157: H7 strains can be revealed by multiple subtyping methods. The emergence of WGS based subtyping method provides high discriminatory power as well as a deeper analysis of virulence factors including horizontally acquired genes and antibiotic resistances.

2.5 The interplay between _E. coli_ O157: H7 and nonpathogenic _E. coli_

The severity of diseases caused by foodborne pathogen _E. coli_ O157:H7 varies, from watery diarrhea, to severe bloody diarrhea, and even to deadly HUS. Highly variable human gut flora and
their ability to amplify Stx when encountering O157 are suspected to be one potential factor in this variable response.

Gamage et al. demonstrated that the susceptibility of nonpathogenic *E. coli* to *stx*-converting phages was the reason for the Stx2 amplification observed in co-culture of either pure 933W or its lysogen with laboratory nonpathogenic strain *E. coli* C600. Then, a collection of *E. coli* isolates from healthy individuals were tested for their susceptibilities to *stx*-converting phages; 4 out of 37 showed toxin amplification. Therefore, 10% of commensal *E. coli* isolates are estimated to amplify toxin production by *E. coli* O157:H7 and a model of how normal intestinal *E. coli* on the production of Stx2 by *E. coli* O157:H7 was proposed (Gamage et al., 2003).

The same research group also tested whether the phenotype of toxin amplification in co-culture could be observed in streptomycin-treated mice using a clinical O157 isolate and three commensal *E. coli* isolates with different characteristics (phage-resistant, phage-susceptible or toxin-neutralizing). Although the Stx level in mice co-colonized with phage-susceptible strain and O157 did not differ significantly from those only administered O157, the colonization of O157 in co-cultured mice was 50-fold reduced, indicating that the commensal *E. coli* may enhance toxin production. Meanwhile, the production of autoinducer, which mediated quorum sensing of *E. coli*, as well as colicin production from the commensal *E. coli* were tested, and neither of them were present (Gamage et al., 2006).

Moreover, the commensal *E. coli* host range for different *stx*-converting phages varied. A collection of human origin *E. coli* isolates from either healthy individuals or patients treated with antibiotics but not infected with O157 was tested. It showed that 933W lysogenized more commensal *E. coli* strains than ØPT32. In addition, only a small percentage of resulting lysogen
showed toxin amplification, the Stx level of which differed even when co-cultured with the same phage (Gamage et al., 2004). This implies that the interplay of O157 and commensal *E. coli* and resulted toxin amplification phenotype may be strain dependent.

A recent study from our lab not only corroborated but also expanded Gamage’s model, by showing that Stx2a amplification occurs but the levels vary among three distinct O157 strains (Sakai, EDL933 and PA2) when co-cultured with nonpathogenic *E. coli* C600. The Stx2a level for Sakai in co-culture was similar to that observed in its monoculture. However, both EDL933 and PA2 produced significantly higher Stx2a in the co-cultures with C600. Interestingly, PA2 which belonged to the hypervirulent clade 8, was the the lowest Stx2a producer in monoculture but the highest toxin producer in co-culture with C600. This was confirmed *in vivo*, where germ-free mice receiving both PA2 and C600 showed more severe signs of kidney damage and higher mortality which were probably due to higher Stx2a production. However, all mice that only ingested the pathogen were visibly healthy. Strains from clade 8 with different *stx2* allelic type were co-incubated with nonpathogenic *E. coli* C600; however, only one of them PA8 belonging to PST2 showed Stx2a amplification, indicating Stx2a amplification in co-culture was not exclusive to clade 8 (Goswami et al., 2015). This study adds to understand the strain-dependent virulence of O157 and its interaction with nonpathogenic *E. coli*.

However, it is possible that other mechanisms besides the phage-mediated one may account for the toxin amplification phenotype. Colicinogenic bacteria which produced the colicin with DNase activity showed Stx amplification when co-incubating with O157 strain. The Stx2 levels in co-culture greatly depended on the dose of the DNase colicin. At certain extent, this colicin was able to enhance the expression of SOS responses related genes, acting similar as phage inducing agent, mitomycin C (Toshima et al., 2007). In addition, the commensal *E. coli* may contribute to
the pathogenicity of O157 not directly through toxin amplification phenotype in co-culture, but by an overexpression of the Stx receptor. Mice were more susceptible to Stx due to the enhanced level of Gb3, the latter of which was associated with an obvious increase in butyrate level after consuming a high fiber diet than a low fiber diet (Zumbrun et al., 2013). Thus, the factor such as metabolites of commensal E. coli may also lead to the enhanced pathogenicity of O157 in co-culture. In summary, current evidence on the mechanisms for toxin amplification phenotype observed for E. coli O157:H7 co-cultured with nonpathogenic E. coli strains is limited by using laboratory strain and the lack of genetic evidence for supporting the previously proposed phage mediated lysis model.

2.6 Conclusion

The foodborne pathogen E. coli O157:H7 raise a great public health concern. Numerous O157 strains have been isolated from cattle, bovine origin food, environment and infected patients, and show strain diversity. Those can produce Stx1, Stx2 or both, yet allelic types like Stx2a or Stx2c are most related to severe disease. As the Stx is essential for fatal HUS development, accurate measurement of Stx2 production by E. coli O157:H7 is necessary. Current widely used ELISA-based methods are constrained in its quantitative ability and expense. Therefore, a quantitative ELISA method which takes affordability, operability, reliability into consideration is needed, and its occurrence will enable future study for more Stx2a quantification and comparison.

That the Stx2 amplification of O157 strains can be enhanced by nonpathogenic E. coli strains has attracted much attention. The exploration of the mechanisms for this phenotype may help explain the O157 strain-level virulence difference and why individual symptoms vary from
asymptomatic to deadly HUS even when infected by the same O157 strain. It appears that both the phage diversity and the interaction of O157 with gut microbiota play important roles in it. Current evidence for a phage-mediated model for explaining toxin amplification are limited in that they were developed through studies with laboratory strains of nonpathogenic E. coli. However, it is still unknown whether the same mechanism will happen with human commensal E. coli. Therefore, investigation of a specific O157 strain and its interaction with different commensal E. coli strains will help to elucidate mechanisms.

Therefore, the objectives of the thesis are: (1) in Chapter 3, describing the development of a quantitative R-ELISA for Stx2 by using only commercially available reagents, and it application in measuring and comparing Stx2a production in a collection of antibiotic induced O157 strains; (2) in Chapter 4, investigating the mechanisms involved in enhanced Stx2a production by E. coli O157:H7 in the presence of different nonpathogenic E. coli.

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Chapter 3 A Quantitative Enzyme-Linked Immunosorbent Assay for Shiga toxin 2a Using Commercially Available Reagents

ABSTRACT

Immunoassays are the widely used methods of quantifying Shiga toxin 2 (Stx2) production by Shiga toxin (Stx)-producing *Escherichia coli* (STEC). We successfully developed a sensitive and specific receptor-based ELISA (R-ELISA) by using all commercially available agents. Hydroxyl acyl ceramide trihexoside, an analogue of Stx2 receptor globotriaosylceramide (Gb3), was used for antigen capture, and we describe several critical steps that must be adhered to ensure repeatability. No cross-reactivity was observed in this assay with Stx1, and linear curves could be constructed using bacterial lysate from an *E. coli* O157:H7 strain producing high levels of Stx2a. Using this method, we quantified Stx2 production by a collection of *E. coli* O157:H7 strains, reporting that toxin production does not necessarily correlate with strain phylogeny.
3.1. Introduction

STEC cause gastrointestinal diseases such as hemorrhagic colitis (HC), and in some cases this develops into the life-threatening hemolytic uremic syndrome (HUS) (Tarr et al. 2005; Scheiring et al. 2008). *E. coli* O157:H7 is a genetically heterogeneous serotype of STEC, responsible for most of the known STEC outbreaks across the globe (Heiman et al. 2015; Locking et al. 2011). Genetic methods, most commonly the lineage specific polymorphism assay (LSPA) (Kim et al., 1999) and clade typing (Manning et al., 2008), have been used to separate isolates by virulence potential and ecology. For example, human isolates are more commonly classified as lineage I and I/II than lineage II (Hartzell et al. 2011; Mellor et al. 2013), and it has been argued that isolates from clades 6 and 8 are more virulent than isolates from other clades (Manning et al. 2008, Iyoda et al. 2014 and Amigo et al. 2015).

Many virulence factors contribute to the pathogenesis of *E. coli* O157:H7 including Stx (Karch et al. 1987; Donnenberg et al. 1993). This is an AB\_5 toxin, comprised of a single A subunit associated with five identical B subunits (Mauro and Koudelka, 2011; Fraser et al. 2004). While the pentamer binds to Gb3 located in the host cell membrane (Waddell et al., 1990), the A subunit functions as a glycosidase, cleaving an adenine nucleotide from 28S rRNA within the 60S subunit. This results in inhibition of protein synthesis, followed by necrosis and cell death (Schüller, 2011). There are two immunologically distinct isoforms of the toxin, designated Stx1 and Stx2, which share 56.8% amino acid identity (Tesh and O’Brien, 1991). Stx2 has a lower affinity for Gb3 than Stx1 (Function et al., 1991), however, Stx2 is more toxic in animal models (Rutjes et al., 2002; Siegler et al., 2003; Tesh et al., 1993) and associated with more severe clinical cases (Kawano et al., 2008). Among the seven subtypes of Stx2 (from Stx2a to Stx2g) (Scheutz et al., 2012), Stx2a and Stx2c are more often associated with strains causing
HUS (Friedrich et al., 2002; Persson et al., 2007). In this manuscript, we will use "Stx2" when collectively referring to its subtypes.

Stx2 producing strains are correlated with the development of HUS (Boerlin et al., 1999) (Ethelberg et al., 2004), leading to the hypothesis that high toxin-producing strains are more likely to cause severe disease symptoms. Several studies have concluded, for example, that lineage I and I/II isolates produce more Stx2 than lineage II isolates (Zhang et al., 2010), and that clade 8 isolates are high toxin producers compared to isolates from other clades (Abu-Ali et al. 2010; Neupane et al. 2011). Most studies measure Stx2 production by semi-quantitative commercial kits (Amigo et al., 2015), qPCR/microarray (Amigo et al. 2015, Abu-Ali et al. 2010; Neupane et al. 2011), or by semi-quantitative western blots (Neupane et al., 2011). Following mRNA quantification, several studies have reported that stx2 transcript levels do not always correlate with Stx2 toxin production (Amigo et al. 2015, Neupane et al. 2011; Yin et al. 2015). Thus, immunoassays are preferred methods for quantifying Stx production.

Several immunologic methods were previously described that use either immobilized antibodies specific for Stx (Ball et al., 1996; He et al. 2013), or a receptor mimic (Ashkenazi and Cleary, 1989; Acheson et al., 1993; Basta et al., 1989) as the antigen capture in the sandwich ELISA. The disadvantage for the former is that it requires two sets of anti-Stx2 antibody from different species for both antigen capture and detection steps, while the latter assay only needs one set of anti-Stx2 antibody for detection. Most commercially available anti-Stx2 antibodies are monoclonal and generated from mice, so we suggest that one drawback preventing most research laboratories from quantifying Stx2 by sandwich ELISA is the limited commercially availability of antibodies from other species. Although commercial kits such as Premier® EHEC has been developed for detecting Stx, they are not designed to be specifically for Stx2 quantification,
because they can not differentiate Stx1 from Stx2. Its high cost also prohibits most laboratories from routinely using it for this purpose. Moreover, previously reported sandwich ELISA by using Gb3 as antigen capture was not developed to be quantitative for Stx2. Therefore, we were motivated to develop a cheap and reliable assay specifically for quantifying Stx2a that required only one set of anti-Stx2a antibody. Our initial attempts to develop a R-ELISA met with several difficulties in consistency and repeatability, an issue echoed in a previous publication (Basta et al., 1989). We suggest here that development of a robust assay would require identifying and standardizing critical steps of this assay, so it can be successfully applied to quantify Stx2a.

3.2 Results

3.2.1 Assay optimization

We started to develop our R-ELISA based on the traditional sandwich ELISA. Although polyclonal anti-Stx2 antibody from rabbit (BEI resource, Manassas, VA) was tried as the antigen capture in our initial attempt, it gave very high background noise. Therefore, we replaced the capture antibody with a Gb3 chemical analogue, hydroxyl acyl ceramide trihexoside (CTH) (Figure 3.1). Mouse anti-Stx2 A subunit monoclonal antibody (Santa Cruz Biotech, Santa Cruz, CA) was selected as the primary antibody. As a result, we were able to reduce the background noise, by giving optical density at 450 nm (OD\textsubscript{450}) less than 0.1 when measuring the negative control.

Using previous publications (Ashkenazi and Cleary, 1989; Basta et al. 1989) as guidelines, several critical steps which were not explicitly stated in those manuscripts were identified and further improved to make our assay robust and quantitative. First, although several Gb3 receptor mimics are available (CTH is available as hydroxyl acyl, non-hydroxyl acyl and lyso-
derivatives), we found that hydroxyl acyl CTH was best at capturing Stx2a at room temperature. Second, although the supplier recommended methanol as the solvent, we found that dissolving hydroxyl acyl CTH first in chloroform: methanol (2:1), and further diluting in methanol to the working concentration gave the best results. Third, we used detachable eight-well polystyrene strip plates instead of traditional 96-well plates. This minimized edge effects, a common observation with ELISA where peripheral wells give a higher absorbance than central wells. Fourth, the coating of R-ELISA plate wells with hydroxyl acyl CTH needs to be performed as rapidly as possible, so evaporation of methanol occurs evenly across the wells. Last, each of the washing steps used to remove the secondary antibody was extended to five minutes to minimize background noise.
**Figure 3.1:** Schematic of R-ELISA used to detect Stx2. The Stx2 capture hydroxyl acyl CTH is added to the detachable eight-well polystyrene strip plate, to which antigen Stx2 specifically binds. Primary antibody binds specifically to Stx2. A horseradish peroxidase (HRP)-conjugated secondary antibody is added, and a positive reaction is detected by a color change after addition of 3,3',5,5' tetramethylbenzidine (TMB).

### 3.2.2 Specificity and sensitivity of Stx2 specific R-ELISA

The specificity of this assay was first tested by using bacteria lysate of Stx1-producing strain previously designated PA24 (Hartzell *et al.*, 2011). The absorbance at 450 nm (A$_{450}$) for bacteria lysate from PA24 is routinely less than 0.1, indicating that no cross-reactivity was observed in this assay with Stx1. As pure Stx2a from BEI resource is available in quite limited quantity, we decided to use lysates from a high Stx2a-producing strain previously designated PA11 (Hartzell *et al.*, 2011) as the toxin source for standard curves. The Stx2a concentration in PA11 supernatants after ciprofloxacin induction was first quantified by using known concentration of pure Stx2a and around 23.2 µg/mL. We routinely obtained a linear range between an A$_{450}$ of 0.3 to 3.2, corresponding to a Stx2a concentration from 23 ng/mL to 363 ng/mL (Figure 3.2).
**Figure 3.2:** Linear curves generated using bacteria lysate supernatant from *E. coli* O157:H7 strain PA11 in R-ELISA. Two-fold dilutions were assayed using R-ELISA, revealing the linear ranges of the assay. The linear equation of the best fit line and R-square are shown.

### 3.2.3 Application of Stx2 specific R-ELISA to quantify toxin in bacteria lysates

This assay was designed specifically for Stx2a. Although Stx2a and Stx2c were reported to vary in their affinities to Gb3 *in vitro*, we expected this assay to detect Stx2c because the primary antibody used targets at the A subunit of Stx2, which is conserved between the two subtypes (Scheutz *et al.* 2012; Strauch *et al.* 2004). Therefore, we next evaluated whether this assay could be applied to measure the Stx2 levels in bacteria lysates from phylogenetically distinct strains. Thirteen strains from six of the known nine clades (Table 3.1) were chosen. These strains consisted of four defined Stx-allelic types: Stx2a only, Stx2c only, Stx1 & 2c and Stx2a & 2c. One strain (PA7) was previously reported (Hartzell *et al.*, 2011) to be stx1- and stx2 negative by PCR, although it was toxin-positive using Premier® EHEC that simultaneously detected both isoforms; and another strain (PA48) could not be categorized by lineage subtype method. We quantified Stx2 in eleven of the thirteen strains (Figure 3.3), with strains PA7 and PA48 under the detection limit. Therefore, this assay could be applied to detect Stx2c levels in bacteria lysates of strains harboring either Stx2c (PA38, 40) or both Stx2c and Stx1 (PA22, 41).

Among them, the highest producer (PA39) had approximately five times more Stx2 production than the lowest strain (PA5). Strains sharing the same lineage, clade or Stx allelic type showed varied Stx2 levels as well. For example, strains PA32, 49 from lineage I produced similar amounts of Stx2 as PA31, but significantly more than lineage I strain PA5. Likewise, among the three strains in clade 8, the Stx2 level in strain PA39 was similar to PA47, however, significantly higher than PA35. Lastly, among strains producing both Stx1 and Stx2a, strain...
PA49 produced significantly higher amounts of toxin than strain PA5, but was not different from PA31.

**Figure 3.3:** Quantification of Stx2 production by *E. coli* O157:H7 strains from various lineages, clades, and toxin profiles. The lineage or clade type is shown below the strain designation, while the Stx profile is differentiated by color. Data is reported as the average concentration ± one standard error. Means that do not share a common letter (a, b, c) are significantly different (Tukey’s test, *p* < 0.05).

### 3.3 Materials and methods

#### 3.3.1 Strains and culture conditions

The fourteen *E. coli* O157:H7 strains (Table 3.1) used in this study were obtained from the Pennsylvania Department of Health and previously characterized by our lab (Hartzell *et al.*, 2011). They were stored in 10% glycerol at -80 °C.
3.3.2 Ciprofloxacin induction and bacteria lysate collection

Each strain was propagated in Luria Broth (LB) at 37 °C with shaking overnight. Cells were diluted to OD\textsubscript{600} of 0.05 in fresh LB broth. To induce toxin expression, ciprofloxacin was added to a sublethal concentration of 45 ng/mL. After an eight-hour incubation at 37 °C with shaking, the culture was centrifuged at 4,000 g for 10 min, and supernatants were filtered through 0.2 µM cellulose acetate filters (VWR, Radnor, PA).

Table 3.1. Characteristics summary of STEC strains used in this study

<table>
<thead>
<tr>
<th>Clade type</th>
<th>Toxin profile</th>
<th>Strain name</th>
<th>Lineage type*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clade 2</td>
<td>Stx2a</td>
<td>PA 32</td>
<td>lineage I</td>
</tr>
<tr>
<td>Clade 2</td>
<td>Stx1 &amp; 2a</td>
<td>PA 5, 31, 49</td>
<td>lineage I</td>
</tr>
<tr>
<td>Clade 5</td>
<td>Stx1 &amp; 2c</td>
<td>PA 22</td>
<td>lineage I/II</td>
</tr>
<tr>
<td>Clade 6</td>
<td>Stx2c</td>
<td>PA 38</td>
<td>lineage I/II</td>
</tr>
<tr>
<td>Clade 7</td>
<td>Stx2c</td>
<td>PA 40</td>
<td>lineage II</td>
</tr>
<tr>
<td>Clade 7</td>
<td>Stx1 &amp; 2c</td>
<td>PA 41</td>
<td>lineage II</td>
</tr>
<tr>
<td>Clade 8</td>
<td>Stx1</td>
<td>PA 24</td>
<td>lineage I/II</td>
</tr>
<tr>
<td>Clade 8</td>
<td>Stx2a &amp; 2c</td>
<td>PA 35, 39, 47</td>
<td>lineage I/II</td>
</tr>
<tr>
<td>Clade 9</td>
<td>Stx1 &amp; 2c</td>
<td>PA 48</td>
<td>Undefined</td>
</tr>
<tr>
<td>Unclassified</td>
<td>Unknown</td>
<td>PA 7</td>
<td>lineage I</td>
</tr>
</tbody>
</table>

* The lineage and Stx profiles of all strains were previously reported by Hartzell et. al (Hartzell et al., 2011).

3.3.3 Stx2 specific R-ELISA

A 0.5 mg/ml stock solution of hydroxyl acyl CTH (Matreya Biosciences, Pleasant Gap, PA) was prepared in chloroform: methanol (2:1, v/v). This stock solution was further diluted to 25 µg/mL in methanol, and was either used immediately or stored at -20°C. Frozen stocks were heated in a 55°C water bath to re-dissolve hydroxyl acyl CTH before use. To coat wells with antigen capture, a volume of 100 µL hydroxyl acyl CTH working solution was added to wells of an eight-well polystyrene strip plate (Thermo Scientific, Waltham, MA) in a chemical fume
hood. Strip plates were rotated manually every 15 minutes until the methanol was fully evaporated. Next, 200 µL blocking buffer [4% bovine serum albumin (BSA) in 0.01 M phosphate buffer saline (PBS) with 0.05% Tween20] was added to each well and incubated at 4 °C for 16 hours. After removing blocking buffer, 200 µL washing buffer (0.01M PBS with 0.05% Tween20) was added to each well and incubated for one minute on a shaking platform. This step was repeated for a total of five washes.

The R-ELISA assay was initiated by adding 100 µL sample into each well, followed by shaking at room temperature for one hour. Wells were washed five times in PBS/Tween20 as described above. Next, 100 µL mouse anti-Stx2 antibody (Santa Cruz Biotech, Santa Cruz CA) was diluted in blocking buffer to 1 µg/mL, added to each well, and incubated with shaking at room temperature for one hour. Wells were again washed five times with PBS/Tween20. Goat anti–mouse HRP conjugated secondary antibody was diluted in the blocking buffer (1 µg/mL, 100 µL) and added to each well, and incubated with shaking at room temperature for one hour. Five washes in PBS/Tween20 followed, however, this time each wash was performed for five minutes. Detection was accomplished using 1-Step Ultra TMB (Thermo-Fischer, Waltham, MA), which was equilibrated to room temperature in a foil-wrapped tube for at least 30 minutes prior to use. TMB substrate (100 µL) was added to each well and incubated with shaking for 10 minutes to allow for color development. Finally, 100 µL of stop solution (2 M H₂SO₄) was added to each well, followed by shaking for 30 seconds. The reading values of A₄₅₀ were obtained using a DU® 730 spectrophotometer (Beckman Coulter, Atlanta, GA).

For all assays, supernatants from E. coli O157:H7 strain PA24, which produces only Stx1, were used for the negative control, and a lysate from a E. coli O157:H7 strain, PA11, served as the positive control. The Stx2a concentration for PA11 stock was quantified by known
concentration of pure Stx2 (BEI Resources, Manassas, VA). The standard curves in R-ELISA were generated using two-fold serially diluted PA11 lysate in PBS. Any OD$_{450}$ above 0.1 was considered as positive. Total protein in each unknown sample was measured by the Bradford assay (VMR Life Science, Philadelphia, PA), following the manufacturer's recommended protocol. Stx2 quantities were reported as µg Stx2/mg total protein.

### 3.3.4 Statistical analysis

All data were reported as the mean from three biological replicates and each sample had three technical repeats. The mean and standard error were calculated in MS Excel. Data were analyzed using linear regression and one-way analysis of variance (ANOVA) in Minitab version 17 (Minitab Inc., State College, PA).

### 3.4 Discussion

We have developed a robust assay for the quantification for Stx2a. This assay is expected to detect Stx2c as well. Although Gb3 have been used previously in immunoassays to capture Stx (Basta *et al.* 1989, Acheson *et al.* 1993 and Togashi *et al.* 2015), we identified several steps that were critical to follow in order to achieve repeatable results. In addition, there are three other modifications from the literature worth highlighting. First, it was reported that the lyso form of Gb3 provided more reliable Stx1 detection, presumably because it is less hydrophobic (Basta *et al.*, 1989). However, during assay development we found that lyso-CTH was less effective at capturing Stx2a than hydroxyl acyl CTH. This could simply reflect differences in affinity of Stx1 and Stx2a for CTH derivatives. Secondly, another group previously reported that they could improve R-ELISA sensitivity by increasing the amount of receptor used to coat wells (Basta *et al.*, 1989). While we saw similar results in initial trials, we decided that 25 µg/ml hydroxyl acyl
CTH was the optimal concentration for detecting Stx2a. Under this concentration, we were able to detect Stx2a as low as 23 ng/mL which met our needs. Moreover, two 96 well R-ELISA plates can be prepared from one vial of commercial available CTH (0.5 mg), instead of one 96 well plate if a higher concentration was used. Lastly, it was reported that the presence of cholesterol alone or along with lecithin caused statistically significant increases in the binding of Gb3 to Stx2 (Gallegos et al., 2012). However, no enhancement of Stx2a capture was found when combining these with hydroxyl acyl CTH during the coating step.

Using R-ELISA, we were able to quantify toxin levels from various E. coli O157:H7 strains, and obtained results that were both confirmed and contrasted with those previously reported. First, we did not notice a correlation between Stx2 production and lineage. It was previously reported that E. coli O157:H7 isolates from lineage II produced less Stx2 than those from lineage I and I/II (Zhang et al., 2010), however, our findings show that the Stx2 levels in strains from lineage II (PA40, 41) were not significantly different from strains belonging to lineage I (PA5, 31, 32, 49) or lineage I/II (PA22, 35, 38, 47). Second, although strains from clade 8 were suggested to produce higher Stx2 levels than isolates from other clades (Abu-Ali et al. 2010; Neupane et al. 2011), our data indicates this is not universal. In our study, clade 8 strain PA35 produced similar amounts of Stx2 as others clades, including clade 2 (PA31), clade 5 (22), clade 6 (PA38) and clade 7 (PA40, 41). Lastly, it was previously suggested that Stx2-only producing strains synthesized more toxin than Stx1/Stx2-producers, due to crosstalk between phage-encoded repressors encoded in different Shiga toxin-converting phages (Serra-Moreno et al., 2008). However, the Stx2a-only strain PA32 produced toxin level that was similar to most Stx1/Stx2-producing strains we tested. It appears likely that phylogeny or toxin profile alone does not explain differences in toxin levels and thus virulence differences observed among
strains. More factors such as colonization, adherence capabilities and other virulence genes, need to be considered as well.

Two strains, PA7 and PA48, produced Stx2 levels that were below the detectable limit using our R-ELISA. Strain PA48 is classified as clade 9, and was previously shown to be LSPA type 311111 (Hartzell et al., 2011). This strain is related to E. coli O157:H7 strain G5101 (Hayes et al., 1995), which is also LSPA 311111 (Liu et al., 2009) and is atypical among pathogens of this serotype in that it is β-glucuronidase-positive. Little is known about the toxin production levels of these strains. PA7 is a strain previously characterized (Hartzell et al., 2011) and identified as PCR-negative for both stx1 and stx2, however the commercial kit that simultaneously detects both subtypes identified this strain as Shiga toxin-positive. Our results here suggest the commercial assay was detecting Stx1, although it is possible that PA7 produces Stx2 level below the limit of detection of our R-ELISA.

While this assay is useful for quantifying Stx2a in the bacterial lysates, we have observed limitations when applying it to environmental samples. In a previous publication (Goswami et al., 2015), we were unsuccessful using this method to quantify toxin levels in cattle mucus, however we could accomplish this using the Premier EHEC kit sold by Meridian Biosciences. Therefore, this R-ELISA may not be practical at the moment for detecting toxin in complex biological matrixes, but is advantageous when one needs to quantify toxin from laboratory samples and it is impractical to generate or obtain antibodies needed for a traditional sandwich ELISA.
3.5 Conclusion

Our R-ELISA provides a reliable way to quantify Stx2a using commercially available components, and it can also be used for detecting Stx2c. The application of this assay can be expanded to other Stx2 subtype once corresponding pure toxin is available. It is a readily easy and cheap way to quantify toxin levels especially for groups that currently assess toxin production by qPCR or other transcription-based methods. We predict a similar quantitative assay can be developed to detect Stx1, although as stated above this may require selecting a different receptor mimic.

3.6 Acknowledgments

We thank Joselyn N. Allen (Pennsylvania State University) for creating the scientific illustration for R-ELISA in this publication; Hillary M. Figler for proofreading this manuscript; Kakolie Goswami and Matreya LLC for technical assistance. This work was funded by USDA-NIFA grant 2010-65201-20619.

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ABSTRACT

Intestinal colonization by the foodborne pathogen *E. coli* O157:H7 causes serious disease symptoms, including bloody diarrhea and severe abdominal cramps. The disease can further develop into hemolytic uremic syndrome (HUS) and hemorrhagic colitis (HC). Synthesis of one or more Shiga toxins (Stx) is essential for HUS and HC development. The genes encoding Stx, including Stx2a, are encoded by a lambdoid prophage in the *E. coli* O157:H7 chromosome. Enhanced Stx2a expression was reported when specific nonpathogenic *E. coli* strains were co-cultured with O157:H7, and it was hypothesized that this phenotype required the former to be sensitive to infection by the Shiga toxin-converting phage. We tested this hypothesis using a previously published method to replace *bamA* (an essential gene and Stx phage receptor) in nonpathogenic *E. coli* strains with the ortholog from *Salmonella enterica*. Such heterologous gene replacement abolished the ability of *E. coli* strain C600 to enhance toxin production when co-cultured with *E. coli* O157:H7 strain PA2, which belongs to the hypervirulent clade 8 cluster. Extracellular loops of BamA (loop 4, 6, 7) were further shown to be important for infection by Stx2a phage. However, gene replacement in other nonpathogenic *E. coli* strain revealed a *bamA*-independent mechanism for toxin amplification. Collectively, these data suggest that multiple mechanisms exist for commensal *E. coli* to increase Stx production when they coexist with *E. coli* O157:H7.
4.1 Introduction

*E. coli* O157:H7 is a well-recognized serotype of pathogen enterohaemorrhagic *E. coli* (EHEC), which has a high incidence (Rangel *et al.*, 2005) and pathogenicity, causing foodborne diseases (Mead, 1999) and leads to a spectrum of symptoms ranging from an asymptomatic carriage, bloody diarrhea, to fatal hemolytic uremic syndrome (HUS) (Tarr *et al.*, 2005). One of the prominent virulence factors of *E. coli* O157:H7 is Shiga toxin (Stx). Stx is an AB₅ toxin comprised of a single A subunit associated with a pentamer of identical B subunits. The B pentamer binds to globotriaosylceramide (Gb3) in host cell membranes (Waddell *et al.*, 1988), and delivers the A subunit into the cytoplasm. In the cytoplasm, the enzymatically active A subunit inhibits protein synthesis by cleaving an adenine nucleotide from 28S RNA within the 60S ribosomal subunit, preventing tRNA binding and protein synthesis, and leading to necrosis and cell death (Sandvig and van Deurs, 1992; Saxena *et al.*, 1989). There are two immunologically distinct Stx, designated Stx1 and Stx2. Stx1 shares 56% identity with Stx2 at the amino acid sequence level (Jackson *et al.*, 1987), but they differ in potencies. In animal models, Stx2 is 400-fold more potent than Stx1 (Tesh *et al.*, 1993), and is more likely to be associated with severe disease outcomes as well as the development of HUS (Kawano *et al.*, 2008). Within Stx2 allele, minor DNA sequence differences further classified them into seven variants, from Stx2a to Stx2g (Scheutz *et al.*, 2012). Epidemiological investigation showed that Stx2a and Stx2c are more frequently related to strains causing deadly HUS (Friedrich *et al.*, 2002; Persson *et al.*, 2007).

The genes encoding Stx are present in prophages (Perna *et al.*, 2001; Hayashi *et al.*, 2001). Prophage has two life cycles, lysogenic and lytic. In the lysogenic state, its genes are amplified along with the dividing bacteria without causing damage to the bacteria. However, SOS response
caused by DNA damaging agents such as antibiotics can trigger it to switch to the lytic state (Zhang et al., 2000). In the lytic state, the prophage excises from the chromosome of E. coli O157:H7, replicates using bacterial machinery, assembles new phage particles and gets released through cell lysis. The switch between the two cycles is controlled by the early regulatory genes. The repressor CI forms a dimer and inhibits transcription from the promoters P_L and P_R. Therefore, the downstream genes are not expressed in the lysogenic state. However, the CI repressor is cleaved when the SOS response is activated, resulting in downstream gene expression. With the production of antiterminator Q, the late promoter P_R' is activated, leading to phage induction, expression of Stx and ultimately bacterial cell lysis (Waldor and Friedman, 2005). During the stx-converting phages lytic infection, BamA (also name YaeT) serves as the phage receptor (Smith et al., 2007). The essential gene bamA, required for the outer membrane protein biogenesis (Wu et al., 2005), is highly conserved among Enterobacteriaceae (Smith et al., 2007). Within species, the extracellular loops of BamA has heterogeneity (Ruhe et al., 2013).

To assess the phylogenetic relationships of E. coli O157:H7 strains, a clade typing method was developed, to determine the relatedness between groups of isolates. Based on 96 single nucleotide polymorphisms (SNPs), more than five hundred E. coli O157:H7 strains were classified into nine distinct clades. Clade 6 and clade 8 have received greatest attention, as strains belong to these two clades were more frequently associated with HUS than other clades (Manning et al., 2008; Amigo et al., 2015). However, it is unclear what accounts for their potential high virulence. As stx-converting phages are critical for understanding toxin production and contribute to the plasticity of E. coli O157:H7 genome, multiple subtyping methods based on phage diversity have been established (Ogura et al., 2015; Yin et al. 2015). After analyzing 22 stx2a-converting phages from a Pennsylvania collection which included several clade 8 strains,
Yin et al. categorized them into three clusters consisted of nine phage sequence types (PST). The three clusters were designated as PST1, PST2 and PST3. Among them, PST2 was a notable cluster, as it not only harbored two clade 8 strains (PA2 and PA8), but also grouped with other non-O157 outbreak strains sharing high HUS rates, including 2011 German outbreak strain O104:H4 and 2006 Norwegian outbreak strain O103:H2 (Yin et al., 2015).

A previous study on E. coli O157:H7 pathogenesis proposed that phage-susceptible nonpathogenic E. coli strains infected with spontaneously induced stx2-converting phages were able to produce more Stx2a, thus leading to enhanced virulence. Ten percent of commensal E. coli isolates are estimated to be able to amplify the Stx production of E. coli O157:H7 in co-culture (Gamage et al., 2003). This was tested in vivo by the same research group. Although the Stx2a levels for mice administrated only E. coli O157:H7 were not significantly different from those co-colonized with both phage-susceptible E. coli strain and O157, the colonization of pathogen in the latter group was much less. It indicated that the commensal E. coli might enhance toxin production of E. coli O157:H7 through phage mediated lysis (Gamage et al., 2006). A recent study corroborated and expanded that model, showing that strain-specific differences among E. coli O157:H7, especially the stx2a-converting phage type, could impact the toxin amplification in co-cultures with laboratory nonpathogenic E. coli C600 (Goswami et al., 2015). Three E. coli O157: H7 strains, namely Sakai, EDL933 and PA2, were co-cultured with nonpathogenic strain E. coli C600 in vitro. Different levels of enhanced Stx2a production for PA2 and EDL933 were observed along with decreased cell counts of E. coli C600 in their co-cultures. However, Sakai did not show increased toxin production in co-culture. Interestingly, PA2 which belonged to the hypervirulent clade 8 was the lowest Stx2a producer in monoculture, but the highest toxin producer in co-culture with E. coli C600. The in vivo study showed that
germ free mouse group receiving both PA2 and C600 had more severe signs of kidney damage and higher mortality along with higher Stx2a levels in their recovered feces. At the same time, mice that ingested only the pathogen were visibly healthy. However, the phenotype of enhanced Stx2a production in co-cultures is not exclusive for all clade 8 strains. Only two of them which contained stx2a-converting phages clustered in PST2 were toxin amplifying strains. Therefore, this would add to our understanding of strain-dependent virulence of O157 as well as generated a compelling argument to reconsider the suitableness of using only the Stx2a level in O157 pure culture to reflect virulence potential.

As the natural resident in the mammalian gastrointestinal tract, E. coli colonizes to approximately $10^8$ organisms in healthy adults. They likely differ in the phage susceptibilities (Gamage et al., 2003) and the potential strategies they use to enhance Stx production in vitro (Toshima et al., 2007; Curtis et al., 2015). A further exploration of the interactions between E. coli O157:H7 and the commensal E. coli is needed to understand how the gut microbiome contribute to affect O157 virulence. Here we hypothesize for a single O157 strain, PA2, multiple mechanisms exist for explaining the enhanced Stx2a productions when co-culture with different commensal E. coli strains.

4.2 Results

4.2.1 Commensal E. coli isolates can contribute to Stx2a amplification of E. coli O157:H7 strain PA2 in co-cultures

As 10% of commensal E. coli are estimated to amplify O157 strain in co-culture (Gamage et al., 2003), we first tested this by using a collection of twelve commensal E. coli isolates and a laboratory strain E. coli C600. Like E. coli C600, four commensal E. coli isolates, namely E. coli
0.1229, 0.1231, 0.1282 and 1.1954, produced significantly more Stx2a in the co-cultures than that in PA2 monoculture after 16hr incubation ($p<0.05$) (Figure 4.1a). The baseline Stx2a produced by PA2 monoculture was $6.10 \pm 0.83 \, \mu g \, Stx2a/mg \, total \, protein$, and highest Stx2a level was in co-culture of PA2+C600, reaching to $95.62 \pm 8.11 \, \mu g \, Stx2a/mg \, total \, protein$. The co-cultures of four commensal *E. coli* isolates with PA2 had $33.72 \pm 1.20; 38.05 \pm 4.00; 91.2 \pm 11.0; 40.34 \pm 1.32 \, \mu g \, Stx2a/mg \, total \, protein$, respectively. These five Stx2a amplifiers showed two different competitive indexes after 16hr co-incubation. In the co-culture of PA2+C600, *E. coli* C600 was outcompeted by PA2, giving a mean cell density of less than 2.2% in the total population. However, the four commensal amplifiers were slightly more favored than PA2 in co-cultures, with average cell density above 60% (Figure 4.1b).

Figure 4.1a:
Figure 4.1b: 

**Figure 4.1:** Stx2a levels and percentage of nonpathogenic *E. coli* strains after co-incubation with *E. coli* O157:H7 strain PA2. PA2 was statically incubated at 37°C, either alone (pink) or with 13 nonpathogenic *E. coli* strains including the laboratory strain *E. coli* C600 (in red). Samples were harvested after 16 hours, R-ELISA (a) and plate counts (b) on sorbitol MacConkey were performed. Error bars were standard error of the mean from three biological replicates. One-way ANOVA was used and the Stx2a levels in co-cultures that marked with an asterisk were significantly higher than that in PA2 monoculture control (Dunnett’s test, *p* <0.05).

As the colicinogenic strain, RR1-E9, has been reported to produce E9 colicin which trigged Stx2 amplification of O157 strain (Toshima *et al.*, 2007), we examined if any stimulator was secreted in the supernatants by these five amplifying strains. As shown in Table 4.1, the Stx2a level for PA2 grown in fresh LB was statistically indistinguishable from those in spent supernatants of *E. coli* 0.1282, 0.1231, 1.1954 and C600 (*p*<0.05), while that of *E. coli* 0.1229 produced significantly more Stx2a. Thus, *E. coli* 0.1229 seems to produce an unknown stimulator for promoting Stx2a amplification. In order to exclude the possibility that commensal *E. coli* only showed the comparable growth profile as PA2 at a single time point of hour 16, their cell densities were monitored at different time points over the 16hr time course. All of them grew as comparable as PA2, giving the same magnitude of colony forming unit (CFU) at each time point (Figure 4.2).
Table 4.1: Fold differences in the Stx2a levels when PA2 grown in the spent supernatant of nonpathogenic *E. coli*

<table>
<thead>
<tr>
<th>O157:H7 Growth Media</th>
<th>PA2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spent supernatant (0.1229)</td>
<td>Saturated</td>
</tr>
<tr>
<td>Spent supernatant (0.1282)</td>
<td>1.6 (±0.3)</td>
</tr>
<tr>
<td>Spent supernatant (1.1954)</td>
<td>1.4 (±0.3)</td>
</tr>
<tr>
<td>Spent supernatant (0.1231)</td>
<td>1.2 (±0.3)</td>
</tr>
<tr>
<td>Spent supernatant (C600)</td>
<td>1.1 (±0.1)</td>
</tr>
<tr>
<td>Fresh LB broth</td>
<td>1</td>
</tr>
</tbody>
</table>

![Growth curve for PA2+0.1231](image)
Figure 4.2: One trial of the growth curves for PA2 and three commensal *E. coli* strains (*E. coli* 0.1282, 0.1231 and 1.1954) over 16hr time course in either monoculture or co-cultures.
4.2.2 The \textit{bamA}-dependent lytic phage infection of \textit{E. coli C600} is the mechanism for Stx2a amplification in co-culture of PA2 and \textit{E. coli C600}

\textit{E. coli C600} was shown to significantly increase Stx2a production of PA2 (Figure 4.1a) and had a much lower cell density than other commensal \textit{E. coli} strains in the co-culture (Figure 4.1b). Hence, we hypothesized that the \textit{stx2a}-converting phages from PA2 mediated lysis of \textit{E. coli C600} and led to more toxin production. A phage resistant strain for \textit{E. coli C600} was needed for testing our hypothesis. By using a similar approach developed previously (Ruhe \textit{et al.}, 2013), we deleted the chromosomal \textit{bamA} in \textit{E. coli C600} which was initially complemented with plasmid containing \textit{E. coli C600} \textit{bamA} (pZS21::\textit{bamA}\textit{\textsubscript{E. coli C600}}) (Figure 4.3a). Subsequently, three derivatives were generated by swapping pZS21-\textit{bamA}\textit{\textsubscript{E. coli C600}} with the plasmid containing \textit{bamA} from \textit{Enterobacter cloacae}, \textit{Salmonella Typhimurium} or \textit{Dickeya dadantii}, respectively (Figure 4.3a). They were named as C600K1 (C600Δ\textit{bamA}:\textit{cat} + pZS21::\textit{bamA}\textit{\textsubscript{E. cloacae}}), C600K2 (C600Δ\textit{bamA}:\textit{cat} + pZS21::\textit{bamA}\textit{\textsubscript{S. Typhimurium}}) and C600K3 (C600Δ\textit{bamA}:\textit{cat} + pZS21::\textit{bamA}\textit{\textsubscript{D. dadantii}}), and showed similar growth profiles as wild type \textit{E. coli C600} did (Figure 4.3b). Their \textit{stx2a}-converting phage susceptibilities were also examined. The wild type \textit{E. coli C600} formed an average of 7.16E+05 plaques. However, none of the three mutants formed any plaques, indicating that all of them were resistant to the \textit{stx2a}-converting phage infection (Figure 4.3c). Moreover, the Stx2a levels in their co-cultures with PA2 were as similar to PA2 monoculture of 1.83 ± 0.17 µg Stx2a/mg total protein, significantly less than that in PA2+C600 \textit{(p<0.05)} (Figure 4.3d). Overall, expression of heterologous \textit{bamA} in phage susceptible \textit{E. coli C600} rendered it resistant to \textit{stx2a}-converting phage infection, suggesting that \textit{bamA}-dependent lytic phage infection of \textit{E. coli C600} was the mechanism explaining enhanced toxin production by PA2 in co-culture.
Figure 4.3a: 

![Diagram](image1)

Figure 4.3b: 

![Diagram](image2)
Figure 4.3c: Schematic diagram for generation of bamA mutants for nonpathogenic E. coli strains (a); growth profiles (b); plaque counts (c) of the wild type E. coli C600 and its three bamA mutants; Stx2a levels (d) after co-incubating them with E. coli O157:H7 strain PA2. Pure PA2 stx2a-converting phages were used in a plaque assay to test their phage susceptibilities. Stx2a levels in PA2 alone or with three bamA mutants, namely C600K1 (C600ΔbamA::cat +pZS21::bamA<sub>Enterobacter cloacae</sub>), C600K2 (C600ΔbamA::cat+pZS21::bamA<sub>Salmonella Typhimurium</sub>), C600K3 (C600ΔbamA::cat +pZS21::bamA<sub>Dickeya dadantii</sub>), were measured by R-ELISA. Error bars were standard error of the mean from three biological replicates. One-way ANOVA was used and Stx2a levels in co-culture that marked with an asterisk were significantly higher than the PA2 monoculture control (Dunnett’s test, p < 0.05).
4.2.3 Extracellular loops of *E. coli* BamA were recognized by stx2a-converting phages during lytic infection of *E. coli* C600

The outer membrane protein, BamA, is highly conserved among species of *Enterobacteriaceae* shown by sequence alignments and topology prediction (Smith *et al*., 2007; Ruhe *et al*., 2013). Its membrane spanning regions shared the greatest conservation, with the lowest conservation located in its extracellular loops, especially loop 4, 6 and 7. Thus, extracellular loop polymorphism was hypothesized to determine the host strain’s phage susceptibility. Ten *bamA*\textsubscript{E.coli} loop variants for C600EE (C600\Delta bamA::cat+pZS21::bamA\textsubscript{E.coli}) was generated after plasmid exchange, and they were categorized into three types (listed in Table 4.2): two mutants with in-frame deletions of loop 4 or loop 6 of *bamA*\textsubscript{E.coli}; three variants with human influenza hemagglutinin (HA) epitope insertion in each loop; and the remaining five were chimeras, with individual loops in *bamA*\textsubscript{E.cloacae} being replaced by the corresponding ones in *bamA*\textsubscript{E.coli}.

Table 4.2: Description of the *bamA*\textsubscript{E.coli} loop variants used in this study

<table>
<thead>
<tr>
<th>Designation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>bamA</em>\textsubscript{A4}\textsubscript{E.coli}</td>
<td>delete residues Pro556–Asn563 within loop 4 of BamA\textsubscript{E.coli}</td>
</tr>
<tr>
<td><em>bamA</em>\textsubscript{A6}\textsubscript{E.coli}</td>
<td>deletes residues Phe675–Lys701 within loop 6 of BamA\textsubscript{E.coli}</td>
</tr>
<tr>
<td><em>bamA</em>HA\textsubscript{4}\textsubscript{E.coli}</td>
<td>introduces an HA epitope into extracellular loop 4 of BamA\textsubscript{E.coli}</td>
</tr>
<tr>
<td><em>bamA</em>HA\textsubscript{6}\textsubscript{E.coli}</td>
<td>introduces an HA epitope into extracellular loop 6 of BamA\textsubscript{E.coli}</td>
</tr>
<tr>
<td><em>bamA</em>HA\textsubscript{7}\textsubscript{E.coli}</td>
<td>introduces an HA epitope into extracellular loop 7 of BamA\textsubscript{E.coli}</td>
</tr>
<tr>
<td><em>bamA</em>Ec\textsubscript{4}\textsubscript{E.coli}</td>
<td>chimeric <em>bamA</em>\textsubscript{E.cloacae} in which the coding sequence for Asp550–Ala567 is replaced with Tyr550–Thr567 from <em>bamA</em>\textsubscript{E.coli}</td>
</tr>
<tr>
<td><em>bamA</em>Ec\textsubscript{6}\textsubscript{E.coli}</td>
<td>chimeric <em>bamA</em>\textsubscript{E.cloacae} in which the coding sequence for Tyr675–Ser693 is replaced with Phe675–Lys701 from <em>bamA</em>\textsubscript{E.coli}</td>
</tr>
<tr>
<td><em>bamA</em>Ec\textsubscript{7}\textsubscript{E.coli}</td>
<td>chimeric <em>bamA</em>\textsubscript{E.cloacae} in which the coding sequence for Ala739–Val752 is replaced with Thr747–Tyr757 from <em>bamA</em>\textsubscript{E.coli}</td>
</tr>
<tr>
<td><em>bamA</em>Ec\textsubscript{4/7}\textsubscript{E.coli}</td>
<td>chimeric <em>bamA</em>\textsubscript{E.cloacae} in which the coding sequence for Asp550–Ala567 and Ala739–Val752 is replaced with Tyr550–Thr567 and Thr747–Tyr757 from <em>bamA</em>\textsubscript{E.coli}</td>
</tr>
<tr>
<td><em>bamA</em>Ec\textsubscript{6/7}\textsubscript{E.coli}</td>
<td>chimeric <em>bamA</em>\textsubscript{E.cloacae} in which the coding sequence for Tyr675–Ser693 and Ala739–Val752 is replaced with Phe675–Lys701 and Thr747–Tyr757 from <em>bamA</em>\textsubscript{E.coli}</td>
</tr>
</tbody>
</table>
No plaques were formed when using \textit{E. coli} C600 variants expressing \textit{bam\textsubscript{A\textsubscript{4}} E. coli} and \textit{bam\textsubscript{A\textsubscript{6}} E. coli} as indicator strains in plaque assay (Table 4.3), suggesting that deletion of either loop 4 or loop 6 resulted in their resistances to \textit{stx2a}-converting phage infection. Similarly, the HA epitope insertion in either loop 4 or 7 of \textit{bam\textsubscript{A} E. coli} abolished its phage susceptibility. However, the mutant with HA epitope insertion in loop 6 was partially susceptible to phage infection by forming 4.40±4E PFU/ml plaques. Moreover, strains expressing chimeras containing \textit{bam\textsubscript{A\textsubscript{E. cloacae}4}}, \textit{bam\textsubscript{A\textsubscript{E. cloacae}6}}, \textit{bam\textsubscript{A\textsubscript{E. cloacae}7}}, or \textit{bam\textsubscript{A\textsubscript{E. cloacae}4/7}} \textit{E. cloacae} were completely resistant to phage infection, which was as the same as the C600K1 expressing intact \textit{bam\textsubscript{A}} from \textit{E. cloacae}. This indicated that neither a single \textit{E. coli} extracellular loop nor combination of loop 4 and 7 of \textit{bam\textsubscript{A} E. coli} was sufficient for the phage resistant \textit{bam\textsubscript{A} E. cloacae} to become phage susceptible. However, one chimera with \textit{bam\textsubscript{A\textsubscript{E. cloacae}6/7}} \textit{E. cloacae} partially restored its susceptibility to phage infection by forming 2.00±4E PFU/mL plaques, approximately one fourth of the virions produced by strains harboring wild type \textit{bam\textsubscript{A} E. coli} either in the chromosome (C600) or plasmid (C600EE), implying that \textit{E. coli} loop 6 and 7 together played an important role in phage lytic infection.

\textbf{Table 4.3}: Plaque counts for using different \textit{E. coli} C600 \textit{bam\textsubscript{A}} derivatives as host strain

<table>
<thead>
<tr>
<th>Host strain</th>
<th>Genotype</th>
<th>Number of plaques</th>
</tr>
</thead>
<tbody>
<tr>
<td>C600</td>
<td></td>
<td>90000</td>
</tr>
<tr>
<td>C600EE</td>
<td>C600\textit{Δbam\textsubscript{A}}::\textit{cat} +pZS21::\textit{bam\textsubscript{A\textsubscript{E. coli}}}</td>
<td>87000</td>
</tr>
<tr>
<td>C00K1</td>
<td>C600\textit{Δbam\textsubscript{A}}::\textit{cat} +pZS21::\textit{bam\textsubscript{A\textsubscript{E. cloacae}}}</td>
<td>0</td>
</tr>
<tr>
<td>C600S1</td>
<td>C600\textit{Δbam\textsubscript{A}}::\textit{cat} +pZS21-\textit{bam\textsubscript{A\textsubscript{A\textsubscript{E. coli}}}}</td>
<td>0</td>
</tr>
<tr>
<td>C600S2</td>
<td>C600\textit{Δbam\textsubscript{A}}::\textit{cat} +pZS21-\textit{bam\textsubscript{A\textsubscript{A\textsubscript{E. cloacae}}}}</td>
<td>0</td>
</tr>
<tr>
<td>C600S3</td>
<td>C600\textit{Δbam\textsubscript{A}}::\textit{cat} +pZS21-\textit{bam\textsubscript{A\textsubscript{HA\textsubscript{E. coli}}}}</td>
<td>0</td>
</tr>
<tr>
<td>C600S4</td>
<td>C600\textit{Δbam\textsubscript{A}}::\textit{cat} +pZS21-\textit{bam\textsubscript{A\textsubscript{HA\textsubscript{E. cloacae}}}}</td>
<td>44000</td>
</tr>
<tr>
<td>C600S5</td>
<td>C600\textit{Δbam\textsubscript{A}}::\textit{cat} +pZS21-\textit{bam\textsubscript{A\textsubscript{HA\textsubscript{E. coli}}}}</td>
<td>0</td>
</tr>
<tr>
<td>C600S6</td>
<td>C600\textit{Δbam\textsubscript{A}}::\textit{cat} +pZS21-\textit{bam\textsubscript{A\textsubscript{E. cloacae}}}</td>
<td>0</td>
</tr>
<tr>
<td>C600S7</td>
<td>C600\textit{Δbam\textsubscript{A}}::\textit{cat} +pZS21-\textit{bam\textsubscript{A\textsubscript{E. cloacae}}}</td>
<td>0</td>
</tr>
<tr>
<td>C600S8</td>
<td>C600\textit{Δbam\textsubscript{A}}::\textit{cat} +pZS21-\textit{bam\textsubscript{A\textsubscript{E. cloacae}}}</td>
<td>0</td>
</tr>
<tr>
<td>C600S9</td>
<td>C600\textit{Δbam\textsubscript{A}}::\textit{cat} +pZS21-\textit{bam\textsubscript{A\textsubscript{E. cloacae}}}</td>
<td>0</td>
</tr>
</tbody>
</table>
In agreement with the plaque assay results, the co-cultures of PA2+C600 produced the highest amount of Stx2a as in PA2+C600EE with an average of 208.34 µg Stx2a/mg total protein. If replacing \( \text{bamA}^{E.\text{coli}} \) with \( \text{bamA}^{E.\text{cloacae}} \), the resulted phage resistant strain C600K1 produced a similar level of Stx2a as in PA2 monoculture of an average of 1.86 µg Stx2a/mg total protein. An increase in Stx2a level was observed for PA2+C600S10 (\( \text{bamA}_{E.\text{cloacae}}^{E.\text{coli}} \)), but it did not significantly differ from that in PA2 monoculture (Figure 3). The PA2+C600S6 (\( \text{bamA}_{HA6}^{E.\text{coli}} \)) produced approximately half the level of Stx2a as in PA2+C600 which agreed with plaque assay results. Together, these results suggest that the three extracellular loops (loop 4, 6 and 7) of \( \text{bamA}^{E.\text{coli}} \) are essential in the \( stx2a \)-converting phage recognition during lytic infection. Thus, the polymorphism within \( \text{bamA} \) among different species affected the host strain’s phage susceptibility.

**Figure 4.4**: The Stx2 levels in co-cultures of \( \text{bamA} \) derivatives containing different bioengineered BamA extracellular loops with PA2. Ten plasmids with different manipulations in BamA extracellular loops were transformed into the \( \text{bamA} \) mutant named C600K1, and the Stx2a...
levels in co-cultures were measured by R-ELISA. Error bars were standard error of the mean from three biological replicates. One-way ANOVA was used and groups that shared the same letter (a, b, c) had no significant difference (Tukey’s test, \( p < 0.05 \)).

### 4.2.4 bamA-independent phage infection of \( E. coli \) 1.1954 may explain for Stx2a amplification phenotype in co-culture with PA2

Whether \( bamA \)-dependent \( stx2a \)-converting phage lytic infection mechanism happened in co-culture of commensal \( E. coli \) and PA2 was examined. The gene knockout system for \( bamA \) requires the target strain to possess at least three different antibiotic susceptibilities, with kanamycin and ampicillin required. The antibiotic resistance patterns for toxin amplifying commensal \( E. coli \) isolates were checked and listed in Table 4.4 and \( E. coli \) 1.1954 was selected as the representative strain for further generation of \( bamA \) gene knockout. Two mutants were generated and called as 1.1954EE and 1.1954K2, containing plasmid with \( bamA \) \( E. coli \) or \( bamA \) \( Salmonella \), respectively.

<table>
<thead>
<tr>
<th></th>
<th>Kan75</th>
<th>Amp100</th>
<th>Cm25</th>
<th>Tet10</th>
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<tr>
<td>( E. coli ) 0.1229</td>
<td>Y</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>( E. coli ) 0.1231</td>
<td>Y</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>( E. coli ) 0.1282</td>
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<td>Y</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>( E. coli ) 1.1954</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
</tr>
</tbody>
</table>

Note: S, sensitivity; Y, yes; N, no; Kan, kanamycin; Amp, ampicillin; Cm, chloramphenicol; Tet, tetracycline.

As expected, the phage resistant strain C600K2 containing \( bamA \) \( Salmonella \) produced baseline level of Stx2a similar to PA2 monoculture which produced, significantly less than in the co-culture of PA2+C600 or PA2+C600EE (\( p < 0.05 \)) (Figure 4.5). However, the Stx2a levels in the co-cultures of two \( bamA \) mutants of \( E. coli \) 1.1954 (1.1954K2 and 1.1954EE) with PA2 was indistinguishable from that in PA2+1.1954, producing an average of 40 µg Stx2a/mg total
protein. This indicates that the bamA was required for phage infection of E. coli C600, however, it is not necessary for commensal E. coli 1.1954.

Figure 4.5: Stx2a levels in the bamA mutants of commensal E. coli 1.1954 and E. coli C600 after co-incubation with E. coli O157:H7 strain PA2. The bamA knockout 1.1954K2 (1.1954ΔbamA::cat+pZS21::bamA<sup>S.Typhimurium</sup>) for commensal E. coli was able to amplify Stx2a, different from C600K2 (C600ΔbamA::cat+pZS21::bamA<sup>S.Typhimurium</sup>) which demonstrated stx2a-converting phage resistance. Error bars were standard error of the mean from three biological replicates. One-way ANOVA was used and groups that shared the same letter (a, b, c) had no significant difference (Tukey’s test, p <0.05).

A previous study showed that certain commensal E. coli strains were able to form lysogens after co-incubating with pure stx2-converting phages in vitro, and these new lysogens produced high levels of phages after spontaneous prophage induction (Iversen et al., 2015). In our study, the Stx2a amplifying strain, E. coli 1.1954, showed growth advantage over PA2 after 16 hr co-incubation, so we suspected it might form a stable lysogen without causing cell lysis and led to Stx2a amplification. Therefore, the lysogenized rate was measured in both E. coli C600 and 1.1954 host backgrounds. After incubating the stx2 mutant of PA2 designated as PA2K (PA2Δstx2::tet) with C600<sup>NalR</sup> for 16hr, the average lysogen forming rate was 0.016% (Figure
4.6), however, no lysogen formed in the *E. coli* 1.1954$^{\text{NalR}}$ host background even using undiluted co-cultures. We extended to examine the lysogen forming rates using other O157 strains whose *stx2a*-converting phages clustered into different phage types. The *stx2a*-converting phage from PA8 belonged to PST2-2, PA28 to PST3 and Sakai to PST1. In the *E. coli* C600 host background, SakaiK (SakaiΔ*stx2*::tet) had the lowest average lysogen forming rate of less than 0.008%, while EDL933K (EDL933Δ*stx2*::tet) gave the highest of 0.021%. No significant difference in the lysogen forming rates was observed among PA2, PA8, PA28 and EDL933. Similar to PA2, no lysogens formed in *E. coli* 1.1954 host background for all tested O157 strains even using undiluted co-cultures.

A previous study has shown that if the preferred phage insertion site in the host chromosome is already occupied, the *stx2*-converting phages will integrate at a second site (Serra-Moreno *et al*., 2008). The observation that no lysogen formed in *E. coli* 1.1954 may be due to the occupancy of its primary insertion sites. Using BLAST, the whole genome sequence contigs of commensal *E. coli* 1.1954 and C600 were screened for the availabilities of six common *stx2a*-converting phage insertion sites including *wrbA*, *sbcB*, *yehV*, *argW*, *yecE* and *z2577* (Serra-Moreno *et al*., 2007). In *E. coli* 1.1954, only *z2577* encoding an oxidoreductase (Koch *et al*., 2003) was found to be occupied, while in *E. coli* C600, none was occupied. Therefore, the inability of *stx2a*-converting phages to lysogenize *E. coli* 1.1954 does not appear to be due to the occupancy of integration sites.
Figure 4.6: Lysogen forming rates of *E. coli* C600 after co-incubation with different *E. coli* O157:H7 strains. The stx2 mutants of EDL933, Sakai, PA2, PA8, PA28 with their stx2a-converting phages from distinct PSTs were co-cultured with *E. coli* C600 for 16hr and the lysogen forming rate in host background of *E. coli* C600 was examined. Error bars were standard error of the mean from three biological replicates. One-way ANOVA was used and groups that shared the same letter (a, b) had no significant difference (Tukey’s test, p < 0.05).

The phage susceptibility of *E. coli* 1.1954 was further investigated. It was not practical to use double layer plaque assay because the addition of ciprofloxacin in the bottom agar prevented *E. coli* 1.1954 from forming a bacteria lawn. This perhaps because ciprofloxacin-inducible prophage exists in the chromosome of *E. coli* 1.1954. After modifying the plaque assay by removing ciprofloxacin, no zone of clearance was observed when using *E. coli* 1.1954 as the indicator strain. Therefore, it is unclear whether *E. coli* 1.1954 is susceptible to stx2a-converting phages.

An alternative way of testing this is introducing a plasmid containing phage repressor *cI* originated from PA2 (pET15b::cI) into both *E. coli* C600 and 1.1954. If the *cI* transformants were phage resistant, zone of clearance would not be observed when spotting stx2a-converting phages from PA2 onto them. As shown in Table 4.5, the wild type C600 and C600::pET15b showed zone of clearances, however, its transformant C600::pET15b::cI did not. This suggested
that overexpression of phage repressor CI in *E. coli* C600 conferred the resistance to phage infection. However, no zone of clearance was observed for *E. coli* 1.1954 and its *cl* transformants.

**Table 4.5**: Spot assay results for *E. coli* C600 and 1.1954 and their *cl* derivatives

<table>
<thead>
<tr>
<th>Strain</th>
<th>Zone of Clearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>C600</td>
<td>Yes</td>
</tr>
<tr>
<td>C600::pET15b</td>
<td>Yes</td>
</tr>
<tr>
<td>C600::pET15b::cl</td>
<td>None</td>
</tr>
<tr>
<td>1.1954</td>
<td>None</td>
</tr>
<tr>
<td>1.1954::pET15b</td>
<td>None</td>
</tr>
<tr>
<td>1.1954::pET15b::cl</td>
<td>None</td>
</tr>
</tbody>
</table>

We next evaluated the Stx2a levels in their co-cultures with PA2. The *E. coli* C600 transformed with empty vector had a significant reduction of average Stx2a production when compared with its wild type (11.27 µg Stx2a/mg total protein vs 50.94 µg Stx2a/mg total protein, *p*<0.05) (Figure 4.7a), but was significantly higher than that in PA2 monoculture (1.524 µg Stx2a/mg total protein, *p*<0.05). The *cl* transformant for *E. coli* C600 produced indistinguishable levels of Stx2a as in PA2 monoculture (2.450 µg Stx2a/mg total protein vs 1.524 µg Stx2a/mg total protein, *p*<0.05). Similarly, the average Stx2a level in PA2+1.1954::pET15b::cl which were similar to that of PA2 monoculture, were significantly lower than that in PA2+1.1954 or PA2+1.1954::pET15b (*p*<0.05) (Figure 4.7b).
Figure 4.7a:

![Figure 4.7a graph showing Stx2a levels in commensal E. coli C600 and 1.1954 and their cl derivatives after co-incubation with E. coli O157:H7 strain PA2. The C600p and 1.1954p were strains transformed with empty plasmid pET15b; C600pcl and 1.1954pcl were strains transformed with pET15b containing cl inserts. Error bars were standard error of the mean from three biological replicates. One-way ANOVA was used and groups that shared the same letter (a, b, c) had no significant difference (Tukey’s test, p < 0.05).]

Figure 4.7b:

![Figure 4.7b graph showing Stx2a levels in commensal E. coli C600 and 1.1954 and their cl derivatives after co-incubation with E. coli O157:H7 strain PA2. The C600p and 1.1954p were strains transformed with empty plasmid pET15b; C600pcl and 1.1954pcl were strains transformed with pET15b containing cl inserts. Error bars were standard error of the mean from three biological replicates. One-way ANOVA was used and groups that shared the same letter (a, b, c) had no significant difference (Tukey’s test, p < 0.05).]
The above results indicated that commensal *E. coli* 1.1954 was phage susceptible and its *bamA* gene was not the key player for explaining the Stx2a amplification phenotype. It is possible that other phage receptors rather than BamA involve in phage lytic infection of commensal *E. coli* strains. Prior to identification of BamA, other outer membrane proteins such as FadL and LamB were hypothesized as the *stx2a*-converting phage receptors (Islam *et al*., 2012; Watarai *et al*., 1998). The protein sequences of LamB and FadL in both *E. coli* C600 and 1.1954 were compared, and it showed that LamB differed by nine amino acids (Figure 4.8a), and FadL by one amino acid (Figure 4.8b). Therefore, future investigations should address whether LamB and/or FadL are alternative phage receptors required for the toxin amplification observed when *E. coli* O157:H7 PA2 and 1.1954 are co-cultured.

Figure 4.8a:

<table>
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<tr>
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</tr>
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<td>421</td>
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<tr>
<td>ADNFLGGSFGRDSDEWTFGAQRME1WW</td>
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</table>
Figure 4.8: The protein alignments of LamB (a) and FadL(b) from *E. coli* C600 and 1.1954. The shared amino acids were highlighted in black.

### 4.3 Discussion

Previous studies described how nonpathogenic *E. coli* increased the Stx2a production of O157 strain by using multiple strategies (Gamage *et al.*, 2003; Gamage *et al.*, 2006; Goswami *et al.*, 2015; Toshima *et al.*, 2007). Our work augmented the understanding of this phenotype by demonstrating multiple mechanisms existed for the interplay of a single O157 strain with different nonpathogenic *E. coli*. In accordance with a former study (Gamage *et al.*, 2003), about 33% of the commensal *E. coli* isolates in our study were able to amplify Stx2a in co-cultures with O157 strain, but they exhibited different competitive indexes from laboratory *E. coli* C600.

As characterized before (Goswami *et al.*, 2015), *E. coli* C600 was outcompeted by O157 strain PA2 after co-incubation, which led to our hypothesis that stx2a-converting lytic phage infected *E. coli* C600 and resulted in toxin amplification. We were able to support this by using
bamA knockouts for E. coli C600 which were resistant to stx2a-converting phage infections. BamA has been reported as the receptor for short-tailed stx2-converting phage (Smith et al., 2007), and its receptor function was previously tested through using either anti-BamA serum, or its over expression in a target organism (Islam et al., 2012a; Smith et al., 2007). The direct evidence through gene knockout was lacking. After adopting a similar genetic approach published before (Ruhe et al., 2013), our study provides the direct genetic evidence showing that BamA is the stx2a-converting phage receptor, and plays an important role in the phage lytic infection of E. coli C600 by O157 in the co-culture. The extracellular loops 6 and 7 of bamA E. coli were shown to be essential for contact-dependent growth inhibition in Ruhe’s study. Although the similar plasmid constructions were used in our study, all three extracellular loop 4, 6, 7 of bamA E. coli are shown to be necessary for phage recognition during the lytic infection. Moreover, the variations within these three loops among species determine whether the corresponding strain is phage susceptible, because the incorporation of both loop 6 and 7 of bamA E. coli into a heterologous bamA E. cloacae changed phage resistant host into partially phage susceptible (Figure 4.4).

In this study, E. coli 1.1954 was selected as the representative strain for the further exploration due to its antibiotic susceptibility profile (Table 4.4), lack of secreted factor stimulating Stx2a production (Table 4.1) and exhibited a competitive index different from E. coli C600 after co-culture with E. coli O157:H7 (Figure 4.1). We excluded the possibility that E. coli 1.1954 was converted into lysogen by stx2a-converting phages during co-incubation. The lysogen forming rates in both E. coli C600 and 1.1954 were measured by using stx2a-converting phages from different O157 strains. Although the most favorable phage insertion sites are available in E. coli 1.1954, no lysogen formed in it for any tested O157 strain. However, in E.
coli C600, a low level of lysogens was observed for all O157 strains. This indicated that lysogen is probably determined by host background more than the source strain of the phage.

Subsequently, we are able to demonstrate that E. coli 1.1954 is phage susceptible by using the phage repressor cl. We suspect E. coli 1.1954 may use other phage receptors instead of BamA during lytic infection. Current studies on the role of FadL and LamB as phage receptors are contradictory. One showed that gene deletion of fadL or/and lamB in E. coli K-12 MG1655 did not affect lytic infection by stx2a-converting phage from Sakai (Islam et al., 2012). Although the similar approach was used in E. coli C600, another study revealed that different phages might use FadL or LamB or both as phage receptors. Perhaps the existence of other potential phage receptor may compensate for the loss of BamA during the stx2a-converting phage infection as we did observe similar Stx2a level in wild type E. coli 1.1954 and its bamA mutant (Figure 4.5).

Initial screening for toxin producing stimulators secreted by the commensal E. coli revealed that one strain, E. coli 0.1229, secretes unknown molecule(s) which promote toxin production of O157 in co-culture. A previous study showed that the colicin E9 produced by some colicinogenic bacteria acted as phage inducing agent, enhanced SOS responses and eventually led to Stx2 amplification of O157 strains in the co-cultures (Toshima et al., 2007). Perhaps, the E. coli 0.1229 can produce colicin-like molecule. Therefore, further exploration of what characteristics it has, how it works and what genes are responsible for it will add to our understanding of this mechanism. Together, this study shows that three mechanisms exist for explaining the toxin amplification phenotype observed in co-cultures of different commensal E. coli with the same O157 strain. Additional future research is needed to advance our understanding on why individuals infected with the same E. coli O157:H7 strain may show the different severity of symptoms.
4.4 Material and methods

4.4.1 Strains and culture conditions

All the strains and plasmids used in the study are listed in Table 1. The *E. coli* O157:H7 strains which were named after “PA” designations were from the Pennsylvania Department of Health and were characterized previously (Hartzell *et al.*, 2011). The commensal *E. coli* strains were obtained from *E. coli* Reference Center (EORC) at Penn State University. The bacteria were routinely grown in LB broth at 37°C, and their culture stocks were kept in 10% glycerol at -80°C. Modified LB broth or modified agar was additionally supplemented with 10mM CaCl₂, and they were prepared for co-incubation experiment.

4.4.2 Co-incubation experiment

To test whether other nonpathogenic *E. coli* strains amplify Stx2a when co-cultured with *E. coli* O157:H7 strain PA2, a random collection of twelve commensal *E. coli* strains from six different O-groups were chosen to do the co-incubation with PA2. Laboratory strain *E. coli* C600 was also selected as the positive control for increased Stx2a accumulation after being co-incubated with PA2. The co-incubation assay was adapted from Gamage *et al* (Gamage *et al.*, 2003). The overnight cultures of PA2 and nonpathogenic *E. coli* strains were separately diluted in LB broth to an OD₆₀₀ of 0.05. 170 µl of either diluted PA2 or nonpathogenic *E. coli* strain was mixed with 850 µl modified LB broth, and added to in six-well plate (BD Biosciences Inc., Franklin Lakes, NJ) as monocultures, serving as controls. The same volume (170 µl) of diluted PA2 and individual nonpathogenic *E. coli* strain were mixed with 680 µl modified LB broth as co-culture. The six well plates had modified LB agar served as the bottom base, on which approximately 1 ml of monoculture or co-culture was added and incubated at 37 ºC. Samples for
enumerating cell density and Stx2 levels were harvested after 16 hour coincubation. Viable cell counts were measured by spreading the harvested cultures on the differential media, Sorbitol MacConkey agar (SMaC), where nonpathogenic *E. coli* was red and *E. coli* O157:H7 was white colonies. The Stx2a production was measured by R-ELISA. Both cell counts and toxin levels were evaluated after three biological replicates. The competitive index of coincubation was reported as percentage of commensal *E. coli* in the total population of co-culture, calculating by the equation:

\[
CI = \frac{\text{(Red colonies on SMaC)} \times 100}{\text{(Red colonies + White colonies on SMaC)}}
\]

### 4.4.3 Stx2a quantification using R-ELISA

We used the assay developed in our lab for measuring the Stx2a level (Yin *et al.*, 2015; Goswami *et al.*, 2015). The detachable 96-well polystyrene microtiter strip plates (Thermo Scientific, Waltham, MA) were coated with 2.5 µg Gb3 analogue, ceramide trihexoside (CTH) (Matreya Biosciences, Pleasant Gap, PA). The plate was blocked with washing buffer (0.01 M phosphate buffer saline with 0.05% Tween20) complemented with 4% bovine serum albumin (Sigma-Aldrich, St. Louis MO) at 4°C overnight. The next day, after washing the plates with washing buffer for five times, diluted or undiluted samples were added in triplicates, and placed on a shaking incubator at room temperature for one hour. The 10 ng monoclonal mouse anti-Stx2 (Santa Cruz Biotech, Santa Cruz CA), which specifically binds to the A subunit of Stx2, was added and incubated at room temperature for one hour. Then, the 10 ng goat anti–mouse secondary antibody conjugated to peroxidase was added and incubated at room temperature for one hour. Detection was accomplished using 1-Step Ultra TMB (Thermo-Fischer, Waltham, MA), which was equilibrated to room temperature in a foil-wrapped tube for at least 30 minutes.
prior to use. TMB substrate (100 µL) was added to each well and incubated with shaking for 10 minutes to allow for color development. Finally, 100 µL of stop solution (2 M H₂SO₄) was added to each well, followed by shaking for 30 seconds. The reading values of A₄₅₀ were obtained using a DU® 730 spectrophotometer (Beckman Coulter, Atlanta, GA). Before adding reagent for each step, the plate was washed by washing buffer for five times. For samples, both co-cultures and monocultures were treated with 6 mg/mL of polymyxin B (PMB) first and incubated at 37 °C for 5 min to lyse cells and get intracellular Stx2a released. After centrifuging them at 4,000 x g for 10 min, the supernatants were collected for immediate usage or storage at -80°C.

For each run, supernatants from *E. coli* O157:H7 strain PA24, which produces only Stx1, is used as the negative control, and the lysate from *E. coli* O157:H7 strain PA11 served as the positive control. The standard curves were generated using two-fold serially diluted PA11 lysate in PBS or pure Stx2 (BEI resources, Manassas, VA). Any A₄₅₀ above 0.2 was considered positive. Total protein in each unknown sample was measured by the Bradford assay (VMR Life Science, Philadelphia, PA), following the manufacturer's recommended protocol. Stx2a quantities were reported as µg Stx2a/mg total protein.

### 4.4.4 Gene knockout assay

The gene knockout for *bamA* in nonpathogenic *E. coli* strains are described below. The target strains were first transformed with plasmid pZS21::*bamA*<sup>E. coli</sup> (Kan<sup>R</sup>). Subsequently, the *cat* cassette was amplified with oligonucleotides *bamA-cat*-For and *bamA-cat*-Rev (see Table 1), and the resulting product was electroporated into *E. coli* C600 competent cells by following previously published one step recombination assay (Datsenko and Wanner, 2000). Transformants were selected on LB agar supplemented with both chloramphenicol (10 µg/ml) and kanamycin (50 µg/ml), and furthered verified by PCR of using primers named *bamA*-VF and *bamA*-VR. The
plasmid exchange used for generating other \( bamA \) derivatives was done by transforming individual plasmid pZS21(Amp\(^R\)) variant carrying different \( bamA \) allele described in Table 1 to replace pZS21::\( bamA \)\( E. coli \) (Kan\(^R\)) in the target strain which already had chromosome \( bamA \) deletion. After incubating for one hour at 37°C, they were plated onto LB agar supplemented with ampicillin (100 µg/ml) and incubated at 37°C for 20 h. The resulting Amp\(^R\) colonies were then cross streaked onto separate LB agar plates supplemented with either kanamycin or ampicillin. Only the colonies which were resistant to ampicillin but sensitive to kanamycin were kept for future usage. In order to generate \( E. coli \) O157:H7 strains’ \( stx2 \) mutants, the tetracycline cassette was amplified with oligonucleotides \( stx2 \)-tet-For and \( stx2 \)-tet-Rev, and the resulting PCR product was electroporated into target strains by following one step recombination assay specific for EHEC strains (Murphy and Campellone, 2003). Transformants were selected by LB agar supplemented with tetracycline (10 µg/ml) and verified by PCR of using primers designated \( stx2 \)-VF and \( stx2 \)-VR.

### 4.4.5 Plaque assay and spot assay

An overnight culture of PA2 was diluted to an OD\(_{600}\) of 0.05 by using LB broth. A final concentration of 45 ng/ml ciprofloxacin was added to it for \( stx2a \)-converting phage induction. After eight hours, the culture was centrifuged at 4,000g for ten minutes and the supernatant was filtered through 0.22 µM cellulose acetate filter (VWR, Radnor PA). The harvest phage lysate was precipitated by adding a ¼ volume of PEG8000/NaCl buffer and stayed at 4°C overnight. The lysate was centrifuged at 4,000 g for one hour, and serial dilutions of phage solution were made in SM buffer (0.1 M NaCl, 50 mM Tris-Cl, 8 mM MgSO4 and 0.01% gelatin). 200 µL of the indicator strain JM109 was added to 100 µL of phage, and further mixed with 6mL modified LB soft agar (0.75% agar). They were added on top of the modified LB agar and incubated at
42°C. After incubating for 16 hours, the plaques were counted. For the spot assay, the target strain was grown in LB broth supplemented with 10 mM MgSO₄ at 37°C for overnight. 100 µL overnight culture was mixed with 3.5 mL melted top agar (1.0% peptone, 0.5% yeast extract, and 0.8 % agar, 0.5 % sodium chloride, 10mM MgSO₄, 1 mM CaCl₂), and overlaid on the modified LB agar plates. 10 µL undiluted phage solution was spotted on top of the center of the top agar. After the phage solution completely dried, the plates were put under 37°C for incubating 12 hours. Zone of clearance would be observed if the target strain was phage susceptible.

4.4.6 Lysogenized rate calculation

The nalidixic acid resistant colonies for E. coli C600 and 1.1954 were selected by spreading their 10 mL overnight cultures onto LB agar plates supplemented with 30 µg/mL nalidixic acid (Nal) and incubated at 37°C for 16 hours. The spontaneous Nal resistant colonies were double checked by being streaked onto LB agar plates containing 30 µg/ml Nal for twice. In our experiment, we found that E. coli O157 strains do not generate spontaneous NalR colonies. The resulted C600 (NalR) or 1.1954 (NalR) was set up in a coincubation assay with PA2Δstx2::tet or stx2 mutants for other E. coli O157:H7 strains. After 16hr incubation, the ten-fold diluted culture was spread on the LB agar plates containing only Nal (30 µg/ml) to enumerate the total number of nonpathogenic E. coli strains, or onto plates containing 10 µg/ml tetracycline (Tet) and 30 µg/ml Nal for selecting the lysogens generated under a specific host background. The lysogenized rate was calculated by using the equation:

\[
\text{Lysogenized rate } \% = \frac{\text{tetR and NalR colonies}}{\text{NalR colonies}} \times 100
\]
4.4.7 Spent supernatant experiment

To test if the nonpathogenic *E. coli* expresses certain molecular that up-regulate Stx2a expression of PA2, the spent supernatant experiment was conducted for the five amplifiers, namely *E. coli* 0.1229, 0.1282, 0.1231, 1.1954 and C600. Those nonpathogenic *E. coli* strains were grown in a shaking incubator at 37°C. After 16 hours, they were centrifuged at 4,000 g for 15 min and filter sterilized using a 0.22 µM cellulose acetate filter (VWR, Radnor PA). The pH was measured and adjusted to 7. An overnight culture of PA2 was diluted to an OD$_{600}$ of 0.05 and added to 1mL spent supernatant. It was also added to 1 mL fresh LB which served as the baseline. Static incubation at 37°C in a six-well plate setup was performed. R-ELISA was done for the PMB treated samples and results were reported as fold difference of A$_{450}$ from PA2 grown in fresh LB.

4.4.8 Data analysis

MS Excel was used to calculate the mean, standard deviation and standard error; Minitab was used for statistical analysis and GraphPad Prism 8 software was used for generating figures.

4.5 Acknowledgement

We thank Dr. Ruhe and Dr. Hayes at University of California, Santa Barbara for providing *bamA* related plasmids; Dr. Koudelka for providing *cI* related plasmids; Hillary M. Figler for proofreading this manuscript. This work was funded by USDA-NIFA grant 2010-65201-2061.

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<th>Table 4.6: Strains, plasmid and primers used in this study</th>
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<tr>
<td><strong>Bacteria strains</strong></td>
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<tr>
<td><em>E. coli</em> O157:H7</td>
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<tr>
<td>PA2 stx2a ; clade 8; PST2-1</td>
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<tr>
<td>Strain</td>
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<tr>
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</tr>
<tr>
<td>PA8</td>
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<td>PA28</td>
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<td>EDL933</td>
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<td>Nonpathogenic $E$. coli</td>
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### Plasmids

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<td>pZS21::bamA&lt;sub&gt;E. coli&lt;/sub&gt;</td>
<td>pZS21 derivative that expresses &lt;i&gt;E. coli&lt;/i&gt; &lt;i&gt;bamA&lt;/i&gt;, Kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Ruhe et al. 2013)</td>
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<td>pZS21::bamA&lt;sub&gt;E. cloacae&lt;/sub&gt;</td>
<td>Expresses &lt;i&gt;bamA&lt;/i&gt; from &lt;i&gt;Enterobacter cloacae&lt;/i&gt; ATCC 13047 (&lt;i&gt;bamA&lt;/i&gt; &lt;i&gt;E. cloacae&lt;/i&gt;), Amp&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>Expresses &lt;i&gt;bamA&lt;/i&gt; from &lt;i&gt;Salmonella enterica&lt;/i&gt; serovar Typhimurium strain LT2 (&lt;i&gt;bamA&lt;/i&gt; &lt;i&gt;LTT&lt;/i&gt;), Amp&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>Expresses &lt;i&gt;bamA&lt;/i&gt; from &lt;i&gt;Dickeya daintii&lt;/i&gt; 3937 (&lt;i&gt;bamA&lt;/i&gt; &lt;i&gt;Dd3937&lt;/i&gt;), Amp&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>pZS21::amp-bamA&lt;sup&gt;P&lt;/sup&gt; derivative that deletes residues Pro&lt;sup&gt;556&lt;/sup&gt; – Asn&lt;sup&gt;563&lt;/sup&gt; within loop 4 of &lt;i&gt;BamA&lt;/i&gt; &lt;i&gt;E. coli&lt;/i&gt;, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>pZS21-bamA&lt;sub&gt;HA&lt;/sub&gt;&lt;sup&gt;E. coli&lt;/sup&gt; derivative that introduces an HA epitope into extracellular loop 6 of &lt;i&gt;BamA&lt;/i&gt; &lt;i&gt;E. coli&lt;/i&gt;, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>Expresses chimeric &lt;i&gt;bamA&lt;/i&gt; &lt;i&gt;E. cloacae&lt;/i&gt; in which the coding sequence for Asp&lt;sup&gt;550&lt;/sup&gt; – Ala&lt;sup&gt;567&lt;/sup&gt; and Ala&lt;sup&gt;739&lt;/sup&gt; – Val&lt;sup&gt;752&lt;/sup&gt; is replaced with Tyr&lt;sup&gt;550&lt;/sup&gt; – Thr&lt;sup&gt;567&lt;/sup&gt; and Thr&lt;sup&gt;747&lt;/sup&gt; – Tyr&lt;sup&gt;757&lt;/sup&gt; from &lt;i&gt;bamA&lt;/i&gt; &lt;i&gt;E. coli&lt;/i&gt;, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>(Ruhe et al. 2013)</td>
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<td>pET15b::&lt;i&gt;cI&lt;/i&gt;</td>
<td>pET15b harboring intact &lt;i&gt;cI&lt;/i&gt; gene from PA2</td>
<td>Koudelka Lab</td>
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### Primers

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Abbreviations: ECRC, <i>E. coli</i> Reference Center at Penn State; Amp<sup>R</sup>, ampicillin resistant; Cm<sup>R</sup>, chloramphenicol resistant; Kan<sup>R</sup>, kanamycin resistant; Tet<sup>R</sup>, tetracycline resistant.
4.6 References


*Escherichia coli* O157:H7 strains harbor at least three distinct sequence types of Shiga toxin 2a-converting phages. BMC Genomics 16, 733-746.


Chapter 5 Conclusions and Future Directions

5.1 Conclusions

An inexpensive quantitative R-ELISA specific for measuring Stx2a was successfully developed by using all commercially available reagents (Chapter 3). The ceramide trihexoside with hydroxyl fatty acid chain was selected as the antigen capture, and gave much better sensitivity than other candidates including current commercially available Stx2 polyclonal antibody. Several critical steps have been identified to enhance its robustness and repeatability. Especially, the utilization of detachable strip plates has greatly prevented the edge effect which is commonly seen in traditional ELISA, and increases the total number of samples that can be tested at one time. The R-ELISA can also detect Stx2c in bacteria lysates. Although limitations were observed when applying it to environmental samples, this cheap, easy and reliable method can be utilized in quantifying Stx2a in bacterial lysates. After comparing Stx2 levels in a collection of *E. coli* O157:H7 strains with the R-ELISA, we demonstrated that the toxin production does not always correlate with strain phylogeny.

We demonstrated 33% of nonpathogenic *E. coli* strains were able to amplify Stx2a when co-cultured with PA2, and two types of competitive indexes were observed (Chapter 4). *E. coli* C600 was outcompeted by PA2, while several commensal *E. coli* strains were present in higher abundance. We proposed three potential mechanisms for Stx2a amplification phenotype and related competitive indexes for the phenomena. For nonpathogenic *E. coli* C600, its *bamA* (an essential gene and Stx phage receptor) has been demonstrated to be important. The replacement of *bamA* in *E. coli* C600 with heterologous *bamA* made it phage resistant and abolished the ability to enhance Stx2a production when co-incubated with PA2. Moreover, the extracellular loops of its BamA (loop 4, 6, 7) were shown to be recognized by *stx2a*-converting phages.
However, the *bamA* knockout in commensal *E. coli* 1.1954 presented a *bamA*-independent mechanism for toxin amplification. Additionally, the commensal *E. coli* 0.1229 produced an unknown molecule(s) in its supernatant to stimulate the Stx2a amplification of PA2 in co-culture. Therefore, three mechanisms may exist to explain the Stx2a amplification phenotype and related competitive indexes.

### 5.2 Future directions

#### 5.2.1 Evaluate the role of other potential phage receptors in *E. coli* 1.1954 for explaining the Stx2a amplification phenotype

In chapter 4, the commensal *E. coli* 1.1954 showed a *bamA*-independent mechanism for toxin amplification, but it was still demonstrated to be phage susceptible. Additionally, we excluded the possibilities that wild type commensal *E. coli* might exert a growth advantage over PA2 in co-culture and produce an unknown stimulator in its supernatant. Although contradictory results have been obtained for other phage receptors (LamB and FadL) in literature, the protein sequence comparison of LamB between *E. coli* C600 and 1.1954 showed some differences, indicating they may be good candidates for future investigation on this topic. Perhaps, existence of LamB or/and FadL would compensate for the loss of BamA in *E. coli* 1.1954 knockout, and still mediate the phage lytic infection. Therefore, generating a double mutant of *bamA* and other potential phage receptor(s) and evaluating its Stx2a change in co-culture with PA2 will test this hypothesis.
5.2.2 Identify the unknown stimulator in *E. coli* 0.1229 supernatant for explaining the Stx2 amplification phenotype

The commensal *E. coli* 0.1229 was shown to secrete an unknown molecule in its supernatant to stimulate the Stx2 amplification of PA2. It has been reported that colicinogenic bacteria showed Stx amplification when co-incubated with O157 strain, and the E9 colicin produced by it could act like the DNA damaging agent, mitomycin C. Future work can identify any molecule that resemble colicin and characterize its chemical nature in *E. coli* 0.1229. Moreover, the corresponding genes responsible for the production of this toxin inducing factor can be investigated. After mastering all of that information, we will be able to carry on to test the prevalence of this gene for encoding similar stimulator among other commensal *E. coli* strains.

5.2.3 Investigate the interactions between PA2 and a cocktail of commensal *E. coli*

In our study, three different mechanisms are proposed to explain the Stx2a amplification phenotype observed for co-cultures of PA2 and commensal *E. coli*. On the one hand, it is unknown if the combination of all phage susceptible commensal *E. coli* as a cocktail will further boost Stx2a production in co-culture with PA2, and whether one of these three mechanisms will take the lead or work cooperatively. On the other hand, it will be meaningful to study the interactions between PA2 and a cocktail composed of both phage susceptible and resistant commensal *E. coli* strains which will more closely reflect real human gut microflora composition.

5.2.4 Characterize and compare the genomes of nonpathogenic *E. coli* strains
As shown in chapter 4, 33% nonpathogenic *E. coli* were Stx2a amplifying strains. Among them, *E. coli* C600 exerted a different competitive index from other commensal *E. coli* strains. In addition, *bamA* was revealed to be critical in *E. coli* C600 but not in *E. coli* 1.1954 for explaining the toxin amplification phenotype, and 0.1229 indicated another mechanism. It is necessary to look into the genomes of nonpathogenic *E. coli* strains and screen for potential gene variances that account for their different phenotypes. Gene markers can be selected for a future prediction on whether a commensal *E. coli* will be a Stx amplifier or not.
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- Donald V. Josephson and Stuart Patton Mentorship Award in Dairy and Food Science 2015
- Honor Student of Graduate University of Chinese Academy of Sciences 2012
- Award for Excellent Student Leadership 2010
- Honor Student of China Agricultural University 2009
- China Agricultural University Excellent Academic Scholarship 2008
- China National Cereals, Oils and Foodstuffs Corporation (COFCO) Excellent Student Award 2008
- The 3rd place of Innovation of Science and Technology Competition 2007
- DuPont Company Excellent Student Award 2007
- Kerry Oils and Grains Company Excellent Student Scholarship 2006