Ecology, Stress Response and Regulation of Virulence Genes in *Escherichia coli*

by

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ABSTRACT

The genetic structure of *Escherichia coli* is diverse in virulence genes that are required for disease pathogenesis. Stress factors presented in nature, agricultural and food production shape the population structure and drive the acquisition of virulence and resistance genes. Shiga toxin producing *E. coli* (STEC) is a severe foodborne pathogen and acquired the Shiga toxin gene (*stx*) by phage lysogenization. The expression and transmission of *stx* are induced and regulated by stress factors. This research aimed to investigate the effect of ecological factors on the genetic variation and regulation of virulence genes in STEC.

The phylogeny and the correlation between virulence and stress resistance genes were analyzed with 13,717 *E. coli* genomes to unravel the determinants that manipulate the genetic structure. The dynamic distribution of virulence and stress resistance genes suggested that the evolution of pathogenic *E. coli* evolved by the acquisition of virulence and stress resistance genes through horizontal gene transfer. Ecological factors contribute to shape the genetic structure by the selective maintenance of resistance mechanisms and virulence factors, which are beneficial for host-and niche-adaptation. Remarkably, acid resistance mediated by urease activity was correlated to gastrointestinal pathogenic *E. coli*, which reflects the adaptation to commensal habitats.

To further understand the effect of environmental factors on the regulation of virulence genes, the expression, regulation and transfer of *stx* at abiotic stress conditions were explored. Hydrogen peroxide and organic acids used as antimicrobials in the food industry induced the expression of *stx*. Moreover, *stx* was differentially expressed at different stress conditions and in different genetic backgrounds. Oxidative stress induced

the expression of *stx* and production of phage through the up-regulation of the RecA-dependent SOS stress response required for DNA repairs. Dehydration results in the generation of reactive oxygen species and induces the oxidative stress in the dehydrated cells. This study demonstrated that drying process resulted in the oxidation of the membrane lipids, and expression of *stx* in STEC. Moreover, the expression of Stx prophage in STEC that survived on dry seeds resulted in the transfer of *stx* to non-pathogenic *E. coli* during seeds germination. The contamination of STEC in low water activity foods compromises food safety by the transmission of virulence genes. In conclusion, this study improves the understanding of the effect of abiotic stress on the *E. coli* population structure and regulation of *stx*, which drives the genetic variation and virulence recombination in the ecological systems.

PREFACE

This thesis is an original work by Yuan Fang, which is written according to the guidelines provided by FGSR.

Chapter 2 is a literature review. My contribution includes collection of information related to the virulence genes, molecular pathogenesis, and host-specification of pathogenic *E. coli*. Dr. Michael G. Gänzle provided suggestions.

Chapter 3 entitled as "Genomic insights to pathogenicity, host adaptation and niche specialization of *Escherichia coli*." Dr. Michael G. Gänzle and Dr. Jinshui Zheng designed this study. Dr. Jinshui Zheng provided the knowledge of bioinformatics, collected and analyzed the genomic data before Huifeng Hu and I were recruited in the project. Huifeng Hu contributed to the bioinformatics analysis and data visualization. My contribution to this work includes data interpretation, literature review and manuscript preparation. Dr. Lynn M. McMullen, Dr. Michael G. Gänzle and Dr. Jinshui Zheng provided suggestions to the manuscript.

Chapter 4 has been published as Yuan Fang, Ryan G. Mercer, Lynn M. McMullen, Michael G. Gänzle (2017), "Induction of Shiga toxin-encoding prophage by abiotic environmental stress in food" Applied and Environmental Microbiology. 83(19): e01378-17. The concepts of the study were developed with Dr. Michael G. Gänzle. I conducted the experiments, analyzed the data and completed the manuscript. Dr. Ryan G. Mercer designed the molecular genetic experiments in this study and edited the manuscript. Dr. Lynn M. McMullen and Dr. Michael G. Gänzle provided suggestions to the experimental design and manuscript editing.

Chapter 5 is in preparation for submission as Yuan Fang, Lynn M. McMullen,

Michael G. Gänzle, "Effect of drying on the oxidative stress response and expression of Shiga toxin prophage in *Escherichia coli*" to Food Microbiology. My contribution to this study includes data generation and manuscript preparation. Dr. Michael G. Gänzle designed the study and provided suggestions to the manuscript composition. Dr. Lynn M. McMullen contributed to the manuscript preparation.

Chapter 6 is prepared for submission as Yuan Fang, Luisa Linda Brückner, Lynn M. McMullen, Michael G. Gänzle "Virulence gene transfer mediated by Shiga toxin phage (Φ11-3088) from *Escherichia coli* O104:H4 in nutrient broth and during sprouts production" to Food Microbiology. Dr. Michael G. Gänzle designed the study. I contributed to the study design, conducted the experiments and prepared the manuscript. Luisa Linda Brückner performed the phage Infection experiments under my mentorship. Dr. Michael G. Gänzle and Dr. Lynn M. McMullen provided suggestions and contributed to the manuscript preparation.

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AAF: aggregative adherence fimbriae

A/E: adhesion and effacing

BFP: bundle-forming pili

CFs: colonization factors

CFA: cyclopropane fatty acids

DA: diffuse adherence

DAF: decay-accelerating factor

DAEC: diffusely adherent *E. coli*

DMSO: dimethyl sulfoxide

EAEC: enteroaggregative *E. coli*

EAST: enteroaggregative heat-stable toxin 1

EDTA: ethylenediaminetetraacetic acid

EHEC: enterohemorrhagic *E. coli*

EIEC: enteroinvasive *E. coli*

EPEC: enteropathogenic *E. coli*

ESBLs: extend spectrum of β-lactamases

ETEC: enterotoxigenic *E. coli*

ExPEC: extra-intestinal pathogenic *E. coli*

Gb_3: globotriaosylceramide

Gb_4: globotetraosyleramide

GC-C: guanylyl cyclase C

HC: hemorrhagic colitis

HUS: hemolytic-uremic syndrome

LEE: locus of enterocyte effacement

LHR: locus of heat resistance

Lpf: long polar fimbriae

LT: heat labile enterotoxin

ST: heat stable enterotoxin

Stx: Shiga toxin

STEC: Shiga toxin producing *E. coli*

ShET1: *Shigella* enterotoxin 1

T3SS: type 3 secretion system

UPEC: uropathogenic *E. coli*

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: General Introduction and Thesis Objectives

1.1. Introduction

Escherichia coli are commensal organisms that inhabit in the large intestine of humans and other warm-blooded animals but the species also contains strains that are pathogenic to humans and animals (Croxen et al., 2013; Tenaillon et al., 2010). Transmission of *E. coli* from feces or water to agricultural products and food leads to the contamination of food products (Croxen et al., 2013). Foods that are contaminated with pathogenic *E. coli* cause foodborne illness associated with mild or chronic diarrhea, and life-threating disease (Pradel et al., 2000).

E. coli are versatile pathogen that are heterogeneous in virulence and stress resistance genes (Rasko et al., 2008). Evolution of pathogenic *E. coli* involves acquisition and recombination of virulence genes (Hazen et al., 2017; Reid et al., 2000). The majority of virulence factors are encoded on mobile genetic island (Cassels and Wolf, 1995; Elliott et al., 2002; Herold et al., 2004). The pathogenesis of *E. coli* is mediated by adherence and colonization on the intestinal mucosa, and/or secretion of effector proteins, enterotoxins or cytotoxins (Croxen and Finlay, 2010; Nataro and Kaper, 1998). Several virulence factors are specific to the host species or host tissue (Gaastra and Svennerholm, 1996). For example, Stx strictly binds to the glycolipid receptors, which are present in humans but are not present in cattle (Pruimboom-Brees et al., 2000). The F18 and F4 fimbriae in enterotoxigenic *E. coli* are explicitly associated with weaning pig diarrhea (Frydendahl, 2002).

Shiga toxin producing *E. coli* is among the most severe pathotypes of *E. coli* that encodes Stx on a lambdoid bacteriophage (Allison, 2007; Herold et al., 2004; Smith et al.,

2014). The phage genome remains in the bacteria as temperate phage, known as prophage, which encodes genomic elements for phage regulation and replication (Feiner et al., 2015). Expression of Stx prophage is regulated by the host stress-response, and induced by environmental stress (Waldor and Friedman, 2005). Induction of Stx prophage results in the production and release of toxin and free phage particles into the environment (Feiner et al., 2015). Phages transfer Shiga toxin genes (*stx*) to any susceptible host by lysogenic infection (Herold et al., 2004). Stx phages mediate the transfer of virulence genes and creation of novel pathotypes (Allison, 2007).

Stress factors that are present in nature, agricultural and food production play an important role in shaping the population structure of *E. coli*. *E. coli* developed a strong resistance to heat and chlorination through the acquisition of locus of heat resistance (LHR) (Mercer et al., 2015; Zhi et al., 2016a). To survive in the acid environment in the gastrointestinal tract, bacteria developed acid tolerance mediated by urease and decarboxylase activities (Merrell and Camilli, 2002). Oxidants such as hydrogen peroxide are naturally produced in the host immune response and used as antimicrobials in food production (Hilgren and Salverda, 2000; Wagner et al., 2001). Moreover, reactive oxidants are generated in cells during dehydration (Licznerska et al., 2015). Oxidants cause damage to proteins, DNA and lipids, and induce the expression of Stx prophage (Cabiscol et al., 2000; Licznerska et al., 2015). Overall, ecological systems are comprised of multiple stressors, which drive the development of stress resistance and adaptation to environmental niches.

Evolution of STEC is mediated by environmental adaptation and transfer of virulence genes by horizontal gene transfer (Allison, 2007). Moreover, the recombination

of virulence genes continues, as exemplified by the novel pathovar *E. coli* O104:H4 that combines the *stx* with the virulence profile of enteroaggregative *E. coli* (Muniesa et al., 2012). Taken together, the selective pressure that STEC experience in their ecological niche shapes their evolution. Understanding this role of the ecology on population genetics and virulence of *E. coli* is essential for control of this organism in the food supply.

1.2. Hypotheses

1) Ecological factors drive the evolution of the population structure of *E. coli* and phylogeny of pathogenic *E. coli.*
2) Stress factors associated with food processing, including drying, acids, oxidants and high-hydrostatic pressure, induce the expression of Stx encoding prophages.
3) Induction of Stx prophages is triggered by environmental stress, and mediates the transfer of *stx* in the food systems.

1.3. Objectives

1) Review of the genotype and molecular pathogenesis of pathogenic *E. coli* (Chapter 2).
2) Investigation of pathogenicity, host adaptation and niche specialization of *E. coli* (Chapter 3).
3) Investigation of the effect of abiotic environmental stress on the expression of Stx prophage (Chapter 4).
4) Investigation of the effect of drying on the oxidative stress response and expression of Stx prophage in *E. coli* (Chapter 5).

5) Characterization of the spectra and rate of phage transduction by Stx phage Φ11-3088 derived from *Escherichia coli* O104:H4 in nutrient broth and during sprout production (Chapter 6).

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CHAPTER 2. Literature Review

2.1. Introduction

Escherichia coli, initially described as *Bacillus coli*, were isolated as commensal intestinal organisms from newborns in 1885 (Escherich, 1988). In the 1940s, a group of strains were classified as pathogenic *E. coli,* which are associated with gastrointestinal illness in humans and animals, and cause high mortality among children less than five years old (Farthing et al., 2013; Pupo et al., 1997). To date, eight major pathogenic groups of *E. coli* and their mechanisms of pathogenicity have been exclusively studied (Croxen et al., 2013; Croxen and Finlay, 2010; Kaper et al., 2004; Levine, 1987). These pathogroups are classified as gastrointestinal pathovars, including Shiga toxin producing *E. coli* (STEC), enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC, including *Shigella*), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC). Uropathogenic *E. coli* (UPEC) is the most common pathogroup that belongs to extra-intestinal pathogenic *E. coli* (ExPEC). Some strains that are classified as ETEC, *Shigella* and EAEC also belong to STEC, because they share the ability to produce Stx (Muniesa et al., 2012; Nyholm et al., 2015; Perna et al., 2001).

Pathogenic *E. coli* have acquired diverse virulence strategies including host-adhesion mechanisms and cytotoxins (Croxen and Finlay, 2010; Kaper et al., 2004) Bacterial colonization on the gastrointestinal surface involves the interaction of adhesion proteins with the epithelial cell receptors (Croxen and Finlay, 2010). Adhesion is achieved by adhesion factors, which are proteins located on the surface of bacteria. These

adhesion factors include fimbriae or pili, and afimbrial surface proteins with distinct morphological structures from fimbriae (Cassels and Wolf, 1995).

Pathogenic *E. coli* co-evolved with commensal *E. coli* through the acquisition of accessory genes to enhance their survival strategies in the stress environment (Ochman et al., 2000; Pupo et al., 1997; Reid et al., 2000). Some strains of *E. coli* are resistant to low pH (Merrell and Camilli, 2002), antibiotics (Ingle et al., 2018) and heat (Mercer et al., 2015). The molecular mechanisms that mediate the resistance of *E. coli* to acids, antibiotics and heat are well studied. This review focused on the mechanism of colonization, virulence factors that contribute to the pathogenesis of major diarrheagenic and extraintestinal *E. coli*; as well as the important stress-resistant mechanisms that influence the ecology and host-adaptation of *E. coli*

2.2. STEC and pathogenesis

STEC cause gastrointestinal diseases and hemolytic-uremic syndrome (HUS) in humans (Antonovskii and Shurukhin, 2005). Over 380 different STEC O:H serotypes have now been isolated, and less than 100 have been associated with human illness, in which a few serotypes frequently cause human infections, including O157:H7, O26, O103 and O111 (Hussein and Bollinger, 2005; Karmali et al., 2003).

The pathogenesis of infection caused by STEC is mediated by colonization on the epithelium surface and production of Stx (Croxen and Finlay, 2010). Stx include type 1 and 2, and are a group of structurally and functionally related exotoxins (Bergan et al., 2012; Fuller et al., 2011; Johannes and Römer, 2010; Melton-Celsa, 2014). STEC produce distinct variants of Stx1 (Stx1a, Stx1c and Stx1d), and Stx2 (Stx2a-g) (Bergan et al., 2012), in which Stx2 variants are 100 times more toxic to humans (Johannes and

Römer, 2010; O'Loughlin and Robins-Browne, 2001). Stx2a and Stx2b, an elastase-cleaved toxin are much more toxic than other variants of Stx2 (Johannes and Römer, 2010). Stx targets cells that expressing glycolipids, which are often on the surface of red blood cells and renal cells in humans, rabbits and pigs (Matise et al., 2003; Richardson et al., 1992; Tarr et al., 2005). Cattle do not produce a glycolipid receptor for Stx in the apparatus targets, including ileum, cecum and rectum, suggesting that cattle are inherently resistant to Stx (Pruimboom-Brees et al., 2000). Administration of purified Stx is able to cause HUS (Stearns-Kurosawa et al., 2010); however it is not realistic to inject the purified Stx from any food or water source, therefore development of HUS relays on the injection of STEC.

In general, STEC are not host-specific; however, Stx2a and 1a are highly prevalent in ruminants compared to other animals, and Stx2e are abundant in swine and responsible for the edema disease in pigs (Fratamico et al., 2004; Hussein and Bollinger, 2005; Imberechts et al., 1992). Stx2e-producing *E. coli* have been isolated from humans with a lower frequency compared with Stx2a-producing *E. coli* (Beutin et al., 2004; Friedrich et al., 2002).

2.2.1. Mode of action of Shiga toxins

Shiga toxins are AB_5 toxins that contain the major enzymatic subunit A and the non-enzymatic homopentameric base formed by B subunits (Melton-Celsa, 2014; O'Loughlin and Robins-Browne, 2001). The B subunits mediate binding to the vascular receptor (Smith et al., 2012a). Stx 1 varies from Stx 2 in both the A (32.2 kDa) and B subunits (7.5 kDa) (Bergan et al., 2012). The A subunits have an N-glycosidase activity that cleaves an adenine on the 28S rRNA, which causes inhibition of tRNA binding

(Johannes and Römer, 2010; Sandvig, 2001). Binding to the cell surface is essential to establish cell intoxication, which is mediated by recognition and affinity between the B subunits and cell glycolipids receptors (Bergan et al., 2012). The B subunits harbour three distinctive binding sites to the trisaccharide moiety of Gb3 in the exoplasmic membrane leaflet (Bergan et al., 2012). Most Stx1 and Stx2 toxins, except for Stx2e recognize Gb3 as cellular receptors (Bergan et al., 2012). In the human, expression of Gb3 is limited to specific cell types, including the kidney epithelium and endothelium, microevascular endothelium in intestinal surface, platelets, and specific sites of B lymphocytes (Engedal et al., 2010). The receptor for Stx2e, a major virulence factor for edema disease of swine, is globotetraosyleramide (Gb_4) instead of Gb_3 (Johannes and Römer, 2010).

The Stx travel in the direction of the trans-Golgi network (TGN), the endoplasmic reticulum (ER) and the cytosol using different endocytosis mechanisms (Johannes and Römer, 2010). After binding, the toxin is internalized to the early endosome by endocytosis (Melton-Celsa, 2014). Internalization of Stx is mediated by clathrin-dependent or dynamic-dependent endocytosis (Johannes and Römer, 2010; Sandvig et al., 2010). Clathrin is an organizing protein that facilitates membrane curvature, and is recruited to endosomes by host proteins (Saint-Pol et al., 2004). In this case, clathrin drives the entry of Stx to endosomes by retrograde transport (Saint-Pol et al., 2004). Dynamic-dependent endocytosis is mediated by tubule membrane invagination, which is required to sort the Stx from early endosomes to TGN (Römer et al., 2007). Sorting from endosomes to TGN is important to intoxication. In the early endosome, A subunits are cleaved by the protease furin to A1 fragment and small A2 fragments, which are bound by disulphide bonds (Bergan et al., 2012). Stx use the ER-associated degradation

machinery to translocate the enzymatic A1 subunit across membrane into the host cell cytosol and cause cell damage by inhibition of protein synthesis (Johannes and Römer, 2010).

Stx are suggested that have adapted to the gastrointestinal environment in the hosts. This hypothesis was assumed based on the evidence, including that the B subunits are resist to low pH and proteases, and with the ability to cross tissues (Johannes and Decaudin, 2005); A subunits are cleaved to A1 and A2 at low pH (Garred et al., 1997; Johannes and Römer, 2010).

2.2.2. Genetic regulation of phage infection

Stx are encoded on lambdoid phage that shares common regulatory components and arrangement with λ phages (Herold et al., 2004). The knowledge of λ phages including the phage replication, integration and excision, lysis versus lysogenic infection, prophage induction can be possibly applied to the regulation and expression of Stx phage (Waldor and Friedman, 2005). The regulation system of Stx encoding bacteriophages shares a mosaic genetic structure particularly in the regulatory region, which is probably due to frequent recombination (Beutin and Martin, 2012; Herold et al., 2004; Ranieri et al., 2014). Phages are obligate parasites to bacteria, and have two life cycles dependent on the genetic interaction with the host (Feiner et al., 2015). Shortly after phage DNA enters the host cells, the decision on the lysis-versus-lysogenization depends upon the Cro, CI, CII and CIII (Grzegorz et al., 2012). Cro functions as a dimer and inhibits the synthesis of repressor CI, which regulates the lytic cycle (Casjens and Hendrix, 2015). CI-III is a regulator that facilitates promoter activities located on the prophage (Grzegorz et al., 2012). Accumulation of CII stimulates the expression of integrase, which catalyzes

the integration of λ phage DNA at specific sites *attB* or Mu, and form a lysogen (Feiner et al., 2015; Oppenheim et al., 2005). Once the lysogen is formed, the prophage is stabilized by CI, which represses promoter pR and pL and inactivates the transcription of the phage late genes encoding proteins for lysis and phage assembly (Casjens and Hendrix, 2015). Stx lambdoid prophage always contains two antagonistic repressors, CI and Cro, but does not always encode other λ phage regulators (Beutin et al., 2012; Smith et al., 2012a).

2.2.3. Induction and regulation of Stx prophage

Stx are encoded in the late region of lambdoid prophage, downstream of Q and upstream of lysis cassette, which are regulated by pR' (Waldor and Friedman, 2005). Q is a antiterminator and interacts with DNA and actives the pR', which results in the expression of Stx, lysozyme, and genes required for assembly of the viable phage (Herold et al., 2004; Waldor and Friedman, 2005). To maintain the lysogen and repress the expression of *stx*, CI repressor is consistently bound to the region of pR (Grzegorz et al., 2012; Waldor and Friedman, 2005). The CI repressor is sensitive to autoproteolysis or autocleavage from the pR region particularly during the interaction with RecA, the major bacterial recombinase (Grzegorz et al., 2012). RecA is overexpressed in the presence of free nucleotide triphosphate or during DNA damage, and it stimulates the autoproteolysis/autocleavage of the repressor LexA, which regulates the SOS stress response (DNA damage response system) (Justice et al., 2008). The SOS response regulates over 30 unlinked genes to facilitate DNA repair, in which the cell-division inhibitor FtsZ is repressed to prevent DNA division and form cell filaments (Quillardet et al., 2003). LexA shows a high degree of homology with several CI repressors, which are also autocleaved by the activated form of RecA (Butala et al., 2009). Autocleavage of CI

results in the activation of lytic life cycle and expression of *stx*, a process called induction (Grzegorz et al., 2012). RecA is crucial to cause the induction of Stx prophage; however it is not the only required factor.

2.2.4. Prophage inducers

Inducers, including UV, mitomycin C, antibiotics, hydrogen peroxide and high hydrostatic pressure, cause the DNA damage, which activates the SOS stress response and induces the expression of lambdoid prophage (Aertsen et al., 2005a; Łos et al., 2010; Łoś et al., 2009). Prophage induction by the above stressors is mediated by the RecA-dependent SOS response (Aertsen et al., 2005a; Janion, 2008; Licznerska et al., 2015). However, induction of prophage can occur without the involvement of the SOS stress response. High concentration of NaCl (200 mM) (Shkilnyj and Koudelka, 2007), EDTA (Imamovic and Muniesa, 2012), lactic acids and HCl induce the Stx prophage without the overexpression of RecA (Chapter 4). The molecular role of the RecA-independent prophage induction has only been observed and explained in detail in a few studies. RcsA and DsrA are transcriptional regulators of capsular polysaccharide synthesis, and involved in the induction of λ and lambdoid prophage in RecA deficient *E. coli* (Rozanov et al., 1998a).

2.3. EPEC

Enteropathogenic *E. coli* (EPEC) produce adhesion and effacing (A/E) lesion on the surface of intestinal epithelium (Croxen et al., 2013; Goosney et al., 2000). The active proteins for the (A/E) lesion are encoded on a 35-kb chromosomal pathogenicity island (PAI), the locus of enterocyte effacement (LEE) (McDaniel et al., 1995). The A/E lesion pathogenesis results in the subversion of actin dynamics within the host, and is mediated

by the interaction between intimin (*eae*) and the bacterial translocated receptor, Tir (Gruenheid et al., 2001; Kenny et al., 1997).

EPEC are further classified into "typical" and "atypical" subtypes determined by the presence or absence of bundle-forming pili (BFP) (Trabulsi et al., 2002). LEE-positive EPEC carrying BFP fall within the subgroups of typical EPEC (tEPEP); BFP-negative EPEC belong to atypical EPEC (aEPEC) (Trabulsi et al., 2002). BFP are type IV pili that produce a characteristic adherence pattern, known as localized adherence (LA) (Bieber et al., 1998; Trabulsi et al., 2002).

2.3.1. LEE positive and negative EHEC

EHEC share the same adhesion of LEE with EPEC, and share the ability to produce Stx with STEC. EHEC are identified as subgroups of EPEC and STEC, and are frequently associated with the hemolytic uremic syndrome (HUS) and hemorrhagic colitis (HC) (Schmidt, 2010). *E. coli* that encode Stx but lack LEE were isolated from stool samples from HUS patients, such as serotypes O91:H21, O104:H4, and O113:H21 (Croxen et al., 2013; Steyert et al., 2012). Therefore, Stx rather than LEE is more important to develop the pathogenesis of HUS (Tarr et al., 2005).

Some LEE-negative EHEC contain a unique auto-agglutination adhesion encoded on *saa* (Newton et al., 2009; Paton et al., 2001; Steyert et al., 2012). The *saa* gene was detected not only in fecal isolates from HUS patients, but also in isolates from healthy individuals, which indicates that *saa* has no strong correlation with HUS (Jenkins et al., 2003). *Saa* was detected in 57% of bovine STEC, suggesting that *Saa* has evolved through adaptive evolution in the bovine gut (Jenkins et al., 2003).

Long polar fimbriae (Lpf) and related long fimbrial proteins are adhesion factors that are encoded either on the chromosome or on the plasmid in both LEE positive and negative EHEC (Doughty et al., 2002; Torres et al., 2007). Lpf are encoded on the chromosome in *E. coli* O157:H7, and contribute to the colonization of the human intestinal epithelium (Torres et al., 2007), but also are significant determinants of the long-term colonization in pigs and sheep (Jordan et al., 2004). Adherence function and pathogenesis of Lpf were first described in *Salmonella enterica* serovar Typhimurium and mediated the cell adhesion to the intestine (Sohel et al., 1993; Weening et al., 2005). The role of Lpf on the adhesion between EHEC and epithelium cells is different, which might depends on the cell types (Desesa et al., 2005; Newton et al., 2004; Tatsuno et al., 2006).

2.4. EIEC/*Shigella*

Shigella is maintained as a separate genus because *Shigella* differs from other strains of *E. coli* with respect to motility and lactose metabolism. Moreover, most strains of *Shigella* acquired the plasmid pINV for cell invasion (Lan et al., 2001; Nataro and Kaper, 1998; Sansonetti et al., 1983). *Shigella* are non-motile and lactose non-fermenting; however, motile and lactose-fermenting *E. coli* that are able to cause cell invasion are known as EIEC (Beld and Reubsaet, 2012; Escobar-Paramo et al., 2003). The biochemical, physiological, pathogenic, and genomic determinants are not different between EIEC and *Shigella,* but confirms that these two genera are identical (Croxen and Finlay, 2010; Pupo et al., 2000, 1997).

Shigella is a highly virulent human-adapted pathogen that causes bloody diarrhea or bacillary dysentery, in particular for infants (Baker and The, 2018). The pathogenesis

of *Shigella/*EIEC is mediated by the invasion to the mucosa, differentiating *Shigella* from other strains of diarrheagenic *E. coli* (Croxen and Finlay, 2010). The Mxi-Spa is a type three secretion system (T3SS) and is encoded on pINV, which is responsible for invasion and apoptosis of macrophages (Nhieu and Sansonetti, 1999; Parsot, 2005). The pathogenesis of *Shigella/*EIEC involves invasion of the host-cell cytoplasm in the colon, replication in the macrophages, and translocation from cell-to-cell, which are facilitated by different effector proteins encoded on the Mxi-Spa apparatus (Croxen and Finlay, 2010; Ogawa et al., 2008; Parsot and Sansonetti, 1996; Schroeder and Hilbi, 2008).

Shigella species generally encode *Shigella* enterotoxin 1 (ShET1); whereas many *Shigella dysenteriae* are more lethal to humans because of the synthesis of Stx1 (Hale, 1991). Gastrointestinal infection caused by *Shigella dysenteriae* can result in the development of HUS (Bergan et al., 2012). Comparable to other STEC, Stx1 in *Shigella dysenteriae* are encoded on a prophage genome; however, the Stx prophage in *S. dysenteriae* is domesticated and lacks essential components for phage replication (Herold et al., 2004; McDonough and Butterton, 1999; Unkmeir and Schmidt, 2000). The selective pressure that maintains Stx prophage in *E. coli* adapted to ruminants and predation by protozoa, which is apparently not relevant for the ecology of human-adapted *Shigella*.

2.5. ETEC

ETEC were frequently isolated from endemic population with 49%, in which 27% was associated with only travel populations (Isidean et al., 2011; Lundgren, 2012). ETEC is diarrheagenic in humans, monogastric animals and calves. ETEC adhere to the small intestine by various surface colonization factors (Cassels and Wolf, 1995), and produce

enterotoxins (Sá Nchez and Holmgren, 2005). ETEC encoded heat labile toxin (LT) and heat stable toxin (ST) were found in 27% and 22% of the ETEC population globally, respectively (Isidean et al., 2011). Secretion of ST causes watery diarrhoea during ETEC infection (Croxen and Finlay, 2010; Kaper et al., 2004; Sá Nchez and Holmgren, 2005). LT and ST are solely or together encoded on plasmids (Peñaranda et al., 1983). ST are small enterotoxins compared to LT (Croxen et al., 2013), and contain two main variants STa (ST-I) and STb (ST-II) (Kaper et al., 2004). ST bind on the guanylyl cyclase C (GC-C) receptor on the intestinal epithelium, and lead to the overexpression of GC-C and accumulation of cGMP (Nair and Takeda, 1997), which stimulates the secretion of chloride (Cl^-) and fluid into the lumen (Wen et al., 1994).

LT are AB_5 toxin and induce watery diarrhea through a different mechanism when compared to ST (Merritt and Hol, 1995). Two classes of LT have been identified, including LT-I and LT-II (Turner et al., 2006). LT-I is more frequently associated with watery diarrhea caused by ETEC compared with LT-II (Croxen et al., 2013). LT-II is proposed to be encoded on a prophage, and differs from LT-I with respect to the genetic structure and host binding receptors (Jobling and Holmes, 2012; Nawar et al., 2010; Spanglert, 1992). The subunits A and B of LT-I are encoded by the operons of *eltA* and *eltB,* respectively (Dallas and Falkow, 1980). The B subunits of LT-I bind to the membrane receptor of host cells, and mediate the internalization of catalytic A subunits into the cell (Dallas and Falkow, 1980). The A subunits of LT-I travel through the endoplasmic reticulum and host cytoplasm, and disrupt the production of AMP in the cell, which leads to the loss of the electrolyte and fluids (Croxen and Finlay, 2010; Kesty et al.,

2004; Nataro and Kaper, 1998). LT was transmitted into the host cells mediated by ETEC-derived vesicles (Kesty et al., 2004).

2.5.2. ETEC adhesion factors

ETEC adhere to enterocytes and colonize on the small intestine through the expression of surface proteins called colonization factors (CFs) (Gaastra and Svennerholm, 1996; Turner et al., 2006). CFs include fimbriae and non-fimbriae adhesion proteins that are strain- and host-specific (Cassels and Wolf, 1995). ETEC are divided into human ETEC and non-human ETEC depending on the CFs (Evans et al., 1975). Most of the CFs are plasmid-encoded (Evans et al., 1975; Krogfelt, 1991; Peñaranda et al., 1983; Thomas et al., 1987). The genetic operons that regulate the biosynthesis of ETEC fimbriae contain genes coding for major and minor subunits, chaperone proteins and membrane usher proteins (Gaastra and Svennerholm, 1996; Krogfelt, 1991). Novel protein EtpA that interactes with flagella was suggested to facilitate the fimbriae adhesion to the host cells in ETEC (Roy et al., 2009).

Human ETEC produce a type IV pilus with a long pilus morphology, also known as longus (Girón et al., 1995). The longus are encoded on plasmids, and frequently co-exist with groups of CFs that are specific to human ETEC (Girón et al., 1995; Qadri et al., 2005). A list of CFs are encoded by human ETEC including CFA/I, CS1 to CS7, CS8 (originally CFA/III), and CS10-CS21 (Pichel et al., 2000). CFA/I has been recognized as the archetype of the largest class of ETEC fimbriae, sharing a similar genetic structure with other variants of CS fimbriae, including CS1, CS2, CS4, CS14 and CS19 (Anantha et al., 2004). CS22 shares the same antigen with CS15 according to the antiserum assay; however, the nucleotide sequence of the structural genes and protein mass of CS22 (*cseA*)

and CS15 are different (Pichel et al., 2000). The *in vitro* adherence assay manifested that all IV fimbriae tested in the study adhered to bovine erythrocytes with no exception (Anantha et al., 2004). Half of the type IV fimbriae including CFA/I, CS4 and CS14 adhered not only to humans, but also to bovine and chicken erythrocytes (Anantha et al., 2004).

The fimbriae types including K88/F4, K99, F18 are commonly found in ETEC and are associated with diarrhea in pigs (Cassels and Wolf, 1995), in which F4 and F18 are strongly correlated to weaning pig diarrhea (Frydendahl, 2002). F4 also known as K88 fimbriae encoded on *fae* operon, are crucial colonization factor associated with porcine neonatal and post-weaning pig diarrhea (Frydendahl, 2002). The F4 (K88) family includes F4ab, F4ac, and F4ad, with distinctive binding-specificity to enterocytes (Guinée and Jansen, 1979; Xia et al., 2015). The F4 fimbriae are encoded on Fae operon, which is composed of major subunits, several minor subunits, regulatory protein, and outer membrane usher (Xia et al., 2015). The genetic structure of F18 is similar to other ETEC fimbriae, and regulated by the Fed operon (Imberechts et al., 1996). Moreover, FedF was determined to be a major functional protein mediating the adhesion to porcine enterocytes (Smeds et al., 2001). K99 or F5 fimbriae facilitate the attachment of ETEC to the mucosal surface of calves, lambs and piglets (Gaastra and De Graaf, 1982), and their biosynthesis have been characterized with eight polypeptides FanA-H (Inoue et al., 1993).

2.6. EAEC

Enteroaggregative *E. coli* (EAEC) are associated with traveller diarrheal (Vila et al., 2000). EAEC produce an aggregative adherence pattern on Hep2 cells (Czeczulin et al., 1999; Nataro et al., 1992). EAEC were isolated from feces of humans, cattle, piglets

and horse (Uber et al., 2006). Presumably, the presence of the plasmid-encoded AAFs confers the aggregative adherence phenotype regardless of the chromosomal background (Czeczulin et al., 1999).

Most of the EAEC strains produce aggregative adherence fimbriae (AAF), encoded on a 60- to 65 MDa plasmid pAA (Dudley et al., 2006). Four different variants of AAF have been defined including AggA (AAF/I), AafA (AAF/II), Agg3A (AaF/III), and Agg4A (AAF/IV) (Bernier et al., 2002; Boisen et al., 2008; Czeczulin et al., 1997; Nataro et al., 1992). AAF/I and AAF/II are encoded by gene clusters *aggABCD* and *aafABCD*, respectively (Czeczulin et al., 1999). The major subunits of AAF/I and II are encoded by gene *aagA* and *aafA* (Czeczulin et al., 1999). AAF/I was rare to be detected in EAEC compared to AAF/II (Vila et al., 2000).

Outer membrane proteins Aat are required for the biosynthesis of AAF (Panchalingam et al., 2012). Aat are part of the protein transporter system, and only identified in EAEC; therefore, it is used as a marker to diagnose EAEC (Aslani et al., 2011; Monteiro et al., 2009; Panchalingam et al., 2012)

2.6.2. Enteroaggregative heat-stable toxin 1 (EAST)

EAST1 is homologue of STa, and associated with post-weaning diarrhea and edema disease in pigs (Frydendahl, 2002;Choi et al., 2001). EAST1 is present in a subset population of EAEC, ETEC and many DAEC as well as commensal *E. coli* and encoded by gene *astA* on the same pathogenic plasmid with AAF (Savarino et al., 1996, 1994, 1993). EAST1 is a pore-forming cytotoxin, which may have similar pathogenesis to STa; however, the mechanism of EAST1 requires further investigation (Croxen and Finlay, 2010).

2.7. DAEC

DAEC are characterized by the formation of diffuse adherence (DA) patterns on the epithelia HEp-2 cells (Scaletsky et al., 1984). DAEC encode diverse fimbriae that belong to Dr adhesion family (Servin, 2014). The Dr adhesion proteins from human pathogenic *E. coli* recognize and bind to the glycosylphosphatidylinositol (GPI)-associated decay-accelerating factor (DAF), which are located in the intestinal and urinary, hematopoietic and endothelial cells in humans (Lublin et al., 2000). Colonization on the urinary and intestinal epithelium by Dr adhesion factors results in a recurrent urinary infection or a gastrointestinal infection in humans (Servin, 2014).

DAEC encode different fimbriae types on mobile genetic operon including Dra, F1845, and Afa fimbriae (Bouguenec and Servin, 2006; Labigne-Roussel et al., 1984; Lalioui et al., 1999; Nowicki et al., 1987). The genetic locus of *daa*A-E is specifically coding for F1845 fimbriae (Loomis and Moseley, 1998). Interestingly, Dr adhesion fimbriae from human isolates are unable to recognize the cell receptor from other animals, such as pigs and rats (Hudault et al., 2004). Afa/Dr shows a specific binding to several reporters on the intestinal epithelium cells (Berger et al., 2004; Servin, 2014). The Afa-related operon was detected in *E. coli* isolated from piglets and calves with diarrhea and septicemia (Harel et al., 1991; Mainil et al., 1997). Animal-associated Afa-VII (*afa-7*) and Afa-VIII (*afa-8*) were identified from bovine isolates (Girardeau et al., 2003; Lalioui et al., 1999). It was suggested that Afa-VIII are not associated with the colonization in the gastrointestinal tract of humans (Le Bouguénec et al., 2001; Lalioui et al., 1999). Fifty percent of *E. coli* positive of animal-specific *afa-8* operon co-existed with uropathogenic-related adhesion factors, including P, S, F17 fimbriae and hemolysis A, which are

virulence factors characterized in human extraintestinal pathogenic *E. coli* (Girardeau et al., 2003).

2.8. ExPEC

ExPEC or UPEC are often isolated from extraintestinal tissues of humans and animals, and considered as one of the major pathogens responsible for urinary infection (Brzuszkiewicz et al., 2006; Stamm and Norrby, 2001). Transmission of ExPEC from oral to gastrointestinal tract is not sufficient to establish the extraintestinal infection and the entry of the organism into an extraintestinal site, such as the urinary tract is required (Flores-Mireles et al., 2015; Russo and Johnson, 2000). Adhesion and invasion to the urinary cells of the hosts are achieved by multiple fimbriae, haemolysin and cytotoxic necrotizing factors (CNF) (Wiles et al., 2008). Virulence factors include α-hemolysin (*hyl*), P (*pap*), S (*sfa*), FIC (*foc*) fimbriae and aerobactin coding for iron-acquisition system were frequently found in patients with urinary tract infection (Tarchouna et al., 2013). P, F and S fimbriae and α-hemolysin are encoded on different pathogenicity islands (Brzuszkiewicz et al., 2006; Oelschlaeger et al., 2002; Wiles et al., 2008). F17 was not classified as UPEC virulence factors; however, F17 positive isolates had a high prevalence of encoding P fimbriae (Girardeau et al., 2003).

2.9. Acid resistance of gastrointestinal pathogens

Gastrointestinal organisms encounter an acid (pH 1.5-3.5) environment in the stomach. Acid tolerance is a primary component for the intestinal organisms to survive within the digestive system in humans and animals (Merrell and Camilli, 2002). Decarboxylase activities and urease activities are major acid resistance mechanisms that contribute to the survival and colonization of gastrointestinal pathogens (Castanie-Cornet

et al., 1999). The decarboxylase utilizes glutamate and arginine to consume protons to maintain the intracellular pH (Castanie-Cornet et al., 1999). Metabolism of urea to carbon dioxide results in an elevation of pH, which balances the proton-motive forces across the membrane and enhances the acid resistance of gastric organisms (Merrell and Camilli, 2002; Stingl et al., 2002). Urease activity contributes to the acid-tolerance in intestinal-pathogens, including *Helicobacter pylori*, *Campylobacter jejuni* (Merrell and Camilli, 2002) and gut microbiota, such as *Lactobacillus reuteri* (Frese et al., 2011; S.Teixeira et al., 2014) . The accumulation of ammonia is toxic for the uroepithelial cells and involved in the urinary tract infection caused by *P. mirabilis* (Flores-Mireles et al., 2015). The urease operon was predominantly identified in EHEC and reported in UPEC (Collins and Falkow, 1988, 1990; Nakano et al., 2001a); however, urease positive *E. coli* did not show enzymatic activity to break down urea *in vitro* (Nakano et al., 2001a).

2.10. Antibiotic resistance, extend spectrum of beta-lactamases (ESBLs)

Resistance to β-lactam antibiotics was first identified in *E. coli,* which produce the β-lactamases to develop the antibiotic resistance (Bradford, 2001). Bacteria developed resistance to a broad-range of β-lactam antibiotics, which is possibly due to the frequent exposure to antibiotics (Andersson and Hughes, 2010). ESBLs are a group of enzymes that break down the β-lactam ring of antibiotics and render the antimicrobial activity of penicillin and cephalosporin groups (Paterson and Bonomo, 2005). According to the Amber molecular classification based on the protein homology, β-lactamases are classified into classes A, C and D, which are described as serine β-lactamase, and class B, known as Metallo-β-lactamase (Ambler et al., 1991). Class A β-lactamase includes enzymes such as TEM-1 and SHV-1 (Bradford, 2001). Up to 90% of ampicillin resistant

E. coli encode genes for TEM-1 β-lactamase (Livermore, 1995a). Currently, the most common genetic variant of ESBL is CTX-M (Paterson and Bonomo, 2005). CTX-M-15 was first identified in *E. coli,* and then spread into other species of *Enterobacteriaceae* (Woodford et al., 2011). Both TEM and CTX can hydrolyze penicillin and cephalosporin (Bradford, 2001); while OXA-β-lactamase hydrolyzes oxyimino cephalosporin (3rd and 4th generation of cephalosporin) (Bush et al., 1995). OXA-1 was found in only 1-10% of *E. coli* isolates (Livermore, 1995a).

The prevalence of ESBLs in *E. coli* depends on the ecological system, where β-lactam antibiotics are wildly used to treat urinary tract infection (Flores-Mireles et al., 2015). This results in a high prevalence of CTX-M (66.7%), *bla-TEM* (40.8%) and *bla-SHV* (20.8%) in UPEC (Sedighi et al., 2015). In wastewater, a total of 17.1% of all ESBLs producing *E. coli* (n=170) were detected, including 4.1% EAEC, 1.8% EPEC, 1.2% EIEC, 1.2% ETEC; no ESBLs positive STEC was identified (Franz et al., 2015). Within those ESBLs positive phenotypes, the CTX-M-15 (41%) and CTM-X-1 were predominate in *E. coli* from wastewater isolates (Franz et al., 2015).

2.11. Locus of heat resistance (LHR)

Development of resistance to heat in *E. coli* contributes to their survival and persistence during food processing, where thermal processing is commonly used to reduce the microorganisms on food products. The LHR confers or correlates to the heat resistance of *E. coli* (Mercer et al., 2015)*, Salmonella enterica* (Mercer et al., 2017)*, Klebsiella pneumoniae* (Saxtorph Bojer et al., 2013)*,* and *Cronobacter sakazakii* (Gajdosova et al., 2011). The predicted genetic reading frame suggested that LHR encodes protein associated with heat shock, envelope stress and oxidative stress

(Mercer et al., 2017). LHR positive *E. coli, S. enteria* were reduced by less than 1 and 1 $\log_{10}$ CFU/mL after treatment with 60 °C for 5 min, while LHR-negative were reduced by higher than 7 $\log_{10}$ CFU/mL after the identical heat treatment (Mercer et al., 2017). LHR-positive isolates were detected after thermal processing during food manufacturing (Dlusskaya et al., 2011). A particularly high prevalence (59%) of LHR positive *E. coli* was quantified in the surviving *E. coli* after chlorination of wastewater (Zhi et al., 2016a). The presence of LHR might ecologically relate to environmental pressure.

2.12. Horizontal gene transfer of virulence and resistance genes in *E. coli*

Horizontal gene transfer plays an important role in the evolution of *E. coli,* particularly for the pathogenic *E. coli* (Nogueira et al., 2009; Ochman et al., 2000; Rabsch et al., 2002). EHEC O157:H7 EDL933 has a 12% difference in gene content compared to the commensal isolate *E. coli* MG1655, which suggests that frequent horizontal gene transfer was involved to increase the genome diversity (Perna et al., 2001). Acquisition of foreign genes is mediated by transformation, transduction and conjugation. Transduction mediated by bacteriophage occurs more frequently to transfer virulence genes, compared to transformation of naked DNA fragments and plasmid conjugation (Ochman et al., 2000). In EHEC, 43% of unique genes are associated with prophage and phage elements (Rasko et al., 2008). Phage play a significant role in the evolution of this pathovar (Fidelma Boyd and Brüssow, 2002). Bacteriophage also encodes virulence genes in other species, including cholera toxin in *Vibrio cholera,* and the T3SS system in *Salmonella* (Fidelma Boyd and Brüssow, 2002). Acquisition of the T3SS system, encoded on pINV in EIEC, from *Shigella* was proposed to contribute to the evolution of EIEC (Lan et al., 2001). Maintenance of pINV is critical to *Shigella* and

EIEC (McVicker and Tang, 2017). Specifically, pINV is a non-conjugative plasmid. The stability of pINV is mediated by the toxin-antitoxin system and dependents on the distinct lifestyle (McVicker and Tang, 2017; Pilla and Tang, 2018). Most of the adhesion factors are encoded on the mobile genetic elements, and the acquisition of adhesion factors is likely driven by the host adaptation (Worley et al., 2018).

Acquisition of stress resistance genes allows microorganisms to extend their ecological niches. Antibiotic resistance genes are transferred across species by plasmids, bacteriophage and transposons (Colomer-Lluch et al., 2011; Ingle et al., 2018; Rashid and Rahman, 2015). The LHR was found in different species (Mercer et al., 2017), which suggests it was acquired by transformation across species.

2.13. Zoonotic transmission

STEC/EHEC has been identified as the only pathogenic groups of *E. coli* that have a zoonotic origin (Karesh et al., 2012). Ruminant animals including cattle, sheep, goats, buffalos and deer are major reservoirs for EHEC, particular for O157 serotypes (Caprioli et al., 2005; Gansheroff et al., 2000). *E. coli* O157:H7 is the most commonly identified serotype of STEC, and is dominant in North American herds ranging from 10-28% of cattle in feedlots (Sargeant et al., 2003). Fecal shedding of EHEC O157, O111 and O26 by cattle is frequent, particularly in the warmer months and after weaning (Karmali et al., 2010). EHEC O157 was isolated from slaughter pigs and birds, but they are not considered to be a true reservoir (Caprioli et al., 2005). STEC that carry Stx2f are frequently isolated from pigeons (Schmidt et al., 2000). STEC can cause disease in young calves, weaning pigs and dogs (Caprioli et al., 2005). Zoonotic transmission of other

pathogroups is not well established. Contaminated food, water and sewage are major vehicles to transmit ETEC, EPEC and EAEC to humans (Croxen et al., 2013).

2.14. Conclusions

The diversity of *E. coli* and their virulence factors are remarkable for food safety and public health. Foodborne illness caused by contaminated food remains a major problem in the food industry. The evolution of pathogenic *E. coli* still continues, as indicated by the EAEC and STEC hybrid strain that caused a major outbreak in Germany in 2011 (Grad et al., 2013b; Hazen et al., 2017). The recombination of virulence traits from two pathogroups infers that a rapid transmission of virulence might occur in the environment. Understanding the factors underlining the evolution and ecology of pathogenic *E. coli* are significant to control the spreading of foodborne infection.

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CHAPTER 3. Genomic Insights to Pathogenicity, Host Adaptation and Niche Specialization of *Escherichia coli*

3.1. Introduction

Escherichia coli are facultative aerobes with extremely diverse serotypes, genetic composition, host-adaptation and pathogenesis (Croxen et al., 2013; Whittam et al., 1993). The majority of *E. coli* isolates are commensal organisms in the intestinal tract of animals but some strains are pathogenic to humans and/or animals (Croxen et al., 2013; Pupo et al., 1997). The prevalence of *E. coli* in the gut microbiota is more than 90% in humans but only 56% in wild mammals, 23% in birds and 10% in reptiles (Gordon and Cowling, 2003; Tenaillon et al., 2010). Pathogenic *E. coli* evolved through gene gain and gene loss from a common ancestor of commensal *E. coli* (Reid et al., 2000). Seven major groups of pathogenic *E. coli* that can cause disease in humans include enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC) and *Shigella*, enteroaggregative *E. coli* (EAEC) (Croxen et al., 2013; Levine, 1987; Pupo et al., 1997). *E. coli* colonized on extraintestinal tract are classified as extraintestinal pathogenic *E. coli* (ExPEC), and of those, *E. coli* that are related to urinary tract infection are known as uropathogenic *E. coli* (UPEC) (Stamm and Norrby, 2001).

Each pathogenic group is defined with a set of specific virulence determinants including adhesion factors and enterotoxins or cytotoxins (Croxen et al., 2013; Croxen and Finlay, 2010), which are mostly encoded on the mobile genetic elements (Ogura et al., 2009; Schmidt and Hensel, 2004). Acquisition of virulence genes by lateral gene transfer contributes to the genomic diversity of pathogenic *E. coli* (Ochman et al., 2000).

Recombination of virulence factors leads to the generation of novel pathovars, such as the Germany outbreak strain enteroaggregative *E. coli* O104:H4, which obtained *stx* by phage lysogenic infection (Muniesa et al., 2012).

To further understand the phylogeny of *E. coli,* six phylogenetic groups, A, B1, B2, D, and E were initially identified using 72 strains with diverse natural origins (Chaudhuri and Henderson, 2012). *E. coli* are considered as host-promiscuous (Gordon, 2003; Gordon and Cowling, 2003; Skippington and Ragan, 2012; Zhi et al., 2016b). However, few studies have indicated a certain level of host-adaptation in *E. coli,* as exemplified with human-adapted *E. coli* O81 clones (Clermont et al., 2008), and cattle-adapted *E. coli* O157:H7 (Kim et al., 1999).

Host specialization has been well established in *Salmonella* at the subspecies level, and at the level of serotypes, which are monophyletic or polyphyletic (Bäumler and Fang, 2013; Laing et al., 2017). For example, *Salmonella enterica* serovar Typhi is a human-specific pathogen; *Salmonella* Cholerasuis is specialized to swine and *Salmonella* Typhimurium is a generalist serotype that causes less severe disease but can switch between humans and animal hosts (Rabsch et al., 2002; Tsolis et al., 1998). Host adaptation of *Salmonella* is associated with both gene acquisition and genome degradation; in particular, host adapted *Salmonella* serovars encode for specific *Salmonella* pathogenicity islands (SPI) (Klemm et al., 2016; McClelland et al., 2004). In contrast, *E. coli* pathovars and the corresponding complement of genes mediating virulence has not been convincingly linked to specific serotypes or phylogroups and virulence genes are considered to be transferred widely by horizontal gene transfer. The evolution of pathogenic *E. coli* occurred at multiple phylogenies (Pupo et al., 2000, 1997),

and is dependent on the acquisition of mobile genetic elements (Ogura et al., 2009; Reid et al., 2000). Virulence factors were part of the pan-genomes, which might contribute to the bacterial survival and environmental adaptation and likely acquired by bacteria driven by the environmental stress. The ecological selection on the development of emerging virulence traits of pathogenic *E. coli* and the relationship between pathotypes, serotype, and phylogenetic position are not well elucidated. To better understand the genetic relationships and the overall genetic structure of *E. coli* and *Shigella,* this study aimed to determine 1) the distribution and co-occurrence of virulence factors and resistance genes in *E. coli* and *Shigella*; 2) the relationship between the phylogeny and genetic variation; and 3) the impact of natural selection and ecology on the genetic shifts and evolution.

3.2. Materials and methods

3.2.1. Data collection and *in silico* serotyping

A total of 13,717 draft genomes including scaffolds and contigs of *E. coli* and *Shigella* were retrieved from GenBank. The serotypes of the genomes were predicted by the comparison of *E. coli* genomes against SerotypeFinder (www.genomicepidemiology.org/) (Joensen et al., 2015). The assembly quality of each genome to the serotype database was assessed using Assembly-stats. The genomes with high assembling quality were used to construct the phylogenetic tree. The local database contains 1,702 genomes that represent non-redundant serotypes.

3.2.2. Phylogenetic analysis

The core-genome phylogenetic tree of *E. coli* and *Shigella* was constructed according to the previously described methods (Zheng et al., 2017). To assure the

consistency and reduce the variation among a total of 1,702 genomes used in this study, all genomes were annotated or re-annotated by Prokka (Seemann, 2014). The divergence or cluster of each phylogenetic branch was calculated based on the substitution rate of the nucleotide sequence among the orthogroups (Yang, 1994). A total of 946 core-genes were identified by the pipeline produced by Roary (Page et al., 2015). The orthologous genes were concatenated using SNP-sites (Page et al., 2016). The maximum likelihood (ML) phylogenetic tree was constructed by RA×ML using the generalized time reversible (GTR) model and the gamma distribution model to estimate the site-specific rate variation (Price et al., 2010; Whelan and Goldman, 2001). Bootstrap values were calculated with 1,000 replicates. The phylogenetic groups were estimated by the phylogenetic structure using RhierBAPS (Tonkin-Hill et al., 2018). Five phylogenetic groups A, B1, B2, D, and E/F were defined.

3.2.3. Detection of virulence factors

The 13,717 draft genomes were translated to protein sequence by Prodigal (Hyatt et al., 2010). The information on virulence factors associated with *E. coli* and *Shigella* were collected from the online database (www.mgc.ac.cn/VFs/main.htm) and downloaded from GenBank. Over 400 virulence genes were imported into the in-house database, including genes encoding adhesion fimbriae, afimbrial proteins, toxins, and its related subtypes and variants. A collection of 13,717 genomes was screened against the database using BLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to detect the presence of known acquired virulence genes in each genome. The positive detection had 60-80% identity and 60% coverage of the amino acid sequence. The multivariate matrix that constitutes the association of virulence factors with each genome was generated, and

transformed into a binary table by in house Perl script in R to indicate the presence or absence of virulence genes alleles.

3.2.4. Detection of urease, locus of heat resistance, and antibiotic resistance

A similar workflow was applied to detect the stress and antibiotic resistance factors. Acid resistance mediated by urease activity was encoded by UreC (Nakano et al., 2001a). Locus of heat resistance (LHR) confers the function of heat resistance, and was found in *E. coli* isolated from a beef processing plant, and wastewater after chlorination (Mercer et al., 2015; Zhi et al., 2016a). The β-lactamase encoding genes $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1}}$, and $bla_{\text{OXA-1}}$ are commonly found in *E. coli* (Bradford, 2001), and were selected to screen against the *E. coli* genomes. The stress resistance genes were extracted from the whole genome of *E. coli,* and followed by the conversion to amino acids by Prodigal. The presence of the protein sequence was determined in each genome of *E. coli* by BLASTn. The positive detection had 60-80% identity and 60% coverage of the amino acid sequence.

3.2.5. Construction of co-occurrence network of Shiga toxins

The sequences of Stx subunits A and B of each type and variant were screened across 13,717 draft genomes as in section 3.2.3. A pairwise co-occurrence matrix of the presence of StxA and StxB was constructed by transformation of the binary Stx gene matrices in R. The co-occurrence of A and B subunits of Stx was visualized using the package of UpSet in R software (Conway et al., 2017). A pairwise occurrence between subunit A and B was visualized by Cytoscope software (Demchak et al., 2014). In the network of virluence genes, the nodes represent genes and the frequency is indicated by numeric values on the edges.

3.2.6. Construction of co-occurrence network of virulence factors

The pairwise occurrence among virulence factors was transformed by R and visualized by Cytoscope software. The edges of the network represent the co-occurrence, and the wildness of the edges was proportional to a given threshold (set to >1000, 450-550, 140-240, 70-111. 40-61, 10-40, <10). To improve the clarity of the network, the subtype information of the same fimbriae types was omitted and consistently labeled with the major family name of the fimbriae. For example, this study included four subtypes of Agg fimbriae and four genes encoding of each Agg variants, which were all included in the database; but only designated with Agg in the table and figures.

3.2.7. Genome size of *E. coli* and *Shigella*

The average genome size of *E. coli* and *Shigella* was calculated by a total of 9,681 and 2,259 genomes, respectively. The significant difference of the genome size between *E. coli* and *Shigella* was performed by T-test with $P<0.05$ using SigmaPlot (v.12.5., Systat Software Inc., UK).

3.3. Results

3.3.1. Phylogenetic structure of *E. coli*

To gain insight into the correlation between phylogeny and the distribution of virulence factors, a core-genome phylogenetic tree was constructed with 13,717 genomes of *E. coli* and *Shigella.* Serotypes of the strains were assigned *in silico* from the whole genome sequence, and 1,702 non-redundant serotypes were identified. The tree with the 1,702 non-redundant serotypes is shown in Figure 3.1. *Shigella* strains were distributed over the whole phylogeny but only few *Shigella* were identified in phylogroups D and B2.

The presence of major virulence factors and resistance genes are labeled for each serotype in the phylogenetic tree. *Shigella* strains with the pINV encoding cell-invasive ability (Nhieu and Sansonetti, 1999) were mainly clustered in two locations in the phylogenetic lineage B1, and also found in lineage A. Plasmids pINV were present in strains of *E. coli* that have not been identified as *Shigella.* The mean of genomes deposited with the designation of *Shigella* was 4.6 Million base pairs (Mbp) was significantly smaller than 5.1 Mbp, which is the mean of genomes deposited with the designation *E. coli* ($P<0.05$).

The antibiotic resistance genes mediated by four classes of β-lactamases were dispersed over the tree; TEM-1 was the most frequently identified. Strains assigned to all phylogroups show a mosaic virulence profile. The phylogenetic lineages B2 and D show a particularly high prevalence of P fimbriae associated with UPEC. LEE or Stx were spread among five lineages with higher prevalence in groups B2, A and B1; however, the combination of LEE and Stx was more abundant in B1. Other virulence factors including ST, LT and Agg were randomly distributed across the tree. Agg fimbriae occurred less commonly and were scattered over the phylogeny. LHR appeared to be strongly associated with phylogroup A and was occasionally located in B1 but excluded from groups D, B2 and E/F. Overall, the virulence factors associated with gastrointestinal pathogens frequently occurred in lineage B1; the LHR was largely confined to group A, which had a lower prevalence of virulence genes compared to B1. The phylogenetic tree of representative serotypes shows a potential relationship between phylogeny and genotypes that encode genes for virulence and heat resistance.

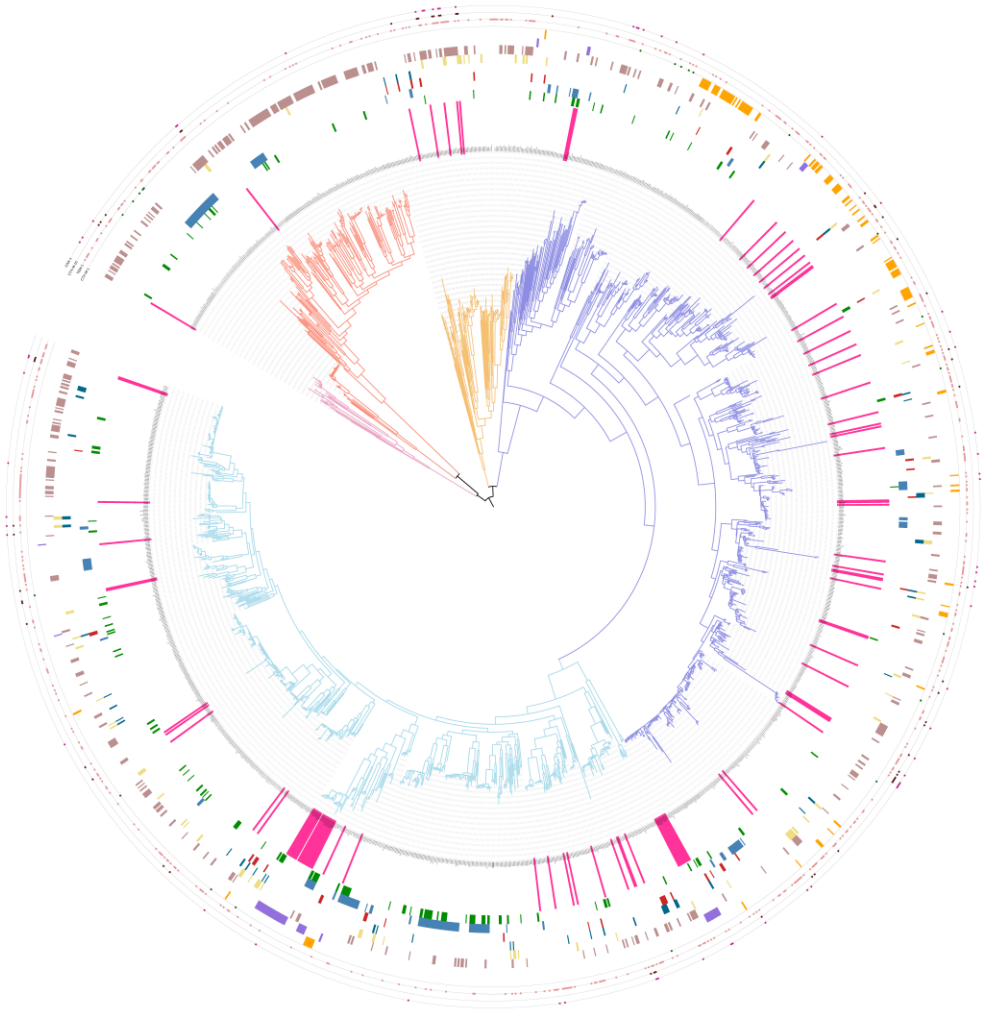

Figure 3. 1. Phylogenetic tree, virulence profile and antibiotic resistance of *Escherichia coli*. A core genome phylogenetic tree was constructed based on 946 core genes using 1719 sequenced genomes. The phylogenetic groups of *E. coli* are color coded on the branches: A, purple; B1, blue; B2, orange; D, pink; E/F, yellow. The figure shows one representative strain of 1702 serotypes. The presence of virulence genes, LHR and β-lactamase are annotated using a color-coded arrangement from the inner to the outer circle of the tree. The serotypes associated with *Shigella* are indicated by the highlighted pink color around the tree. The virulence genes are color-coded from the inner to the outer layer of the tree with the following: Stx, green; LEE, blue; ST, red; LT, navy-blue; Agg, yellow; Pap, rosy-brown; pINV, purple; LHR, orange. The β-lactamase resistance genes are color-coded with a smaller icon located in the outermost layer of the tree.
OXA-1, violet red; CTX-M-15, dark maroon; TEM-1, light pink; CTX-M-1, green.

Therefore, a comprehensive co-occurrence analysis within the interactions among different types of toxins and/or adhesion factors was conducted to address the relationship of ecology and phylogeny in combination with virulence factors.

3.3.2. Co-occurrence of Shiga toxin subunit A and B

Both Stx type 1 and type 2 are encoded on the phage genome, and are composed of major subunits A and B (Trachtman et al., 2012). Genomes of STEC often encode for two Stx; moreover, lambdoid prophages have a flexible genome structure that is characterized by frequent recombination (Smith et al., 2012a). Figure 3.2 shows the combination of Stx subunits A and B, including the mismatch of A and B subunits from different Stx variants. The combinations of at least two subunits are shown in Figure 3.2.A. A single and co-existence of three subunits were detected due to incomplete genome sequencing or alignments (Figure 3.2.A). Figure 3.2.B. only shows the pair-wise co-occurrence of subunits A and B. Subunits derived from Stx2d were frequently recombined with other types of Stx and often with Stx2a. Overall, the prevalence of mismatch of A and B subunits was lower compared with the frequency of the correct-pairs. Therefore, the subunit A was representative of the intact Stx in the subsequent analysis.

3.3.3. Co-occurrence of featured virulence factors derived from STEC, ETEC, EAEC and EIEC

Genes with a high prevalence in a specific pathotype were used as screening markers to identify the pathogroups of an unknown isolate. A network of the co-occurrence of Stx, LT, ST, Agg, LEE and pINV was constructed to illustrate the cross-interaction between different pathotypes (Figure 3.3). In the network, the nodes are

labeled with the respective virulence factors, and the edges represent co-occurrence. Heat-labile enterotoxin (LT) is composed of subunits A and B, which are encoded by genes *eltA* and *eltB* (Spanglert, 1992). The pINV was associated only with Stx1A but not with other Stx. LT was associated mainly with Agg and LEE was associated mainly with Stx2a, Stx1A, and Stx1d.

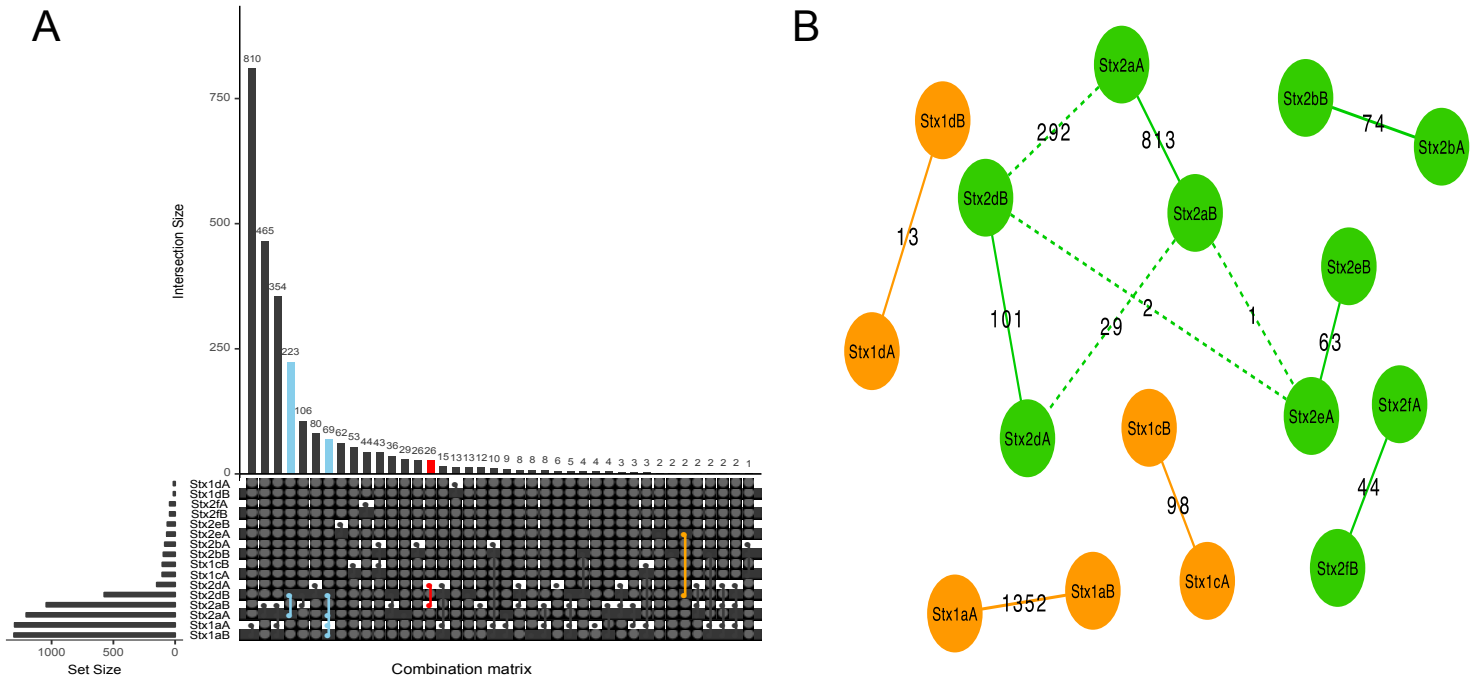

Figure 3. 2. Co-occurrence of Shiga toxin subunits A and B. The co-occurrence of Stx subunits was constructed from 2881 *E. coli* genomes. **A.** Frequency of the co-occurrence of Stx subunits. Each row of the matrix table represents a Stx subunit. In the matrix, cells with the same color in each column illustrate that the corresponding subunits occurred in the same genome. The mixed pairs of Stx subunits are highlighted in blue, red or yellow. The co-occurrences of Stx subunits are shown by the intersection size. The set size indicates the total number of genomes encoding the Stx subunits corresponding to each row in the table. **B.** Pair-wise co-occurrence of Stx subunits. Subunits of Stx 1 and Stx 2 are color-coded with orange and green, respectively. The co-occurrence of two Stx subunits is connected by solid lines if the two subunits belong to the same subtype of Stx. Dash lines indicate the Stx subunit A was mixed pair with other variants of subunit B. The number of times that two subunits have occurred is shown between the two subunits.

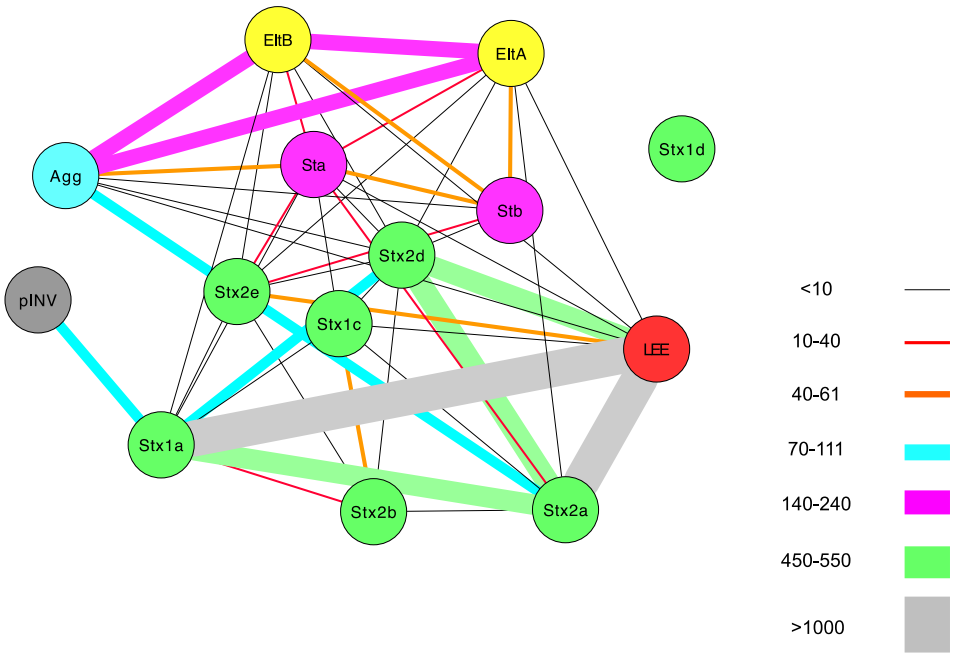

Figure 3. 3. Co-occurrence network of genes encoding toxin and adhesion factors of pathogenic *Escherichia coli*. The network was built with 6585 sequenced genomes from Genbank sequence database from NCBI. Solid lines connect genes that occurred together in the same genome. The width of each line is proportional to the number of times that the two virulence factors occurred as shown in the figure legend.

3.3.4. Distribution of adhesion and virulence factors in pathogenic *E. coli.*

Colonization on the epithelium mucosa is crucial to establish the infection of pathogenic *E. coli*. To investigate the prevalence of adhesion factors in Stx producing *E. coli*, enterotoxigenic *E. coli* and *Shigella* species, the co-occurrence of virulence factors and adhesion fimbriae within 13,441 genomes is shown in Table 3.1. Flagellin, long polar pills (Lpf), and FIC fimbriae were ubiquitously distributed in all pathotypes of *E. coli.* Bundle-forming pili (Bfp) were rarely found together with STEC and ETEC.

Saa, the auto-agglutinating adhesion was subsequently determined in various LEE-negative EHEC (Jenkins et al., 2003; Paton et al., 2001). Saa was only found in STEC, but not in Stx1d and 2e producing *E. coli*. Fimbriae associated with human- and animal-ETEC co-occurred with heat labile enterotoxin (LT) and heat stable enterotoxin (ST) but show a distinct virulence combination with Stx. Fimbriae associated with human-ETEC did not co-occur with Stx; however, fimbriae in animal-ETEC co-occurred with different variants of Stx. In particular, the F18 fimbriae was predominant in 70% of *E. coli* encodes Stx2e. F4 (K88) operon was not strain-specific, but more frequently detected in ETEC, STEC (Stx1c, Stx1d, Stx 2d) and *Shigella*.

Aggregative adhesion fimbriae were frequently combined with heat LT, and STa. AAF type I were predominant in LT and STa positive *E. coli*; however, AAF type II were not found in all the phylogroups. EAEC associated membrane protein Aat co-occurred with Stx2a and LT. Fimbriae type P, S, FIC and F17 fimbriae in UPEC co-occurred with Stx, LT and ST. In STEC, the P fimbriae were highly co-evolved with Stx1d-producing *E. coli.* The F1C fimbriae were found in all the intestinal pathogenic *E. coli,* except for *Shigella*. DAEC encodes a group of fimbriae, in which Afa was more frequently found in all pathogroups compared to Dr and F1845. The F1845 fimbriae were only detected in the same genome with Stx1c and Stx2b. The Dr fimbriae were not correlated with the other types of toxic encoding genes.

3.3.5. The abundance of stress resistance in pathogenic *E. coli*

To unravel the pressure from stressors that exist in nature on the diversity of stress resistance genes, the prevalence of selected genes contribute to acid, heat and antibiotic resistance were detected. The most striking association of resistance mechanisms and

virulence were observed with urease, *bla*$_{\text{OXA-1}}$ and the LHR. The LHR was present in 2.4% of *E. coli* genomes but only 74 of those 326 genomes also carried virulence factors. Presence of the LHR in *E. coli* genomes excludes all other virulence factors except Agg, LEE, Pap, LT and ST (Figure 3.4). More than 90% of urease-positive genomes also encoded LEE, and most of those genomes also carried Stx1a, Stx2a or Stx2d. Genomes encoding Stx rarely encode genes associated with ESBLs (Figure 3.4). The *bla*$_{\text{TEM-1}}$ occurred in association with all virulence factors, except Stx1d and Stx2b. The majority of genomes encoding a combination of Agg and Stx also encoded the β-lactamase CTX-M-1 and TEM-1.

TEM-1-β-lactamase was relatively abundant in EHEC, EAEC, ETEC, and *Shigella* (Figure 3.4. panel B), but pathogenic *E. coli* were not a major attributor of β-lactamase resistant isolates (Figure 3.4. panel B). Most of the Agg and Stx bearing genomes were sequenced from the foodborne outbreak caused by EAHEC in Germany in 2011. About 50% of genomes with *bla*$_{\text{OXA-1}}$ also contained *Shigella* invasion plasmid pINV but *bla*$_{\text{OXA-1}}$ was rarely associated with other virulence factors.

Table 3. 1. Frequency of co-occurrence of virulence factors and adhesion factors. The frequency were calculated as % of strains that encode toxin as indicated in the columns and adhesion factor indicated in the corresponding row to the total number of strains that encode toxins.

Virotype			Shiga toxins							Heat labile Enterotoxin		Heat Stable Enterotoxin		*Shigella* T3SS
		# of strains	Stx1a 1409	Stx1c 108	Stx1d 13	Stx2a 1402	Stx2b 96	Stx2d 666	Stx2e 70	EltB 275	EltA 354	Sta 169	Stb 136	IpaH 1887
Flagellin	Fml	13441	99.5	97.2	100	99.8	94.8	99.4	98.6	96.7	97.5	100	100	97.2
tEPEC	Bfp	332	0.4	0	0	0.7	0	0.2	0	5.8	0	0	0	0.1
aEPEC/ EHEC	Lpf	6776	64.7	81.5	61.5	39.5	82.3	28.0	51.4	53.1	60.5	59.8	56.6	94.5
	Eae	2772	80.4	1.9	0	76.8	0	69.4	1.4	3.3	2.3	3.6	0	0
	Saa	162	4.9	10.2	0	7.0	10.4	8.3	0	0	0	0	0	0
ETEC	Cs4	38	0	0	0	0	0	0	0	1.8	5.6	1.2	0	0
	Cs17	69	0	0	0	0	0	0	0	4.0	9.9	0	0	0
	CFA/I	57	0	0	0	0	0	0	0	4.0	4.0	10.1	0	0
	Cs19	49	0	0	0	0	0	0	0	6.9	10.7	1.8	0	0
	Cs22	15	0	0	0	0	0	0	0	0	0	0	0	0
	Lng	110	0	0	0	0	0	0	0	14.2	15.3	10.7	0	0
	F18	384	0	0.9	7.7	2.5	2.1	5.3	70	4.4	3.7	23.1	23.5	0
	F4 (K88)	717	0.9	13.0	15.4	1.6	14.6	2.3	2.9	17.8	16.9	24.9	21.3	5.6
	K99	22	0	0	0	0	0	0	0	0	0	4.7	0	0
EAEC	Agg	580	0	0	0	5.8	0	0.2	1.4	53.5	62.7	34.9	1.5	0
	Aat	209	0	0	0	4.9	0	0	0	3.3	6.8	0	0	0
	Aaf	104	0	0	0	0	0	0	0	0	0	0	0	0.1
UPEC	P fimbriea	2845	1.9	1.9	38.5	2.4	6.3	2.4	4.3	7.3	5.4	4.7	2.9	0
	FIC	8633	88.5	71.3	84.6	95.7	70.8	91.6	54.3	55.3	61.3	58.0	63.2	1.4
	S fimbriea	732	0	1.9	0	0.1	1.0	0	0	0	0	0	0	0
	F17	383	0.1	0.9	38.5	0.1	1.0	0.3	0	0	0	0	0	0
DAEC	Afa	573	1.8	2. 8	15.4	0.4	4.2	1.7	1.4	1.8	3.1	3.6	0	0.4
	Dra	240	0.1	0	0	0.1	0	0.3	0	0	0	1.2	0	0
	Daa	191	0.21	10.2	0	0.1	10.4	0.2	0	0	0	1.2	0	0

Figure 3. 4. Co-occurrence frequency of resistance genes (Res gene) with virulence factors (VF). The frequency of the number of genomes that encode both VF and Res genes to the total number of genomes encoding resistance genes (panel A) or to the total number of genomes encoding virulence gene (panel B). Resistance genes are color-coded as follows: UreC, yellow; LHR heat resistance, purple; resistance genes of β-lactamase resistance including CTX-M-1, blue; CTX-M-15, green; TEM-1, black; and OXA-1, pink

3.4. Discussion

E. coli are widespread gut commensals of vertebrates (Gordon and Cowling, 2003; Gordon, 2003), but also diverse pathogens with a growing emergence of novel pathotypes (Croxen et al., 2013). As such, it is intriguing to explore the transition from commensalism to pathogenicity. Due to the significant impact of strains of EHEC on food safety and agricultural economics, pathogenic strains of *E. coli* have been intensely studied but fewer studies have focused on commensal strains and non-food outbreak strains (Tenaillon et al., 2010). This results in a strong sampling bias towards pathogenic strains, and more specific human-illness related pathogenic strains, which is also the limitation of the in *silico* analysis of this study. The database of *E. coli* genomes constructed in this study consisted of 20.7% UPEC, 20% of EPEC, 10% ETEC, 6.6% EAEC, 7.4% DAEC, and 2.4% of tEPEC, which skewed the sequencing database towards an abundance of pathogenic groups. This is a consequence of prioritizing clinical and veterinary disease. However, the genome database is unbiased on the perspective of serotypes as the genomes were collected from 1,702 non-repetitive serotypes to present the complete phylogeny of *E. coli* and the limitation of sampling bias is partially compensated by analysis of a large number of genomes. To make an unbiased interpretation, the discussion focused on the factors underlying the ecological and evolutionary contexts.

E. coli are commonly defined with O and H antigen, which represent cell-wall lipopolysaccharide component, and flagella filaments, respectively (Hussain, 2015). Virulence factors are associated with a broad range of serotypes; however, the combinations of virulence were specific for each pathogroup and limited to a narrow

range of serotypes (data not shown). The influence of the co-selection of serotypes and virulence genes on the evolution of the *E. coli* phylogeny is not well demonstrated.

Genetic variation and genome reduction are evolutionary processes, which are commonly observed in the specialization of host adaptation (Duar et al., 2017; Lo et al., 2016). *E. coli* have a high degree of genome plasticity, particular for pathogenic *E. coli,* which contain an average of 4,721 genes, but only 2,000 genes are part of the core genome (Hendrickson, 2009). Commensal *E. coli* have a significantly smaller genome size than pathogenic *E. coli,* which contain additional 500-1,000 genes (Rasko et al., 2008). Acquisition of virulence factors from genetic elements rather than genome deletion is attributed to the evolution of *E. coli* pathovars (Rasko et al., 2008; Reid et al., 2000). Niche- or host-adaptive evolution is manipulated by gene gain and loss (Klemm et al., 2016; McClelland et al., 2004). Host-adaptation in *Shigella* involved genome degradation (Feng et al., 2011; this study). Low virulence diversity of *Shigella* infers narrow ranges of niche- or host-adaptation, but instead confers a strict host-adaptation (this study). *Shigella* clones have arisen from multiple independent lineages of *E. coli* in this study, and was confirmed by previous studies (Pupo et al., 2000; The et al., 2016).

The phylogenetic analysis from this study manifested a link between phylogeny and P fimbriae associated with ExPEC. Similar research confirmed that the ExPEC related virulence genes are more frequently found in phylogroups B2 and D, while they were also found among other lineages in the phylogeny (Bingen-Bidois et al., 2002; Escobar-Páramo et al., 2004; Johnson et al., 2001; Johnson and Stell, 2000; Picard et al., 1999). *Salmonella* also reveals similar pattern of fimbriae distribution, where two

fimbrial operons *stf* and *lpf* were only conserved among clade A but not in clade B (den Bakker et al., 2011).

The link between intestinal pathogens and phylogeny is not as strong as the phylogenetic correlation with extra-intestinal pathogens. Phylogroups B2 and D were more specialized to the endothermic vertebrate, while A and B1 appeared to be generalists (Carlos et al., 2010; Gordon, 2003; Gordon and Cowling, 2003). Human commensals were more often within groups A and B; however, the distribution varies depending on the diet and body mass of animals, climate and geographic origins (Escobar-Páramo et al., 2006; Gordon and Cowling, 2003; Tenaillon et al., 2010). *E. coli* are generally adapted to the large intestine of mammals; however, the precise lifestyle and ecological niches corresponding to the phylogeny are unclear.

Intestinal pathogens were located among the phylogenetic tree that was constructed with the core-genome or multi-locus enzyme electrophoresis (Escobar-Páramo et al., 2004; this study). Different hosts represent different ecological niches, and the mechanisms related to niche-adaptation vary from host to host. A recent study developed a bio-marker based method and investigated the host-adaptation of *E. coli* between humans and cattle (Zhi et al., 2016b). Cattle are natural reservoirs of EHEC, in particular of serotype O157:H7 (Karmali et al., 2010), which encodes intimin and Stx (Schmidt et al., 1995). In this study, a strong correlation between phylogeny and EHEC was not confirmed; however, the specialization of co-occurrence of LEE and Stx was demonstrated. Co-occurrence of LEE and Stx infers a co-selection between host and genotypes during the interaction of bacterial adhesion factors and intestinal epithelium receptors (Cleary et al., 2004). Specialization with a combination of colonization factors

and toxins were also found in human- and animal-adapted ETEC (Gaastra and De Graaf, 1982; Mentzer et al., 2014; this study). Specifically, a combination of Stx and colonization factors associated with human ETEC was not identified in this study. However, the pig-diarrheal-associated F18 was highly correlated with Stx2e, which are specific to diarrhea and edema in pigs (Fratamico et al., 2004; Frydendahl, 2002). The strong co-occurrence of virulence factors predicts that ecological force or host properties could play a role in shaping genetic variants. The distribution of colonization factors encoded in ETEC was clustered in strains, which share a similar genetic background (Mentzer et al., 2014). The evolution of pathogenic *E. coli* is related to the uptake and retention of virulence factors into the *E. coli* genome via horizontal gene transfer (Escobar-Páramo et al., 2004; Reid et al., 2000). A similar scenario was found in *Salmonella,* where the distribution of *Salmonella* pathogenic islands (SPI) and fimbriae adhesion is specific to specific serovars and phylogenetic clades, indicating the role of niche specialization (den Bakker et al., 2011; Jacobsen et al., 2011). Specifically, SPIs were distinct in human-adapted *S.* Typhi and cold-blooded animal adapted *S. arizonae*; fimbriae types, combination of virulence factors (SPI-18 and CtdB-islet) are differentially distributed in serovars associated with clade A and B, which might involves host-specific colonization and fitness benefits, respectively (den Bakker et al., 2011).

Phylogenetic analysis from this study reveals a strong correlation between phylogroup A and niche adaptation by the acquisition of locus of heat resistance (LHR), which mediates multiple stress resistance mechanisms (Mercer et al., 2015). Strains from group A possess a low level of intestinal virulence genes, particularly in strains associated with LHR (~14kb). The low frequency of LHR co-existing with virulence

factors could be related to environmental adaptation. Specifically, bacteria might not acquire and maintain the LHR if it did not benefit for their survival in the ecological system. Moreover, the maintenance of large genetic elements also consumes overall fitness cost (Rajon and Masel, 2013). However, environmental stress dominates the selective force of resistance genotypes even if resistance comes with the cost of host fitness, as exemplified by the persistence of antibiotic-resistant bacteria in the environment without antibiotics (Andersson and Hughes, 2010; Andersson and Levin, 1999; Levin et al., 1997). Environmental stress on the selection of LHR was illustrated by Zhi et al. (2016), who found that over 50% of chlorine-resistant strains isolated from wastewater possessed the LHR, which was exclusive to phylogroup A (Zhi et al., 2016a). The phylogeny and ecology of *E. coli* carrying LHR further demonstrated that LHR is associated with environmental-adapted hosts, which are most divergent from commensals in animal (Zhi et al., 2016a), and pathogenic *E. coli* (this study), both of which are generally adapted to the gut of warm-blooded animals (Gordon and Cowling, 2003). In addition, EHEC appeared to be co-evolved with the acid resistance mechanisms regulated by urease activity (Nakano et al., 2001b); however, the evidence on the acid resistance regulated by urease operon *in vivo* is deficient (Nakano et al., 2001a). Urease activity confers acid resistance to the gastric pathogen *Helicobacter* and the animal commensal *Lactobacillus reuteri,* and is essential for these organisms to colonize their respective hosts (Ferrero and Lee, 2009; Krumbeck et al., 2015; Scott et al., 1998; Stingl et al., 2002). To prove the fitness preference of LHR and urease, additional competition experiments involving the comparison of the fitness of the wild types strains and LHR or urease deficient strains in the natural environment or low pH, are necessary.

Lateral gene transfer produces an extremely dynamic structure in genome composition, and is driven by environmental pressure (Ochman et al., 2000). The majority of the virulence factors were encoded on mobile genetic elements, such as the pathogenicity island of LEE, plasmid-borne fimbriae and phage-encoded Stx (Gamage et al., 2004; Schmidt and Hensel, 2004). Lateral gene transfer contributes to the acquisition of virulence genes of many pathogens (Groisman and Ochman, 1996; Hacker et al., 1997).

Resistance to β-lactam antibiotics including penicillin, ampicillin and cephalosporin is regulated by the ESBLs, which are predominant in Gram-negative bacteria (Bradford, 2001). Ampicillin resistance mediated by β-lactamase type TEM-1 was first identified in *E. coli* and contributed to ampicillin resistance in over 90% of *E. coli* (Bradford, 2001; Livermore, 1995b). High prevalence of *Shigella* in human-adapted pathogens shows a high prevalence of OXA class of β-lactamase enzymes, mediating the antibiotic resistance to 3rd or 4th generation of cephalosporin (Siu et al., 2000; Connor et al., 2015; this study). Lateral gene transfer of plasmid-born ESBL could have occurred between commensal *E. coli* and *Shigella* in the human gut as *Shigella* are frequently exposed to the intestinal commensals in humans (Rashid and Rahman, 2015). Other antibiotics are used to treat traveler's diarrhea caused by EAEC or ETEC (Croxen et al., 2013) and it is still used as an effective treatment against *Shigella/*EIEC in developing countries (Siu et al., 2000). Overall, stress factors have a significant force on the lateral gene transfer of stress resistance across the phylogenetic groups of *E. coli* (Davies and Davies, 2010).

3.5. Conclusions

This study illustrated the co-evolution between virulence factors and stress

resistance strategies on the niche- and host-adaptation. Dynamic distribution of virulence indicated that the evolution of pathogenic *E. coli* evolved by horizontal gene transfer. The *E. coli* population exhibits a high diversity on the combination of various genotypes shaped by environmental stress and antibiotics. The relationship between the phylogeny and presence of LHR manifests the acquisition of LHR might relate to the evolution of environmental-adaptation.

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CHAPTER 4. Induction of Shiga toxin Prophage by Abiotic Environmental Stress in Food

4.1. Introduction

STEC cause the life-threatening hemolytic uremic syndrome (HUS), which is associated with acute renal failure and significant mortality (O'Loughlin and Robins-Browne, 2001; Tarr et al., 2005). Stx include Stx1 and Stx2, in which Stx2 causes more sever damage to renal cells (O'Loughlin and Robins-Browne, 2001). Stx are N-glycosidases that remove one adenine from 28S rRNA, block binding of amino-acyl-tRNA to the ribosome, and thus inhibit protein synthesis, which induces apoptosis of human renal cell (Karpman et al., 1998; Obrig et al., 1987). The genes encoding Stx (*stx*) are located on a lambdoid prophage (Waldor and Friedman, 2005). The production of Stx is dependent on the expression of the Stx-encoding prophage. Induction to the lytic cycle lyses the host cell, which releases toxin and phage particles (Shimizu et al., 2009a; Waldor and Friedman, 2005). Stx-phages can create novel STEC by lysogenic infection. A STEC outbreak with more than 4000 cases and 50 deaths in Germany 2011 provides a prominent example for the evolution of novel pathotypes by transduction of *stx* genes (Frank et al., 2011; Laing et al., 2012). The outbreak strain is a novel pathotype with the serotype O104:H4, combining the virulence factors of STEC and enteroaggregative *E. coli* (EAEC) (Laing et al., 2012). Stx2-phages can also establish lysogeny in non-STEC including enteropathogenic, enteroinvasive and enterotoxigenic *E. coli* and commensal *E. coli* strains *in vitro* (Iversen et al., 2015; Leonard et al., 2016; Mora et al., 2011). This demonstrates that Stx2-phages have a broad host range among strains of *E. coli*.

Production of Stx2-phages and Stx2 are controlled by the phage late gene promoter pR' (Waldor and Friedman, 2005). The lysogenic state is maintained by the phage repressor CI (Johnson et al., 1981; Neely and Friedman, 1998). Expression of *stx2* and prophage induction results from proteolysis of the CI repressor. Many agents that induce prophages also induce the SOS response (Kimmitt et al., 2000; Matsushiro et al., 1999). Induction of SOS response is due to the auto-cleavage of the LexA repressor by the RecA nucleoprotein, which is formed by RecA and single stranded DNA (Little, 1993). Auto-cleavage of LexA depresses over 30 genes for DNA repair and inhibition of cell division (Quillardet et al., 2003). CI repressors are auto-proteolysis as a results of the formation of a complex of RecA and single stranded DNA, leading prophage induction (Roberts et al., 1978).

Environmental stress encountered by *E. coli* in the human intestine or in food processing also induces Stx prophages (Aertsen et al., 2005a; Ehrmann et al., 2001; Wagner et al., 2001). Human neutrophils in the intestine release antibacterial molecules including H_2O_2, which stimulates the production of Stx and phage (Wagner et al., 2001). The Stx-phage released in the intestine may transduce to commensal strains and thus amplify toxin production and exacerbate disease symptoms (Iversen et al., 2015). High hydrostatic pressure (HHP), a physical method of food preservation, also induced Stx prophages (Aertsen et al., 2005a; Łoś et al., 2009). Prophage induction in any of the ecological niches populated by STEC drives horizontal gene transfer by release of infectious phage particles that allow transduction of *stx* to non-Shiga toxin producing strains, and thus disseminates genes coding for the Stx in intestine and food systems (Cornick et al., 2006; Nyambe et al., 2017). This study aimed to evaluate the effect of

different stresses on the induction of Stx2 prophage and the SOS response in an outbreak strain that caused a significant mortality and morbidity in Germany (Frank et al., 2011; Laing et al., 2012). To quantify *stx2a* expression, *stx2a* was replaced with the gene (*gfp*) that encodes for green fluorescence protein in STEC O104:H4. Flow cytometry was used to measure the fluorescence signals to determine the stress response of the *E. coli* culture at the single cell level.

4.2. Materials and methods

4.2.1. Bacterial strains and growth conditions

E. coli O104:H4, *E. coli* O104:H4 Δ*stx2a*:*gfp*:*amp*^{r} and *E. coli* DH5α (Table 4.1) were grown aerobically at 37°C on Luria-Bertani (LB) medium (BD, Mississauga, ON, Canada). Ampicillin (100 mg/L) and chloramphenicol (34 mg/L) were added for maintenance of plasmids pUC19 or pKOV, respectively. Stationary-phase *E. coli* were obtained by overnight incubation at 37°C; exponential phase cultures were prepared by subcultures (0.01%) of overnight cultures and incubated to an OD_{600} 0.4 to 0.5.

4.2.2. Generation of *E. coli* O104:H4 Δ*stx2*:*gfp*:*amp*^{r}

The method for *stx2a* replacement with *gfp*:*amp*^{r} was adapted from Link et al. (1997). Primers and plasmids used in this study are listed in Tables 4.1 and 4.2. PCR reactions were carried out using Taq DNA polymerase (TaKaRa Bio, Shiga, Japan). Two homologous sequences A and B in the upstream and downstream of *stx2a* from *E. coli* O104:H4, respectively, were amplified with the primers that contain restriction sites on both ends. The homologs A and B were digested with BamHI and XbaI; Xbal and PstI, respectively.

Table 4. 1. Bacterial strains and plasmids

Strains	Description	Reference or source
E. coli O104:H4	Stx2 producing and enteroaggregative strain *E. coli*, related to Germany outbreak in 2011	(Liu et al., 2015)
E. coli O104:H4 Δ*stx2*: *gfp* :*amp*^{r}	*E. coli* O104:H4 with replacement of *stx2* to *gfp* and *amp*^{r}	This study
E. coli DH5α	Cloning host for plasmids	New England Biolabs
Plasmids	**Description**	**Reference or source**
pUC19	*lacZ* promoter; cloning vector; Amp^r	New England Biolabs
pEGFP	*lac* promoter, carry *gfp*	(Ehrmann et al., 2001)
pKOV	Temperature sensitive pSC101; SacB, Cm^R	(Link et al., 1997)
pUC19-A	pUC19 plasmid contains a homolog A (954 bp) in the upstream of *stx2* in *E. coli* O104: H4; Amp^r	This study
pUC19-B	pUC19 plasmid contains a homolog B (852 bp) in the downstream of *stx2* in *E. coli* O104: H4; Amp^r	This study
pUC19-A:B	pUC19 plasmid contains two homologs A:B in the up- and downstream of *stx2* in *E. coli* O104:H4; Amp^r	This study
pUC19A:*gfp*:B	pUC19 plasmid contains two homologs and *gfp*; Amp^r	This study
pUC19A:*gfp*:*amp*^{r}:B	pUC19 plasmid contains two homologs and *gfp* and *amp*^{r}; Amp^r	This study
pKOVΔ*stx2:gfp:amp*^{r}	pKOV plasmid contains two homologs and *gfp, amp*^{r} and 1.8 kb flanking region of *E. coli* O104:H4, Amp^r and Cm^r	This study

Amp^r: ampicillin-resistance gene; Cm^r: chloramphenicol-resistance gene

The resulting fragments were sequentially ligated into vector pUC19 to generate pUC19A:B. The fragment of *gfp* was extracted from gels with NcoI and XbaI digested plasmids (pEGFP), and ligated into pUC19A:B to create pUC19A:*gfp*:B. The *amp*^{r} was amplified by PCR from pUC19, and digested with NruI. The resulting fragments were purified with a gel purification kit (Qiagen, Mississauga, ON, Canada), and ligated into pUC19A:*gfp*:B. The allele exchange cassette A:*gfp*:*amp*^{r}:B was sub-cloned into a low copy number plasmid pKOV as a BamHI/PstI insert. The resulting recombinant plasmid pKOVΔ*stx2a:gfp:amp*^{r} was transferred into *E. coli* O104:H4 by electroporation and the

transformed strains were recovered at 30°C for 1 h. The cells were plated onto chloramphenicol-LB plates and incubated at 43°C for plasmid integration into the chromosome. The resulting colonies were serially diluted in 0.85% NaCl, plated onto 5% (wt/vol) sucrose-LB plates and incubated at 30°C for allelic exchange. Colonies grown on 5% sucrose-LB plates were replica plated on to chloramphenicol-LB and 5% sucrose-LB plates at 30°C to screen the *E. coli* O104:H4 Δ*stx2a*:*gfp:amp*^{r}. Gene replacement was confirmed by PCR amplification and sequencing.

4.2.3. Quantification of gene expression of *stx2a* and *gfp*

Mitomycin C was used to induce Stx2 prophage (Otsuji et al., 1959; Wagner et al., 1999). Exponential phase cultures were centrifuged and re-suspended in LB broth with the addition of mitomycin C (Sigma-Aldrich, St Louis, MO, USA) to a concentration of 0.5 mg/L, followed by incubation at 37°C for 1 h. Corresponding treatment of cultures without the addition of mitomycin C served as control. After treatment, cells were harvested from samples and controls to quantify gene expression. RNA was isolated using RNAprotect bacteria reagent and the RNAeasy mini kit (Qiagen Inc., Valencia, CA, USA) and was reverse transcribed to cDNA by QuantiTect reverse transcription kit (Qiagen Inc., Valencia, CA, USA) following the manufacturer's protocols. The expression of genes in *E. coli* O104:H4 and its mutant was quantified using SYBR green reagent (Qiagen Inc., Valencia, CA, USA) and performed with real-time PCR (7500 Fast, Applied Biosystems, Foster City, CA, USA). Negative controls included DNase-treated RNA and no-template controls. The gene coding for glyceraldehyde-3-phosphate dehydrogenase A (*gapA*) served as the reference gene. The gene expression ratio of *stx2a* in *E. coli* O104:H4 (wildtype) relative to *gfp* in *E. coli*

O104:H4 Δ*stx2:gfp:amp*^{r} (mutant) at induced and control conditions were calculated according to Pfaffl (2001), with re-arrangements to account for the different PCR efficiencies of *gfp* and *stx2a* amplification:

$$\text{ratio} = \frac{\frac{E_{stx}^{CP_{stx}}}{E_{gfp}^{CP_{gfp}}}}{E_{gapA}^{(CP_{wildtype}-CP_{mutant})}},$$

where *E* is the PCR efficiency of PCR reactions with primers targeting *stx2a, gfp,* or *gapA* and CP is the threshold cycle for *E. coli* strains at induced and control conditions, respectively. The primers used for quantification of gene expression are listed in Table 4.2. Results are presented as means ± standard deviation (SD) for five biological replicates.

Table 4. 2. Primers used in this study

Primers (forward, F; Reverse, R)	Direction: Sequence (5'-3') containing restriction site (underlined)	Restriction site
	Primers used for cloning	
Homolog A, upstream of *stx2*	F: TAGGATCCCTGTATAGGTAAACGCCTC	BamHI
	R: TATCTAGATACCATGGTACACTTCATATACACCTGGT	XbaI, NcoI
Homolog B, downstream of *stx2*	F: GTTCTAGAGTGCAGTTTAATAATGACTGAGGCATAACCTG	XbaI
	R: TACTGCAGGCGGCCGCGCTAACTGTTTAATTCG	PstI
amp^r	F: ATGTATCGCGATCTAAATACATTCAAA	NurI
	R: ATGTATCGCGAGAGTAAACTTGGTCTG	NurI
	Primer used for confirming mutant strains	
Stx2A', F	TTATATCTGCGCCGGGTCTG	
Stx2B', R	ACCCACATACCACGAAGCAG	
	Primer used for quantification of gene expression	
stx2	F: TATCCTATTCCCGGGAGTTT	
	R: TGCTCAATAATCAGACGAAGAT	
gfp	F: TTCTTCAAGTCCGCCATG	
	R: TGAAACGGCCTTGTGTAGTATC	
gapA	F: GTTGACCTGACCGTTCGTCT	
	R: TGAAACGGCCTTGTGTAGTATC	
recA	F: ATTGGTGTGATGTTCGGTAA	
	R: GCCGTAGAGGATCTGAAATT	

4.2.4. Inactivation of *E. coli* by physical and chemical stressors.

Exponential phase cultures were harvested and treated with heat, oxidative, acid stress, or pressure. To compare the diverse physical and chemical stressors, treatment intensity and times were selected to reduce cell counts by 90 to 99%. Cells were heated by heating 100 µL of culture to 50°C for 5 min in a PCR cycler; control cultures were maintained at 37°C. Samples were kept on ice before determination of cell counts. Oxidative stress was induced by the addition of H_2O_2 (Sigma-Aldrich, St Louis, MO, USA) to a final concentration of 2.5 mM, followed by incubation for 40 min at 37°C. Control cultures were maintained for 40 min at 37°C without additives. Acid stress was determined with LB broth adjusted to pH 2.5 with 1 M HCl (Sigma-Aldrich, St Louis, MO, USA) or pH 3.5 with 1 M DL-lactic acid (Sigma-Aldrich). Cells were re-suspended in LB broth at pH 2.5, and incubated for 20 min at 37°C, or in LB broth at pH 3.5 and incubated for 5 min at 37°C. Control cultures were maintained in LB broth at 37°C. Pressure treatments were performed as described previously (Chen and Gänzle, 2016). In brief, 120 µL of culture were transferred into sterile 3.3 cm tubing (E3603, Fisher Scientific, Akron, OH, USA) and heat-sealed at both ends. The samples were treated at 200 MPa and 20°C for 7 min. The rate of compression and decompression were 270 MPa/min. Control cultures were transferred to plastic tubing, heat sealed, and incubated at ambient pressure and temperature for 15 min. Cell counts after treatments with heat, acids, HHP and the respective controls were determined by surface plating of appropriate dilutions on LB agar. H_2O_2 treated samples were diluted with 0.2% sodium thiosulfate (wt/vol) (Sigma-Aldrich) as a H_2O_2 neutralizer; all other samples were diluted with 0.85% NaCl. Reduction in bacterial counts for all stress treatments were expressed as

$\log_{10}(N_0/N)$, where N_0 and N are cell counts from untreated and treated cultures, respectively. Results are presented as means ± standard deviation for three biological replicates analyzed in duplicate.

4.2.5. Membrane permeability

Stress conditions determined in Section 4.2.4. were used to induce the *E. coli* O104:H4 Δ*stx2:gfp:amp*^{r} for 3 h, which was lethal for over 90-99% of the cell population. Therefore, the membrane-impermeable nucleic acid binding dye propidium-iodide (PI) (Sigma-Aldrich) (Comas and Vives-Rego, 1997; Gant et al., 1993) was used to label cells with permeable membranes and indicate the level of damages during 3 h. The optimum PI concentration was determined by literature data, and microscopic assessment of the fluorescent intensity of stressed cells after mixing with serial two-fold dilutions of PI. For analyses of membrane permeability, PI was added to the treated samples and the respective controls to a final concentration of 20 μM and incubated at room temperature in the dark for 10-15 min.

4.2.6. Flow cytometric determination of GFP and PI fluorescence and forward scatter

Exponential phase cultures were treated with mitomycin C (0.5 mg/L), heat (50°C), HCl (pH 2.5), lactic acid (pH 3.5), or H_2O_2 (2.5 mM) over 3 h. Pressure treated samples were compressed to 200 MPa for 7 min, followed by incubation at 37°C for 3 h. The parameters for pressure treatment were adapted from a previous study (Chen and Gänzle, 2016). The 3 h exposure aims to determine whether continuous stress will affect the efficacy of induction. Control samples were treated in the same manner without stressors. Details of stress treatments are described in section 4.2.4. Flow cytometry was

performed using BD LSR-Fotessa™ X-20 (BD Biosciences, San Jose, CA, USA) equipped with a 488 nm excitation from a blue air laser at 50 mW, and a 561 nm excitation from a yellow air laser at 50 mW to excite green (530±30 nm) and red fluorescence (586±15 nm). The light scattered was measured by two detectors. The detector located along with the path of laser measured the forward scatter light. The detector located at 90 ° to the laser measured the side scatter light. Forward scattered light is proportional to the volume of the cells and was used to evaluate cell filamentation (Gant et al., 1993; Koch et al., 1996; Martinez et al., 1982). Treated cultures and controls (200 µL) were diluted with 1 mL 0.85% NaCl for mitomycin C, heat, acid, or pressure treated samples, or with 1 mL PBS containing 0.2% sodium thiosulfate (wt/vol) (Sigma-Aldrich) for H_2O_2 treated samples. Samples were collected at a 20 min interval during 3 h, and kept on ice before flow cytometry. Samples were diluted with FACS buffer (1% PBS, 2% FCS, 0.02% sodium azide) to exclude pH effects on GFP fluorescence, and to maintain the running speeds at no more than 5,000 events per sec. Sample injection and acquisition were started simultaneously and continued until 10,000 events were recorded. FCS files were extracted from FACDiva 8 software and analyzed by FlowJo software (Tree Star, Ashland, Oregon, USA). Samples treated with mitomycin C were analyzed with respect to GFP fluorescence intensity and forward light scatter. Samples treated with heat, H_2O_2, acids and pressure were analyzed with respect to GFP and PI fluorescence intensity. The gating of forward light scatter (FSC) and GFP fluorescence was set manually to account for more than 96% of the cells in controls as normal sized and GFP negative (Figure 4.1). The gating for GFP and PI fluorescence intensity was set manually to account for more than 97% of the cells in all controls as GFP and PI negative (Figure

4.2). Samples were divided into four sub-populations and the percentage values of four sub-populations for each treatment were calculated. Data are presented as means ± standard deviation for three biological replicates.

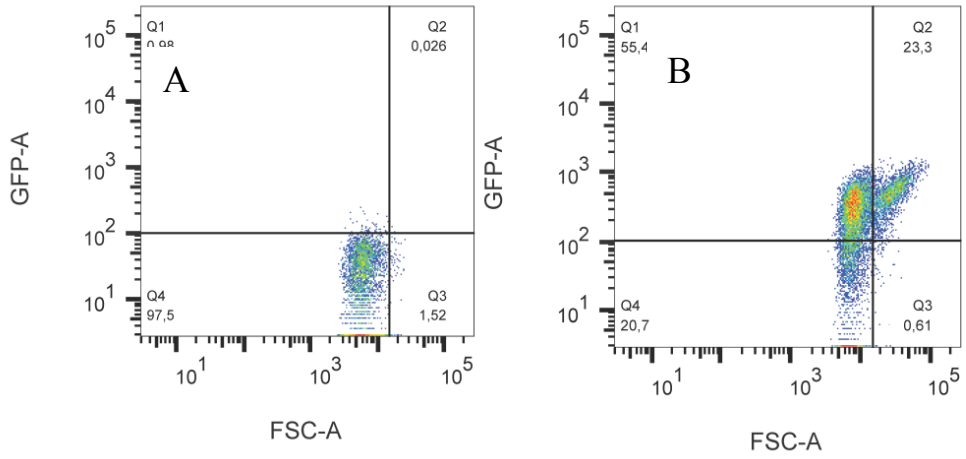

Figure 4. 1. Dot plot of Forward Scatter (FSC) and GFP fluorescence for *E. coli* O104:H4 Δ*stx2:gfp:amp*^{r} grown in LB broth (panel A) or treated with mitomycin C (0.5 mg/mL) for 180 min (panel B). The population was divided into four sub-populations by FSC and GFP reference lines. The reference lines were determined from untreated samples, where at least 96% of the population was GFP negative as shown in Figure 1A. Percentage value (%) of four populations (Q1: FSC-/GFP+; Q2: FSC+/GFP+; Q3: FSC+/GFP-; Q4: FSC-/GFP-) were automatically calculated and presented on the corner of the panels.

4.2.7. Determination of phage production by spot agar assay

The production of infectious phage in control cultures and stressed cultures was determined by filtration of phage particles, followed by spot agar assay (Andersson and Hughes, 2014; Iversen et al., 2015). Exponential-phase *E. coli* O104:H4 Δ*stx2:gfp:amp*^{r} and wild type *E. coli* O104:H4 were obtained by subculture in LB broth containing $CaCl_2$ (1 mM) and $MgSO_4$ (10 mM). *E. coli* strains were stressed with mitomycin C (0.5 g/L), H_2O_2 (2.5 mM) overnight at 37 °C, or stressed with HCl (pH 2.5) and lactic acid (pH 3.5)

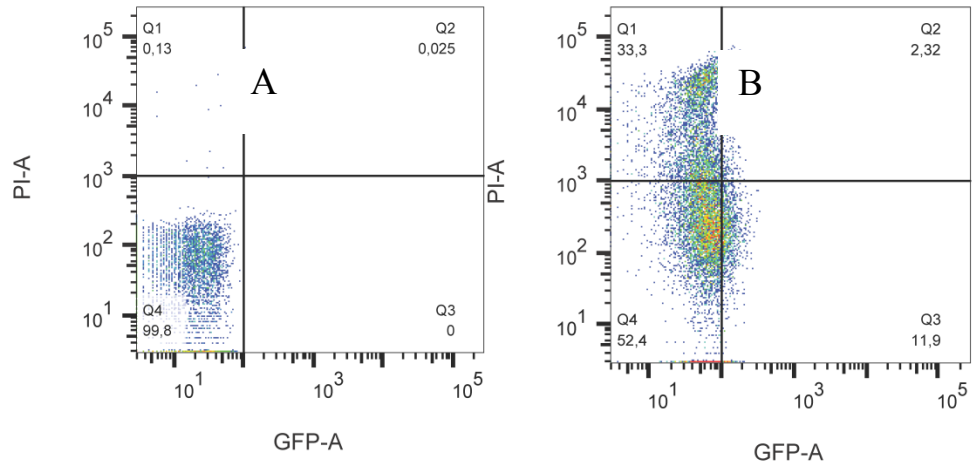

Figure 4. 2. Example of a dot plot of GFP fluorescence and PI fluorescence for *E. coli* O104:H4 Δ*stx2*:*gfp*:*amp*^{r} grown in LB broth (panel A) or treated with HCl (pH 2.5) for 20 min (panel B). The population was divided into four subpopulations by GFP and PI reference lines. The reference lines were determined from untreated samples, where at least 97% of the population is GFP and PI negative. Percentage value (%) of four populations (Q1: GFP-/PI+; Q2: GFP+/PI+; Q3: GFP+/PI-; Q4: GFP-/PI-) were automatically calculated and presented on the corner of the panels.

for 20 min, then neutralized in LB broth and incubated overnight at 37°C. Treatment with H_2O_2 (2.5 mM) for 40 min at 37 °C was not sufficient to induce enough viable phage to form a plaque by spot agar on lawn assay. Cultures incubated in LB without additives served as control. Phage particles were obtained by centrifugation at 5,311×g for 10 min followed by filtration through 0.22 μm filters to remove bacterial cells. *E. coli* DH5α served as recipient strain for phage infection. Stationary-phase *E. coli* DH5α was suspended in LB broth containing $CaCl_2$ (1 mM) and $MgSO_4$ (10 mM). A volume of 100 μL of culture was mixed with 3 mL of warm 0.6% LB agar and poured on normal LB agar. Ten microliters of culture filtrate from mutant and wild type strains were spotted on the recipient strains after solidification on the top agar. The clear zone formed after incubation of the plate at 37°C overnight was recorded to indicate the production of phage particles.

4.2.8. Quantification of expression of *recA*

Exponential phase culture of *E. coli* O104:H4 Δ*stx2a:gfp:amp*^{r} was treated with LB broth containing: mitomycin C (0.5 mg/L) for 1 h; H_2O_2 (2.5 mM) for 40 min and HCl or lactic acid for 20 min. The stress treatment using H_2O_2 (2.5 mM) for 40 min and HCl for 20 min was same as the sub-lethal stress determined in section 4.2.4. The expression of *recA* relative to un-induced cells was determined with *gapA* as the housekeeping gene as described above. The time used for the gene quantification was selected based on the expression of GFP by flow cytometry, at the point where there was the greatest GFP expression (Figure 4.4, 4.6 and 4.7), except for mitomycin C. Treatment of cells for 1 h with mitomycin C was sufficient to induce prophage expression (Figure 4.4).

4.2.9. Microscopic determination of cell morphology

Cultures were grown to exponential phase, and treated with 0.5 mg/L mitomycin C for 3 h, and with other stressors for different treatment times of up to 1 h (2.5 mM H_2O_2 and HCl, pH 2.5); 20 min (lactic acid, pH 3.5) or 40 min (200 MPa). Treatment time showing the greatest extent of GFP expression (Figure 4.4, 4.6 and 4.7) was selected for analysis of cell filamentation. According to Figure 4.4, greatest extent of filamentation was detected after treatment with mitomycin C for 3 h. The conditions for other stress treatments were same as the conditions used to determine the relative gene expression. At each time point, approximately 20 μL of bacterial culture was examined using transmission light microscopy (Axio Imager M1m microscope,Carl Zeiss Inc.). The images were acquired at 100 × magnification with an AxioCam M1m camera and Axio Vision version 4.8.2.0 software (Carl Zeiss Inc., Göttingen, Germany).

4.2.10. Statistical analysis

The gene expression is reported as average values of 4 or 5 independent experiments and represented by means ± standard deviation. Significant differences of *stx2a* to *gfp* gene expression between induced and control conditions were assessed with a t-test. The results from *recA* gene expression after stress treatment were analyzed by one-way analysis of variance and statistical differences among treatments were determined by Tukey's test using SAS (SAS Institute Inc., Cary, NC, USA). Significant differences among samples or treatments were denoted at an error probability of 5% ($P<0.05$).

4.3. Results

4.3.1. Generation and validation of *E. coli* O104:H4 Δ*stx2:gfp:amp*^{r}

To quantify the expression of the *stx2a* during stress, *stx2a* was replaced by *gfp* as fluorescent reporter gene; *amp*^{r} was used as a selection marker to screen mutants. PCR amplification and sequencing confirmed that the *stx2a* was replaced with *gfp* and *amp*^{r} in *E. coli* O104:H4 Δ*stx2:gfp:amp*^{r}. The transcription of *gfp* and *stx2a* in response to stress was determined by reverse transcription (RT) qPCR. Mitomycin C increased expression of *stx2a* and *gfp* about 5 fold (Figure 4.3). The expression of *stx2* in the wild type strain relative to *gfp* expression in the mutant strain at stress and control conditions was 0.76 ± 0.034 and 0.68 ± 0.26, respectively. Expressions of *stx2a* and *gfp* at induced and un-induced conditions were not different ($P>0.05$), demonstrating that the relative expression of *gfp* was equivalent to the expression of *stx2a* in response to mitomycin C.

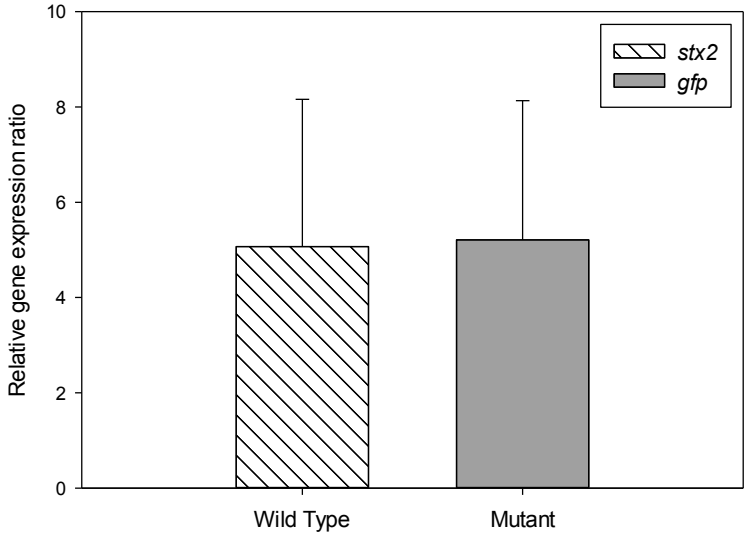

Figure 4. 3. Quantification of gene expression of *stx2a* in *E.coli* O104:H4 and *gfp* in *E. coli* O104:H4 Δ*stx2a*:*amp*^{r}*:gfp* after induction with 0.5 mg/L mitomycin C for 1 hour**.** Data represent means ± standard deviation for four independent experiments. Relative gene expression ratio value for *stx2a* (dash bar) and *gfp* (grey bar) are 5.071±3.089 and 5.213±2.921, respectively. There is no significant difference between the gene expression level of *gfp* and *stx2a* response to stress (P>0.05).

4.3.2. Quantification of prophage induction by mitomycin C

Mitomycin C causes cross-links of double-stranded DNA, activates the SOS response, and induces cell filamentation and phage production (Castellazzi et al., 1972; Howard-Flanders et al., 1964). Exposure of *E. coli* O104:H4 Δ*stx2a*:*gfp:amp*^{r} to mitomycin C for 2 and 3 h induced *gfp* expression in 17 and 56% of cells of the population, respectively (Figure 4.4). Of the cells that expressed GFP after 3 h of treatment with mitomycin C, 19.4% were also filament, confirming that mitomycin C induced the SOS response.

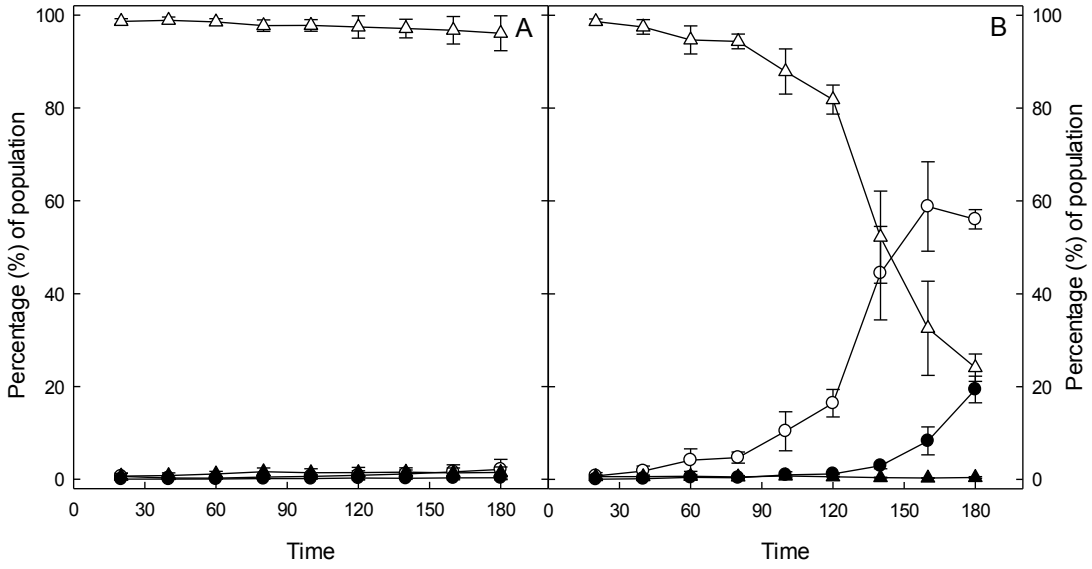

Figure 4. 4. Quantification of *gfp* expression in *E. coli* O104:H4 Δ*stx2a*:*gfp:amp*^{r} in LB, or after induction by mitomycin C. Exponential phase *E. coli* O104:H4 Δ*stx2a*:*gfp* were incubated in LB (panel A), or incubated after addition of mitomycin C to a concentration of 0.5 mg/L (panel B). At each time point, bacterial culture was harvested and the proportion of GFP fluorescent and filament cells was quantified by flow cytometry. ○,● GFP positive cells; △,▲ GFP negative cells. Open symbols, normal sized cells, closed symbols, filament cells. Data represent means ± standard deviation for three independent experiments.

4.3.3 Stress resistance of *E. coli* O104:H4 Δ*stx2a*:*gfp:amp*^{r}

The effect of environmental stress on the expression of *stx2a* and induction of the Stx2-prophage was evaluated with stressors related to food preservation, which included heat, oxidative and acid stress, and pressure. To compare prophage expression in response to different stressors, each stress was applied to reduce bacterial cell counts by 1-2 log (CFU/mL) (Figure 4.5).

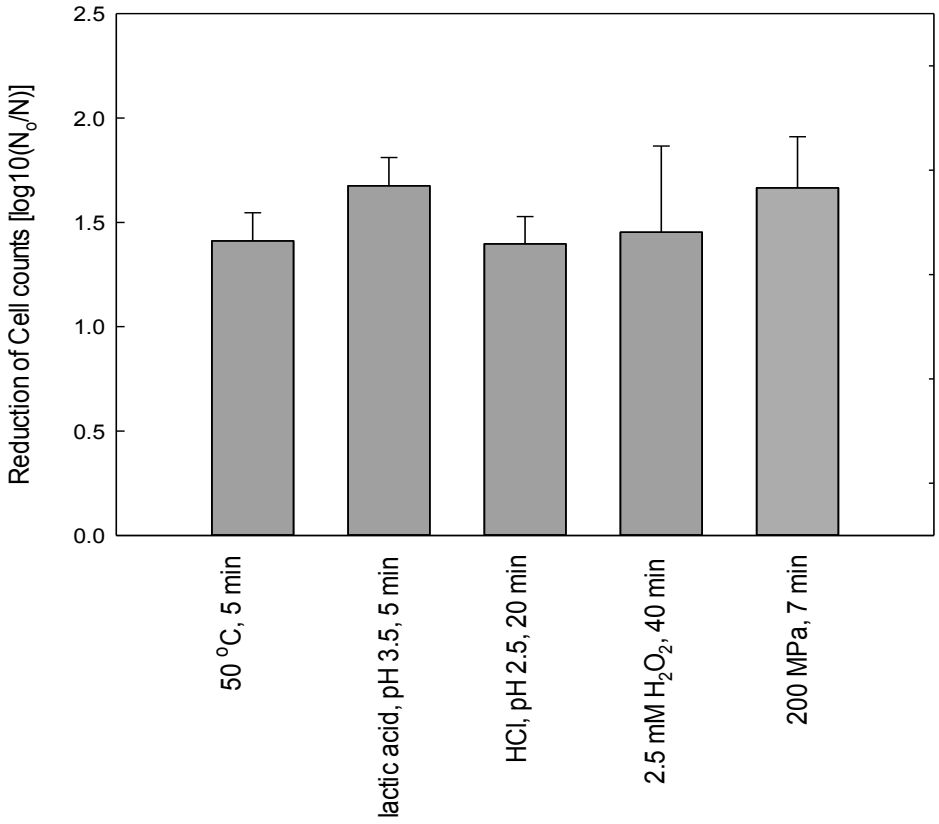

Figure 4. 5. Reduction of cell counts of *E. coli* O104:H4 Δ*stx2a*:*gfp:amp*^{r} after exposure to heat, acid, oxidative stress, or to 200 MPa HHP. Data represent means ± standard deviation or three independent experiments.

4.3.4. Effect of heat and hydrogen peroxide on membrane permeability and GFP expression in *E. coli* O104:H4 Δ*stx2*:*gfp:amp*^{r}

Prophage expression and loss of membrane permeability were simultaneously assessed at the single cell level by flow cytometric determination of GFP and PI fluorescence, respectively. The proportions of the GFP-expressing and PI permeable population were measured over 3 h of exposure to stress (Figure 4.6), corresponding to the time required for full GFP induction with mitomycin C. In control cultures, less than 3% of the cells expressed GFP, or were stained with PI (data not shown). Exposure to 50 °C for 3 h increased membrane permeability in 64% of the cells but did not induce GFP

(Figure 4.6A). Exposure to 2.5 mM H_2O_2 for 3 h increased membrane permeability in less than 10% of cells and induced GFP in 3% of the cells (Figure 4.6B). Of note, treatment of *E. coli* O104:H4 Δ*stx2a*:*gfp:amp*^{r} with H_2O_2 for 3 h reduced viable cell counts by more than 6 log (CFU/mL) without increasing membrane permeability.

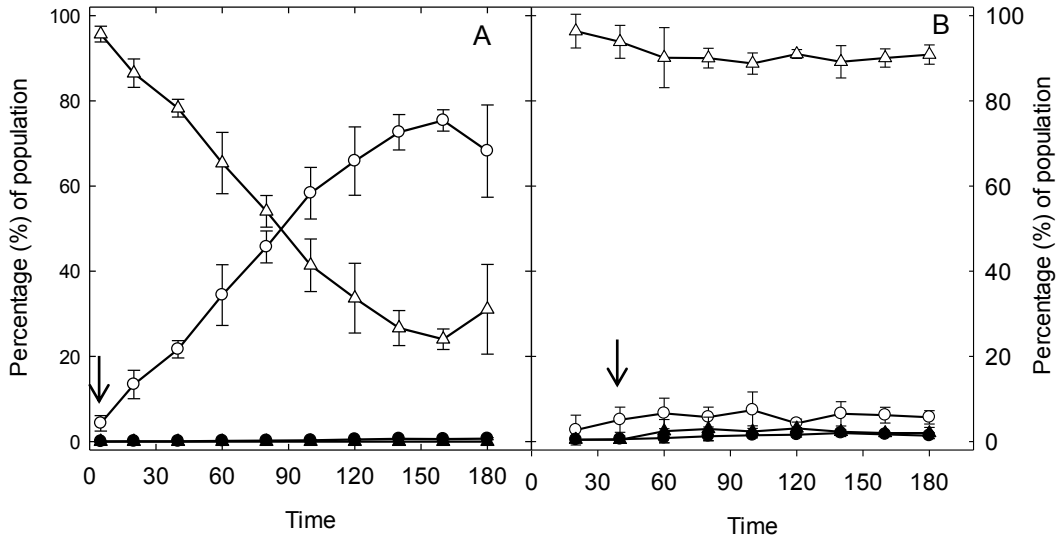

Figure 4. 6. Effect of heat and H_2O_2 on *gfp* expression and membrane permeability in *E. coli* O104:H4 Δ*stx2a*:*gfp:amp*^{r}. Exponential phase *E. coli* O104:H4 Δ*stx2a*:*gfp:amp*^{r} were incubated in LB broth at 37°C (not shown); at 50 °C (panel A) or at 37°C with the addition of 2.5 mM H_2O_2 (panel B) for 3 h. At each time point, bacterial culture was diluted and stained with PI. GFP and PI fluorescence were quantified by flow cytometry. Control conditions did not alter the GFP expression or the membrane permeability of cells (same as Figure 4.4.A and data not shown). ○,● PI permeable; △,▲: PI impermeable; open symbols: GFP-negative; closed symbols: GFP-positive. ↓indicates the time when treatment reduced cell counts by 1-2 log (CFU/mL). Data represent means ± standard deviation for three independent experiments.

4.3.5. Effect of acids on membrane permeability and GFP expression in *E. coli* O104:H4 Δ*stx2a*:*gfp:amp*^{r}

The effect of HCl and lactic acid on GFP expression and membrane permeability in *E. coli* O104:H4 Δ*stx2a*:*gfp:amp*^{r} is shown in Figure 4.7. The GFP intensity is affected by low pH, but the intensity can be regained by transferring cells to a neutral pH (Hansen et al., 2001). Samples treated with low pH were transferred into the neutralized buffer.

HCl induced GFP in a higher proportion of cells (12%) when compared to lactic acid, which induced GFP in 6% of cells. During treatment with HCl, a GFP positive and PI negative population appeared after 20 to 40 min of treatment but decreased from 12 to 0.5% during subsequent incubation. Correspondingly, a GFP and PI positive population increased from 2 to 17% between 40 and 180 min of incubation (Figure 4.7A). A similar trend was observed for lactic acid treatment, where the GFP positive and PI negative population dropped to less than 1% after 40 min treatment, while the GFP and PI positive population increased to 12.7% (Figure 4.7B). These data indicate that GFP was expressed

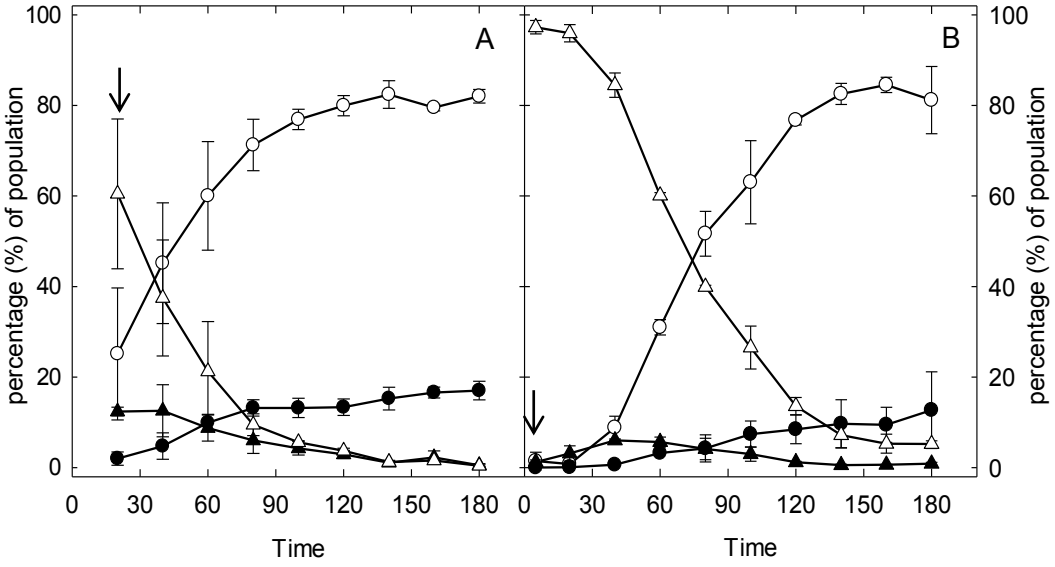

Figure 4. 7. Effect of HCl and lactic acid on *gfp* expression and membrane permeability in *E. coli* O104:H4 Δ*stx2a*:*gfp:amp*^{r}. Exponential phase *E. coli* O104:H4 Δ*stx2a*:*gfp:amp*^{r} were incubated in LB broth (not shown) incubated in LB acidified with HCl to pH 2.5 (panel A), or incubated in LB acidified with lactic acid to pH 3.5 (panel B) for 3 h. At each time point, bacterial culture was diluted and stained with PI. GFP and PI fluorescence were quantified by flow cytometry. Control conditions did not alter the GFP expression or the membrane permeability of cells (Figure 4.3.A and data not shown). ○,● PI permeable; Δ,▲: PI impermeable; open symbols, GFP-negative; closed symbol: GFP-positive. ↓indicates the time when treatment reduced cell counts by 1-2 log (CFU/mL). Data represent means ± standard deviation for three independent experiments.

first but GFP expressing cells lost membrane integrity and likely viability during subsequent incubation at acid conditions.

4.3.6. Effect of pressure on membrane permeability and GFP expression in *E. coli* O104:H4 Δ*stx2a*:*gfp*:*amp*^{r}

The effect of pressure on GFP expression and membrane permeability in *E. coli* O104:H4 Δ*stx2a*:*gfp*:*amp*^{r} is shown in Figure 4.8. Control cultures were heat-sealed in sample tubing and stored at ambient pressure and temperature; here, the membrane integrity was compromised in around 10% of the population even without pressure treatment (Figure 4.8.A). After treatment with 200 MPa for 7 min, 2% of the population was positive for GFP and PI (Figure 4.8.B). During recovery at ambient pressure and 37 °C for 3 h, the proportion of cells with damaged membranes decreased owing to membrane repair, or to the growth of surviving cells, and GFP positive cells were not detected.

4.3.7. Effect of acids and H_2O_2 on phage production in wild type *E. coli* O104:H4 and *E. coli* O104:H4 Δ*stx2a*:*gfp*:*amp*^{r}

To determine whether induction of *gfp* and *stx2a* also leads to release of infectious phage particles, spot on lawn assays were carried out after induction with diverse stressors (Table 4.3). Filtrates isolated from control cultures and acid-stressed cultures of the mutant and wild type strains did not contain infectious phage particles. In contrast, phages isolated from wild type and mutant strain after mitomycin C and H_2O_2 treatments lysed bacterial lawn *E. coli* DH5α and formed plaques, indicating the presence of infectious phages.

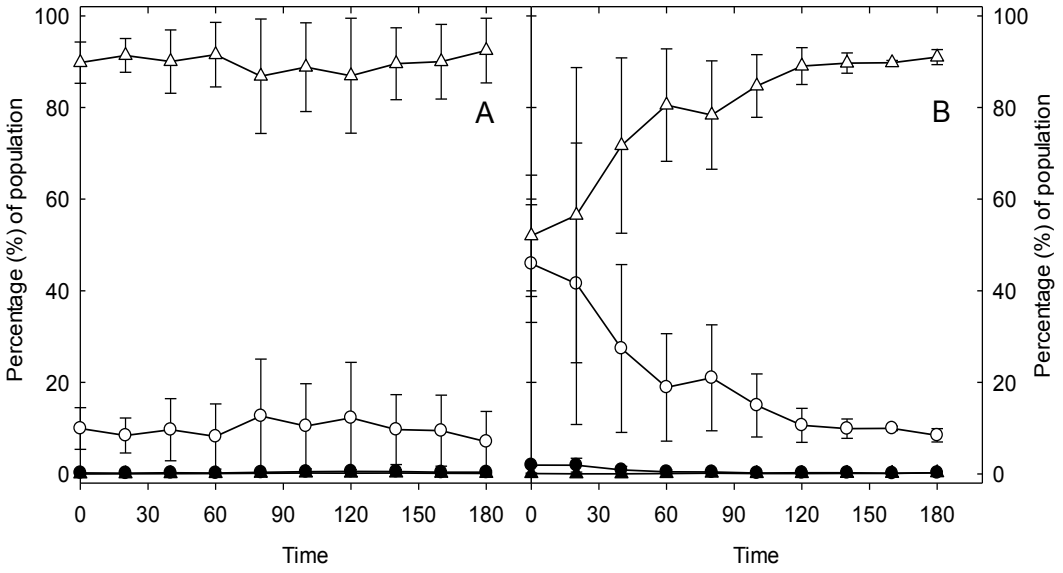

Figure 4. 8. Effect of pressure on *gfp* expression and membrane permeability in *E. coli* O104:H4 Δ*stx2a:gfp:amp*^{r}. Exponential phase *E. coli* O104:H4 Δ*stx2a:gfp:amp*^{r} in LB broth were heat-sealed in tubes and incubated at 37°C (panel A), or heat sealed in tubes, incubated at 200 MPa at 20°C for 7 min, followed by incubation at 37°C and ambient pressure during 3 h (panel B) . At each time point, bacterial culture was diluted and stained with PI. GFP and PI fluorescence were quantified by flow cytometry. ○,● PI permeable; Δ,▲: PI impermeable; open symbols, GFP-negative; closed symbol: GFP-positive. Pressure treatment reduced cell counts by 1-2 log (CFU/mL). Data represent means ± standard deviation for three independent experiments.

Table 4. 3. Detection of infectious phage particles in culture filtrates of *E. coli* O104:H4 and *E. coli* O104:H4 Δ*stx2a:gfp:amp*^{r}. The presence of infectious phage particles was assessed by a spot agar assay.

Treatments	Presence of infectious phage particles in culture filtrate	
	E. coli O104:H4	*E. coli* O104:H4 Δ*stx2a:gfp :amp*^{r}.
No-stressed control	−	−
Mitomycin C	+	+
H_2O_2	+	+
HCl	−	−
Lactic acids	−	−

+, observation of a clear halo indicative of infectious phage particles
−, growth in the presence of culture filtrate, indicative of the absence of infectious phages

4.3.8. Cell morphology of stress-treated cells

To determine whether GFP expression in *E. coli* O104:H4 Δ*stx2a:gfp:amp* is linked to the SOS response, the morphology of untreated and stressed cells were

compared by microscopic observation. The SOS response inhibits cell division and causes filamentation (Castellazzi et al., 1972; Howard-Flanders et al., 1964). No morphological changes were observed after treatment with HCl (pH 2.5), lactic acid (pH 3.5) and HHP (200 MPa) (Figure 4.9). However, after the treatment with mitomycin C and H_2O_2, some of the cells formed filaments (Figure 4.9E & F).

4.3.9. Effect of environmental stress on *recA* expression

To further confirm the link between GFP expression and the SOS response in response to stress, expression of *recA* in *E. coli* after stress treatment was compared with non-stress treatment (Figure 4.10). Mitomycin C and H_2O_2 induced expression of *recA*; however, acid treatment did not affect *recA* expression. These results indicate that mitomycin C and H_2O_2 activated the SOS response, which corresponds to the microscopic observation of cell filamentation as an indicator of induction of the SOS response (Figures 4.9 and 4.10). However, stressors inducing *recA* did not overlap with conditions inducing GFP. GFP expression requires longer induction time.

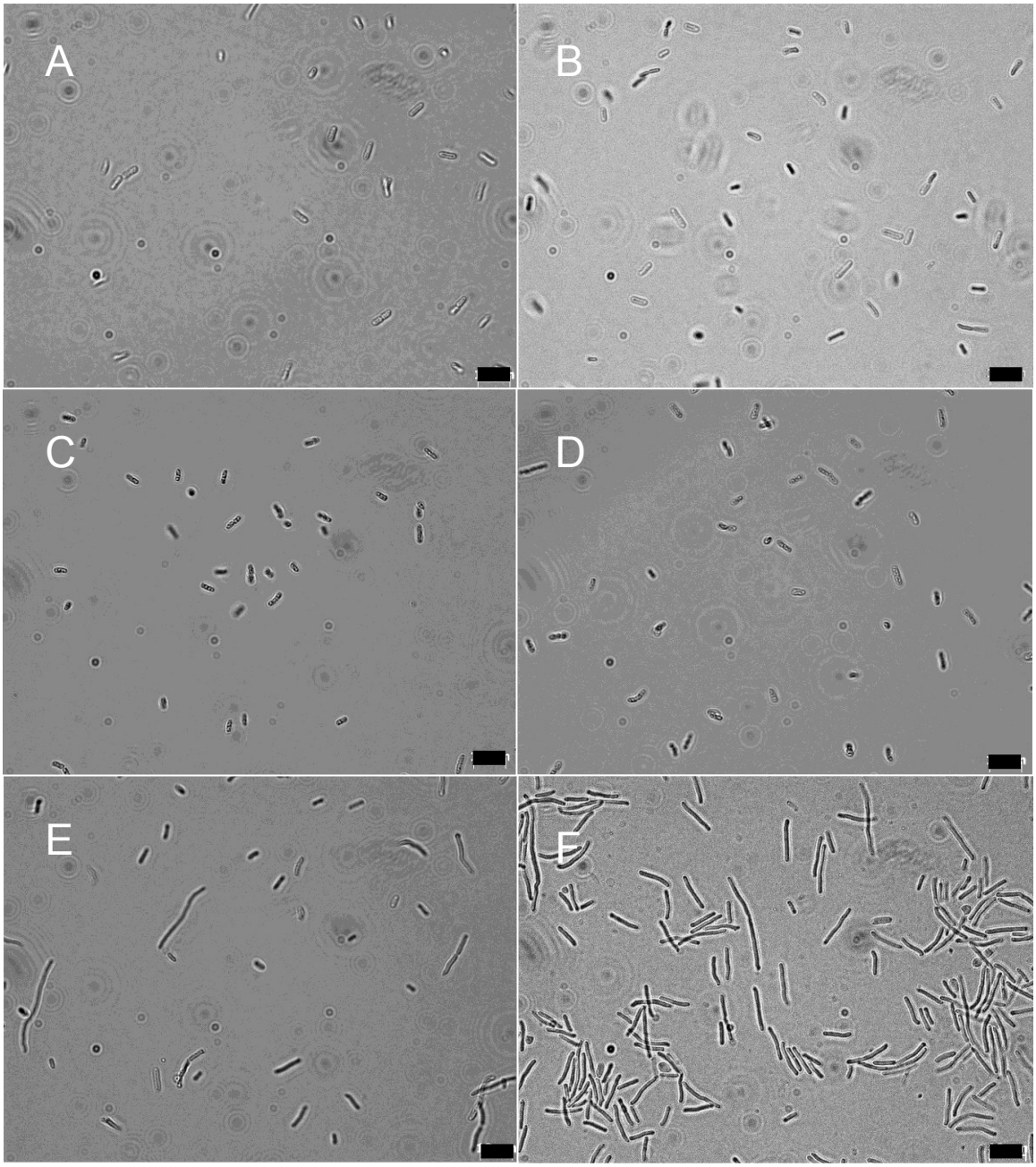

Figure 4. 9. Effect of stress on cell morphology of *E. coli* O104:H4 Δ*stx2a*:*gfp:amp*^{r} as observed by phase contrast microscopy. Panels represent exponential phase cells (Panel A), or exponential phase cells treated with 200 MPa for 40 min (Panel B); with HCl for 1 h (Panel C); with lactic acid for 20 min (Panel D); with 2.5 mM H_2O_2 for 1 h (Panel E); or with mitomycin (0.5 mg/mL) for 3 h (Panel F). One thousand cells were observed, representative for 5 pictures. Three biological replicates were done for each treatment. Scale bars represent 10 μm.

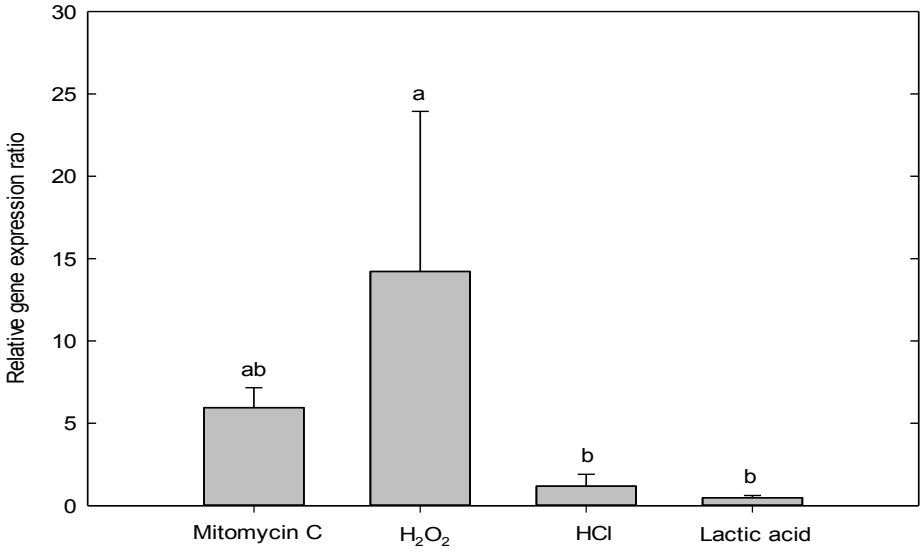

Figure 4. 10. Expression of *recA* in *E. coli* O104:H4 Δ*stx2:gfp:amp*^{r} after stress treatments. Relative gene expression was quantified by RT-qPCR with *gapA* as housekeeping gene and exponential cultures in LB broth as reference conditions. Exponential phase cultures were treated with LB broth containing mitomycin C (0.5 mg/L); H_2O_2 (2.5 mM) for 40 min; acidified LB broth with HCl (pH 2.5) or lactic acid (pH 3.5) for 20 min. Values for different treatments that do not share a common superscript are significantly different (P<0.05). Data represent means ± standard error for the mean for four independent experiments.

4.4. Discussion

The induction of the Stx prophage regulates toxin and phage production, which are integral to the virulence of STEC (Waldor and Friedman, 2005). This study developed a method for simultaneous detection of the expression of Stx2 prophages, changes of membrane permeability, and the induction of the SOS response. This novel tool was used to assess the role of environmental and food-related stressors on the induction of Stx2-prophage and the SOS response.

Classical techniques for the evaluation of prophage induction employ plaque assays (Łoś et al., 2009; Zhang et al., 2000), protein assays (Iversen et al., 2015; Shimizu et al., 2009a) and quantification of gene expression (Herold et al., 2005); however, these

methods inform only on prophage induction at a population level. Using a fluorescent reporter gene to replace *stx2a* was done in previous studies to investigate the induction of Stx2 prophages (Łos et al., 2010; Shimizu et al., 2009a). This study generated a single-cell detection method using flow cytometry to assess the induction of Stx2-prophage and the SOS response by quantification of GFP and determination of cell morphology (Mason et al., 1995). This study also combined the fluorescence probe, PI, with flow cytometry to measure membrane permeability. Treatment with H_2O_2 reduced cell counts without disrupting membrane permeability. The link of prophage induction to the SOS response was further confirmed by quantification of *recA* expression. Mitomycin C was used as a positive control to induce both the prophage (Aertsen et al., 2005a; Herold et al., 2005) and the SOS response (Aertsen and Michiels, 2005). Results from flow cytometry, microscopic observation of the cell morphology, and the quantification of *recA* expression all confirmed that mitomycin C induced the SOS response (Figures 4.4, 4.9. and 4.10.). Using the same method above, the study found that HCl and lactic acid induced the Stx2-prophage without overexpression of *recA,* indicating that mechanisms of acid-induced Stx2-prophage induction differ from induction by H_2O_2 or mitomycin C. RecA-dependent cleavage of the phage repressor CI is considered necessary for induction of the prophage. The peroxide-induced SOS response leads to cell filamentation (Imlay and Linn, 1987). RecA-dependent gene regulation (Imlay and Linn, 1987; Palma et al., 2004; Vanbogelen et al., 1987) and prophage induction (Łos et al., 2010; Łoś et al., 2009) are well documented. The present study additionally documents RecA-independent induction of the Stx2-converting prophage. RecA-independent induction may relate to conformational changes of the repressor at low cytoplasmic pH. At low pH, SOS

response repressor (LexA) undergoes structural reorganization, causing LexA self-cleavage independent of RecA (Sousa et al., 2006). Since the amino acid sequence of the C-terminal domain of the lambdoid repressor shares roughly 50% similarity with the sequence of LexA, a similar pattern of repressor tetramerization may account for the induction of expression of genes encoded by Stx2-prophages in acid-stressed cells.

The single-cell detection of Stx2-prophage expression also demonstrated that prophage induction occurred only in a fraction of the STEC population. The results affirm the bacterial "altruism" hypothesis proposed by Los et al. (2013) who proposed that only a small fraction of the STEC population switches to the lytic cycle in response to stress. This sacrifice of a small percentage of cells produces sufficient Stx to kill eukaryotic unicellular predators, and warrants survival of the remainder of the population (Łoś et al., 2013). Protozoa kill their bacterial prey with reactive oxygen species; oxidative stress also induces Stx production as a self-defence mechanism for STEC (Arnold and Koudelka, 2014; Łoś et al., 2013). H_2O_2 (2.5 mM) induced the expression of Stx2 and production of the Stx2-phage by a small percentage of the population (this study). This result conforms to prior data that H_2O_2 induced the prophage in up to 1.6% of STEC cells (Łoś et al., 2009). Protozoa occur in significant numbers in the intestinal microbiota of ruminants. It was hypothesized that predation by protozoa in the ruminant intestinal tract provides selective pressure for maintenance of Stx2-prophages in commensal *E. coli* and thus results in a high proportion of STEC in cattle (Imamovic et al., 2010).

STEC may encounter low pH during gastric transit, in food fermentations, and after application of acids as a pathogen intervention step in meat production. Stomach

acidity provides a barrier against bacterial infection, where the pH can be as low as 1.5-3.0 (Bonanno et al., 2017; Kidd, 2011). Lactic acids are produced by bacteria during food fermentation and are commonly used as food processing aids (Bonanno et al., 2017; Gill, 2009). The present study found that HCl of pH 2.5 and lactic acid of pH 3.5 induced the expression of *stx2a*; however, low pH did not result in the release of infectious phage particles. Induction of Stx2-prophages by 2.5% sodium citrate also induced Stx expression without the formation of infective phage particles (Lenzi et al., 2016). Low pH interferes with phage infectivity by inhibition of the formation of Stx2-prophages, or by inactivation of phages after correct assembly and release (Imamovic and Muniesa, 2012; Rode et al., 2011). Induction of Stx production without the release of infectious phages may benefit *E. coli* in defense against predatory protozoa (Imamovic et al., 2010) but is not of concern for food safety.

Contaminated food and water are major vehicles for STEC infection of humans. Ruminants and particularly cattle are primary reservoirs of STEC, and thus are a major source of STEC contamination of foods and water (Russell et al., 2000). Lactic acid (2%) and peroxyacetic acid containing 1-5% H_2O_2 is used commercially in North America to decontaminate the surface of beef carcasses and fresh vegetables (Gill, 2009; Hilgren and Salverda, 2000). Stx phages were found in high numbers on processed beef and salad (Imamovic and Muniesa, 2011). Because pathogen intervention steps in food processing include acid, oxidative stress and pressure, it is conceivable that induction of the Stx2-prophage occurs in food processing (Aertsen et al., 2005a; Łos et al., 2010; Łoś et al., 2009).

4.5. Conclusions

In conclusion, the present study developed a method to achieve simultaneous quantification of prophage induction and physiological characteristics of STEC at the single cell level. H_2O_2 and low pH induced the Stx prophage; induction by acid stress was not dependent on RecA or the SOS response and did not result in the release of infectious phage particles. Oxidative stress and acid stress are encountered in the human and animal gastrointestinal tract and in food processing. H_2O_2 treatment of foods contaminated with STEC may induce the Stx2 prophage and thus promote Stx2 phage transduction. Heat treatment eliminates STEC without inducing the Stx-prophage. This study improved knowledge on the mechanism of prophage induction in *E. coli* by different stressors. Because genomes of Stx-prophages are highly variable, the study also enables further investigation to understand the ecological features of STEC and different Stx-prophages in different ecological niches.

4.6. References

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CHAPTER 5. Effect of Drying on the Oxidative Stress Response and Expression of Shiga toxin Prophage in *Escherichia coli*

5.1. Introduction

The survival of pathogenic bacteria in low water activity (a_w) foods is a threat to food safety; low-infectious dose pathogens including *Salmonella* and Shiga toxin producing *Escherichia coli* are of particular concern. Bacteria in the dry state resist heat and other interventions, which makes the decontamination of low a_w foods challenging (Beuchat et al., 2013; Finn et al., 2013; Gruzdev et al., 2011). STEC caused numerous outbreaks associated with low a_w foods including nuts, fenugreek seeds and dry cured meats (Beuchat et al., 2013). The production of Stx by STEC results in severe illness, such as hemolysis, kidney failure, and neurological symptoms (O'Loughlin and Robins-Browne, 2001; Trachtman et al., 2012). Stx is encoded in the late region of a prophage (Iii et al., 1999). Induction of prophage and production of Stx are repressed by the prophage repressor CI but induced by environmental stress including oxidative stress (Chapter 4; Licznerska et al., 2015; Waldor and Friedman, 2005).

The accumulation of compatible solutes mediates the tolerance of eukaryotic and bacterial cells to desiccation (Crowe, 2001). Among the compatible solutes, trehalose is particularly effective; it stabilizes not only cytoplasmic proteins but also the lamellar structure of the phospholipid membrane (Crowe and Hoekstra, 1992; Leslie et al., 1995). The generation of reactive oxygen species (ROS) after drying induces oxidative stress in dehydrated cells (França et al., 2007). In metabolically active cells, ROS are formed during respiration but rapidly reduced by enzymes or antioxidants, such as catalase, superoxide dismutase, glutathione, or thioredoxin (Cabiscol et al., 2000). Dehydration

compromises the permeability barrier of the cytoplasmic membrane and inactivates enzymes, which promotes the accumulation of ROS (França et al., 2007). The accumulation of ROS in dehydrated yeast and plant cells contributes to protein denaturation, DNA damage, and lipid peroxidation (França et al., 2007, Garre et al., 2010).

Membrane fluidity and integrity are crucial to protect macromolecules in dry cells. Unsaturated fatty acids in the membrane are prone to oxidation by ROS (Castro et al., 1996; Crowe et al., 1989). Upon lipid peroxidation, ROS are formed as byproducts and sustain the autocatalytic oxidation of membrane lipids, which compromises the cell survival during desiccation (Castro et al., 1996; Laguerre et al., 2007). Cyclopropane fatty acids (CFA) are more stable to ROS than unsaturated fatty acids as the double bond is converted to the cyclopropane ring (Grogan and Cronan, 1997). Conversion of unsaturated fatty acids to CFA maintains the membrane fluidity in stress conditions and increases bacterial resistance to heat, high pressure, oxidative stress, and freeze-drying (Chen and Gänzle, 2016; Muñoz-rojas et al., 2006; Zhang and Rock, 2008). Desiccation induces oxidative stress, which may trigger the expression of *stx* in STEC (Licznerska et al., 2015); however, the role of oxidative stress in the survival of dehydrated bacteria and particularly its effect on the expression of Stx in STEC are poorly documented.

Fluorescence-based methods to measure the ROS are commonly used because the manipulation is straightforward (Gomes et al., 2005; Laguerre et al., 2007), but only a few studies determined oxidation of membrane lipids in bacterial cells. C_{11}-BODIPY$^{581/591}$ is a membrane soluble fluorescein lipid analog and sensitive to oxidation by peroxide radicals (Laguerre et al., 2007). The oxidation of probe results in a

green shift of its fluorescence (Borst et al., 2000; Drummen et al., 2002; Gomes et al., 2005; Laguerre et al., 2007; Yoshida et al., 2003). This study aimed to develop the flow cytometry-based methods using C_{11}-BODIPY$^{581/591}$ to evaluate lipid oxidation in *E. coli*. The method was used to explore the effect of drying, compatible solutes and CFA on the oxidation of membrane lipids. The expression of *stx* at low a_w environment was investigated with a strain of *E. coli* O104:H4 Δ*stx2a*:*gfp*:*amp*^{r}, which was constructed with green fluorescence protein (GFP) as a reporter of *stx2a* (Chapter 4). The effect of drying on the oxidative stress and prophage induction during drying was further confirmed by relative gene expression.

5. 2. Materials and methods

5.2.1. Bacterial strains and culture conditions

Strains of *E. coli* used in this study included *E. coli* O104:H4 Δ*stx2a*:*gfp*:*amp*^{r}, an outbreak strain with *stx2a* replaced with *gfp* (Chapter 4; Muniesa et al., 2012); *E. coli* AW1.7, a non-pathogenic and heat resistant beef isolate (Dlusskaya et al., 2011) and *E. coli* AW1.7 Δ*cfa,* which has an in-frame unmarked deletion of the CFA synthase (Chen and Gänzle, 2016). Strains of *E. coli* were streaked from frozen stock cultures and sub-cultured in Luria-Bertani (LB) (BD, Mississauga, ON, Canada) broth and incubated overnight at 37 °C and 200 rpm. The protocol for preparing dry cells was established for *Salmonella enterica* (Uesugi et al., 2006). Bacterial culture (100 µL) was plated on the surface of LB agar and incubated overnight at 37 °C. Cells were removed from the surface of the agar by washing with 1 mL of 0.85% NaCl (Fisher Scientific, Ottawa, ON, Canada). The resulting cell suspension had a cell count of 10-11 $\log_{10}$ CFU/mL.

5.2.2. Preparation of dried bacterial cells

Bacterial culture was washed twice with 0.85% NaCl and re-suspended in 0.1% peptone water (BD) or solutions of 1 or 10% trehalose (Fisher Scientific) in water (wt/vol). Bacterial suspensions (20 µL) were transferred into sterilized glass vials (12 mm by 30 mm by 4.6 mm; Sigma-Aldrich, St Louis, MO, USA) and dried under vacuum in a desiccator for 3-4 h or air dried in an anaerobic system (model 1025/1029, ThermoForma, Fisher Scientific). After drying, samples were transferred to an air-tight container containing saturated NaCl solution to achieve a_w of 0.75 and incubated at 37 °C for 18 h (Mathlouthi, 2001). Samples dried in the anaerobic chamber were transferred in an airtight container with two BD GasPak™ (Fisher Scientific).

5.2.3. Membrane lipid oxidation assay based on C_{11}-BODIPY$^{581/591}$

The stock solution of C_{11}-BODIPY$^{581/591}$ (Thermo Fisher Scientific, Waltham, MA, USA) was dissolved in dimethyl sulfoxide (DMSO) to achieve a concentration of 1 mM. Cells were suspended in 10 mM citrate buffer (pH 7) and incubated with 10 µM C_{11}-BODIPY$^{581/591}$ in the dark for 30 min at 37 °C and 200 rpm. The outer membrane from bacteria was disrupted with ethylenediaminetetraacetic acid (EDTA) and lysozyme before staining as described to increase the solubility of C_{11}-BODIPY$^{581/591}$ into the cytoplasm lipid membrane (Gänzle et al., 1999). In brief, cells were washed twice with ice-cold 50 mM Tris×HCl (pH 8.0) containing 20% (wt/vol) sucrose. Addition of 0.2 mL lysozyme solution (5 mg/mL lysozyme in 0.25 M Tris×HCl, pH 8.0) and 0.4 mL EDTA (0.25 M, pH 8.0) were added to the cell suspension, followed by incubation for 30 min at 37 °C and 200 rpm. After incubation, cell pellets were suspended with citrate buffer and incubated with C_{11}-BODIPY$^{581/591}$ for 30 min at 37 °C with shaking. To expose the cells

to oxidative stress, *E. coli* cells were firstly treated with H_2O_2 (50 mM) for 30 min, and then followed the staining protocols with C_{11}-BODIPY$^{581/591}$.

5.2.4. Flow cytometric determination of fluorescence

The method to perform flow cytometric analysis is described in section 4.2.6. *E. coli* cells treated with C_{11}-BODIPY$^{581/591}$ were analyzed with respect to green and red fluorescence intensity. The gating of green and red fluorescence intensity was manually set to include more than 99% of the cells from non-stained controls as green and red fluorescence negative (Figure 5.1). Four subpopulations including non-oxidized, oxidized and fully oxidized, fully oxidized and non-stained were divided by two reference-lines shown in Figure 5.1. The fully oxidized population was combined with oxidized and non-oxidized population in Figure 5.2 and 5.3 because the value of oxidized population was negligible.

5.2.5. Microscopy determination of cell morphology

E. coli AW1.7 was dried with peptone and followed the staining protocol including treatment with EDAT and Lysozyme in section 5.2.3. The cell morphology was observed to confirm the cell integrity after lysozyme treatment. A volume of 10 μL of cell suspension was transfer onto the microscopy slides and examined using fluorescence microscopy (Axio Imager M1m microscope, Carl Zeiss Inc.). The images were acquired at 100 × magnification with an AxioCam M1m camera and Axio Vision version 4.8.2.0 software (Carl Zeiss Inc., Göttingen, Germany).

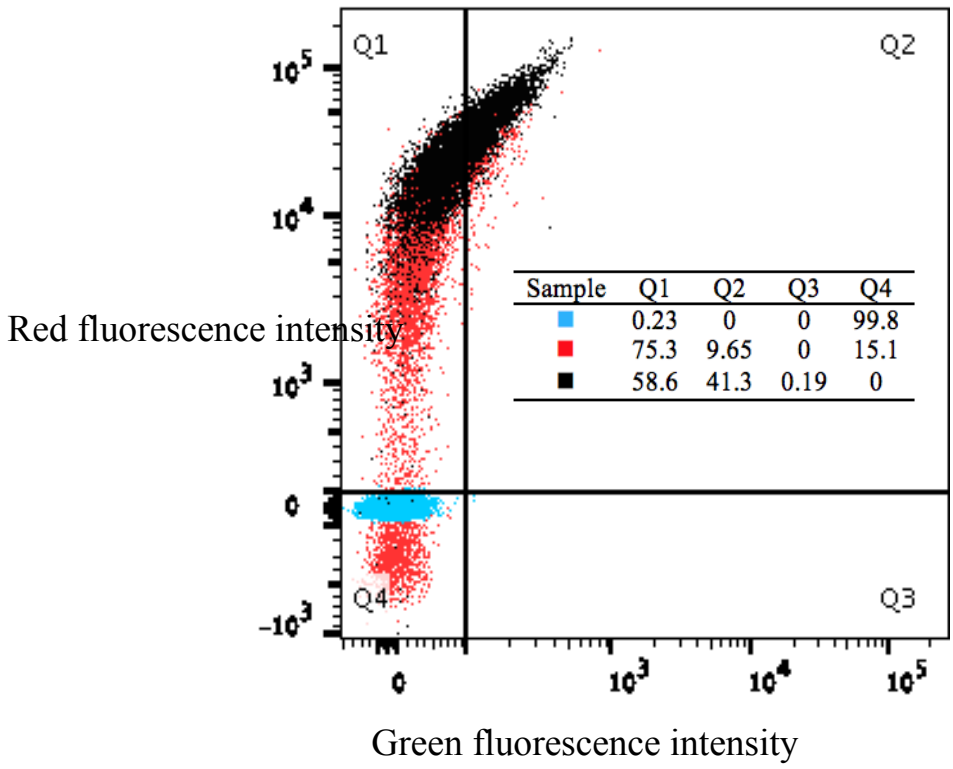

Figure 5. 1. Dot plot of red fluorescence and green fluorescence for *E. coli* AW1.7 not stained with C_{11}-BODIPY$^{581/591}$ (blue symbols) and stained C_{11}-BODIPY$^{581/591}$ (red and black clusters). Stationary phase culture was treated with H_2O_2 (50 mM) for 30 min, treated with EDTA and lysozyme and stained with C_{11}-BODIPY$^{581/591}$ (10 μM) for 30 min (black symbols). Untreated bacteria were treated with EDTA and lysozyme, and then stained with C_{11}-BODIPY$^{581/591}$ (red symbols). The population was divided into four sub-groups by reference lines for green and red fluorescence. The reference lines were set to include at least 99% of unstained cells that was negative for green and red fluorescence. The inset shows the % of cells in the three samples that were not oxidized (Q1); oxidized and non-oxidized (Q2); fully oxidized (Q3), or not stained (Q4).

5.2.6. Quantification of Stx prophage expression

The method to quantify GFP expression in *E. coli* O104:H4 Δ*stx2a*:*gfp*:*amp*^{r} was described in section 4.2.6. A culture of *E. coli* O104:H4 Δ*stx2a*:*gfp*:*amp*^{r} was dried in 0.1% peptone water, or 10% trehalose solution in a vacuum desiccator. Dry cells were equilibrated to a_W 0.75 as described above. Stationary phase *E. coli* O104:H4 Δ*stx2*:*gfp*:*amp*^{r} in LB media served as a control. After drying, *E. coli* cells were suspended in 1 mL LB broth, and analyzed by flow cytometry to determine the

population of cells exhibiting green fluorescence. The gating of GFP fluorescence was set manually to account more than 99% of the cells in controls as GFP negative. Two subpopulations including GFP positive and GFP negative were detected. Cells expressing GFP indicates the expression of the Stx prophage. Data are presented as means ± standard deviation (SD) for three biological replicates.

5.2.7. Determination of viable cell counts

Dry cells were serially diluted and surface-plated onto LB agar, followed by incubation at 37 °C for 24 h. Bacteria without drying served as controls. Reduction of cell counts after drying was expressed as $\log_{10}(N_0/N)$ with N_0 representing the cell counts of the wet cell without drying and N representing the cell counts after drying. Results are means ± SD for four biological replicates.

5.2.8. Quantification of gene expression of dry cells by RT-qPCR

Desiccated cells were rehydrated with 1 mL LB broth and incubated at 30 °C for 2 h. Total RNA was isolated from dry and stationary phase cultures of *E. coli* O104:H4 Δ*stx2a*:*gfp*:*amp*^{r}. RNA was reverse transcribed to cDNA using QuantiTect reverse transcription kit (Qiagen). The gene expression was performed with real-time qPCR (7500 Fast; Applied Biosystems, Foster City, CA, USA) using primers target *gfp*, *recA*, *soxR*, *oxyR* and *rpos* (Table 5.1). Negative controls included DNase-treated RNA and no-template controls. The gene coding for glyceraldehyde-3-phosphate dehydrogenase A (*gapA*) served as the reference gene. The gene expression ratio of target genes to *gapA* in *E. coli* O104:H4 Δ*stx2a:gfp:amp*^{r} after drying and rehydration relative to the bacteria at control conditions were calculated according to the method described by Pfaffl (Pfaffl, 2001). Stationary phase growing cells in LB broth serve as a control condition. The ratio

of gene expression was normalized by transformation with $\log_2$. The value of the relative gene expression of target genes is higher than 1, which is considered as overexpression. Data are presented as means ± SD for four biological replicates.

Table 5. 1. Primers used for relative gene expression

Primers (forward, F; Reverse, R)	Direction: Sequence (5'-3')	Size (base pair)
Primer used for quantification of gene expression		
stx2	F: TATCCTATTCCCGGGAGTTT	200
	R: TGCTCAATAATCAGACGAAGAT	
gfp	F: TTCTTCAAGTCCGCCATG	200
	R: TGAAACGGCCTTGTGTAGTATC	
gapA	F: GTTGACCTGACCGTTCGTCT	116
	R: TGAAACGGCCTTGTGTAGTATC	
recA	F: ATTGGTGTGATGTTCGGTAA	200
	R: GCCGTAGAGGATCTGAAATT	
oxyR	F: CGGTCCAACTGTGGGAATCA	195
	R: CTGGAAGATGAGTTGGGCGT	
soxR	F: GTACCCTGTGATGAGCCGTT	203
	R: ATCAGGAACCCGCCAATACC	
rpos	F: TGCTGGGATAGAGACAGGCA	209
	R: TATCGCGATGCCACGAATGA	

5.2.9. Statistical analysis

The data from membrane lipid oxidation and reduction of cell counts after drying and Stx prophage induction were analyzed by two-way and one-way analysis of variance (ANOVA), respectively. Statistical differences among treatments were determined by Tukey's test with $P<0.05$ using SAS (SAS Institute Inc., Cary, NC, USA).

5.3. Results

5.3.1. Development and validation of C_{11}-BODIPY$^{581/591}$ based flow cytometric assays for quantification of membrane lipid oxidation in *E. coli*

To validate the method based on C_{11}-BODIPY$^{581/591}$, the permeability of the outer membrane to C_{11}-BODIPY$^{581/591}$ was assessed with *E. coli* cells. The availability of

E. coli AW1.7 Δ*cfa* allowed the comparison of membrane lipid oxidation in the wildtype strain *E. coli* AW1.7 and their mutant. The disruption of CFA synthase replaces CFA in the membranes of *E. coli* AW1.7 by an equivalent amount of unsaturated fatty acids (Chen and Gänzle, 2016). The lipid probe C_{11}-BODIPY$^{581/591}$ is soluble in the membrane lipid bilayer (Drummen et al., 2002); however, the outer membrane of Gram-negative bacteria prevents the access of hydrophobic dyes to the cytoplasmic membrane. Accordingly, less than 50% of cells of *E. coli* were stained with C_{11}-BODIPY$^{581/591}$. Treatment with EDTA and lysozyme was added to increase the permeability of the outer membrane to lipophilic C_{11}-BODIPY$^{581/591}$. EDTA and lysozyme significantly enhanced the membrane permeability to C_{11}-BODIPY$^{581/591}$ and decreased the proportion of unstained cells (Figure 5.3). The cell appears spherical under the microscopy (Figure 5.2.), which confirms the formation of protoplasts.

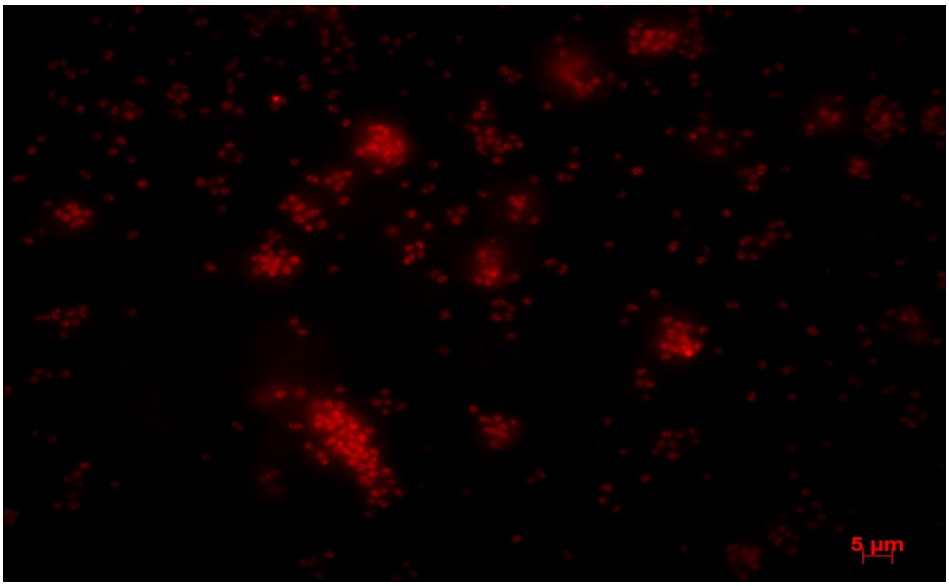

Figure 5. 2. Cell morphology of *E. coli* AW1.7 after treatment with EDTA and lysozyme, and C_{11}-BODIPY$^{581/591}$ under the fluorescence microscopy. Samples were observed with 10 µL of stationary culture with cell density of 10-11 $\log_{10}$ CFU/mL. Three biological replicates were done for each treatment. Scale bars represent 5 µm.

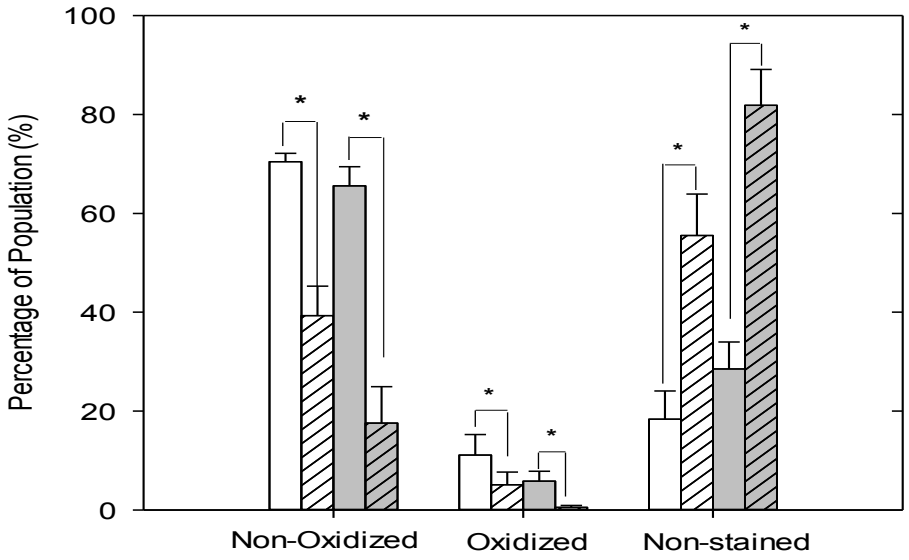

Figure 5. 3. Percentage of *E. coli* cells incorporated with C_{11}-BODIPY$^{581/591}$ and (solid-bars) or without (hatched-bars) the treatment of EDTA and lysozyme. Stationary phase cultures of *E. coli* AW1.7 (white bars) and *E. coli* AW1.7 Δ*cfa* (grey bars) were directly incubated with 10 μM C_{11}-BODIPY$^{581/591}$ for 30 min. The significant differences between EDTA and lysozyme treated cells and untreated cells are indicated by single asterisk (*). Data are means ± standard deviation for three independent experiments.

Treatment with EDTA for 1 h or overnight did not enhance the permeability of C_{11}-BODIPY$^{581/591}$. Specifically, 27% and 11% of the population had incorporated C_{11}-BODIPY$^{581/591}$ after treatment with EDTA for 1 h in the wild type and the mutant of *E. coli* AW1.7, respectively (n=1). The non-oxidized population of cells treated with EDTA and lysozyme was significantly higher than those without EDTA and lysozyme treatments (Figure 5.3). This suggested that adding lysozyme was necessary to achieve a high level of incorporation of C_{11}-BODIPY$^{581/591}$ in the cells.

To determine the effect of oxidative stress on the fluorescence of C_{11}-BODIPY$^{581/591}$, cultures of *E. coli* were treated with H_2O_2, labeled with C_{11}-BODIPY$^{581/591}$, and analyzed by flow cytometry (Figure 5.4). *E. coli* AW1.7 Δ*cfa* and *E. coli* AW1.7 contain unsaturated fatty acids and CFA in the membrane, respectively, as

major components of the cytoplasmic membrane (Chen and Gänzle, 2016) were used to determine the effect of unsaturated fatty acids and CFA on the lipid peroxidation. Without oxidation, the proportion of oxidized C_{11}-BODIPY$^{581/591}$ in wild type and mutant were below 20%. After treatment with H_2O_2, oxidized C11- BODIPY$^{581/591}$ was increased to 48% and 21% for *E. coli* AW1.7 and AW1.7 Δ*cfa*, respectively. Oxidation of C_{11}-BODIPY$^{581/591}$ indicated that *E. coli* AW1.7 Δ*cfa* were less sensitive to oxidation than *E. coli* AW1.7. Overall, the C_{11}-BODIPY$^{581/591}$-flow cytometry assay indicates the oxidation of membrane lipids.

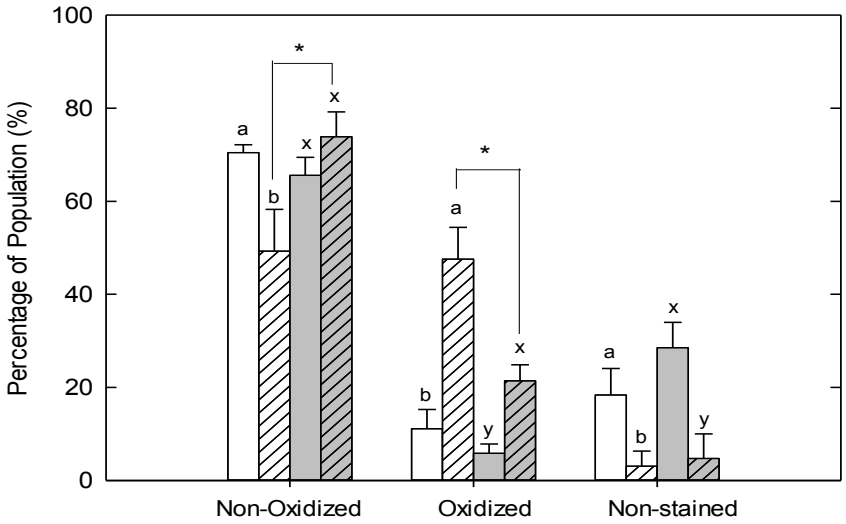

Figure 5. 4. Percentage of C_{11}-BODIPY$^{581/591}$ labeled cells of *E. coli* AW1.7 (white bars) and *E. coli* AW1.7 Δ*cfa* (grey bars) after oxidation by H_2O_2. Stationary phase culture was treated with H_2O_2 (50 mM) for 30 min (hatched bars) and stained with C_{11}-BODIPY$^{581/591}$ (10 μM); untreated cultures after incubation in LB media for 30 min (solid bars) served as control. The significant difference between *E. coli* AW1.7 and *E. coli* AW1.7 Δ*cfa* is indicated by an asterisk (*) (P<0.05). Values for the same strain treated under different conditions that do not share a common superscript differ significantly (P<0.05). Data are means ± standard deviation for three independent experiments.

5.3.2. Effect of drying on the lipid peroxidation

To explore the oxidation of membrane lipids during drying, bacteria were labeled with C_{11}-BODIPY$^{581/591}$ and analyzed by flow cytometry (Figure 5.5). Cultures dried in

0.1% peptone under aerobic conditions show that C_{11}-BODIPY$^{581/591}$ was extensively oxidized, and the proportion of cells with oxidized membrane lipids was above 60% in *E. coli* AW1.7 (Figure 5.5.A) and AW1.7 Δ*cfa* (Figure 5.5.B). In comparison to cultures dried in peptone solution, the percentage of cells with oxidized probe decreased in both strains of *E. coli* after drying in 1% and 10% trehalose. In the presence of 10% trehalose, drying under aerobic or anaerobic conditions did not change the proportion of oxidized probe. The presence of CFA in the membrane of *E. coli* AW1.7 did not increase level of lipid peroxidation after desiccation in comparison to *E. coli* AW1.7 Δ*cfa*. In contrast, hydrated cells of *E. coli* AW1.7 were more prone to oxidation by H_2O_2 (Figure 5.4).

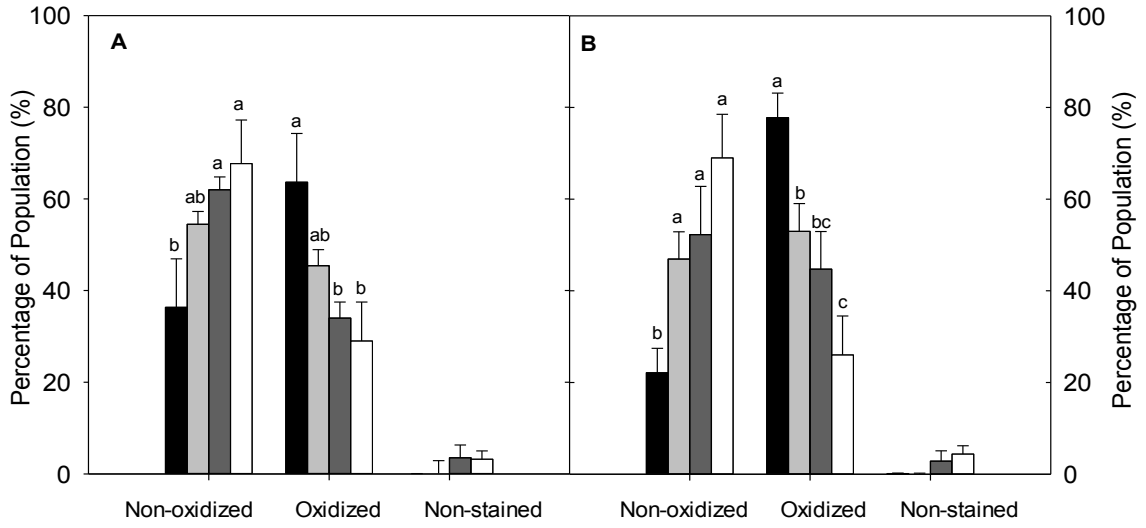

Figure 5. 5. Percentage of C_{11}-BODIPY$^{581/591}$ labeled cells of *E. coli* AW1.7 (panel A) and *E. coli* AW1.7 Δ*cfa* (panel B) after drying. *E. coli* cells were air-dried with 0.1% peptone (black bars), 1% trehalose (grey bars), 10% trehalose (dark grey bars), and dried with 10% trehalose at anaerobic conditions (white bars). No significant differences between *E. coli* AW1.7 and *E. coli* AW1.7 Δ*cfa* after treatment at the same conditions (P<0.05). Values for the same strain treated under different conditions that do not share a common superscript differ significantly (P<0.05). Data represent means ± standard deviation of the means for three independent experiments.

5.3.3. Effect of drying on bacterial survival

Oxidation of membrane lipids compromises the dry survival of *E. coli*. Viable cell count was determined with *E. coli* AW1.7 and *E. coli* AW1.7Δ*cfa* after drying with the conditions used to determine the lipid peroxidation (Figure 5.6). Cultures dried with 0.1% peptone water were most sensitive to oxidation and least resistant to survive after desiccation. Drying in the presence of trehalose, or absence of oxygen reduced the oxidation of C_{11}-BODIPY$^{581/591}$ as shown previously; these conditions also improved the survival of *E. coli*. Under aerobic conditions, *E. coli* AW1.7 Δ*cfa* was more sensitive to drying and re-hydration than *E. coli* AW1.7.

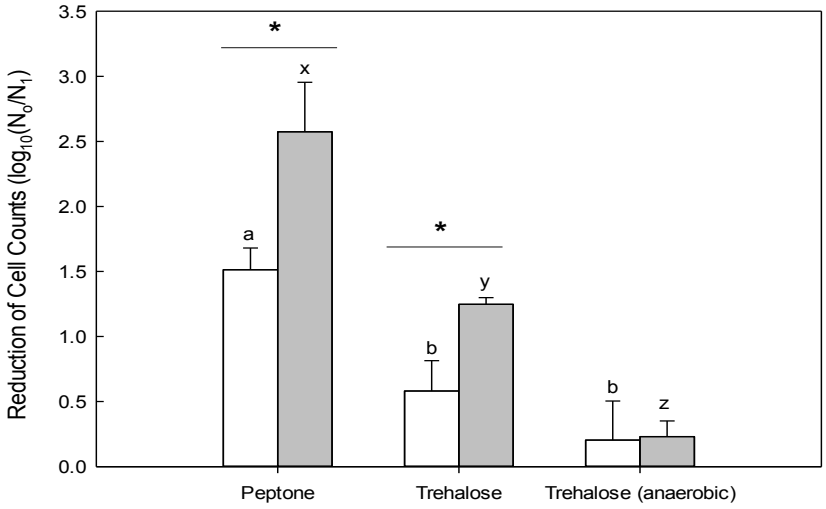

Figure 5. 6. Reduction of cell counts of *E. coli* AW1.7 (white bars) and *E. coli* AW1.7Δ*cfa* (grey bars) after drying in different matrices under aerobic or anaerobic conditions. Liquid cultures were suspended in 0.1% peptone or 10% trehalose before drying in a vacuum desiccator and equilibration to a_w 0.75. The significant difference between *E. coli* AW1.7 and *E. coli* AW1.7Δ*cfa* after the same treatment is indicated by an asterisk (P<0.05). Values for the same strain treated with different conditions that do not share a common superscript are significantly different (P<0.05). Data are means ± standard deviation for at least three independent experiments.

5.3.4. Induction of Stx prophage by drying

Drying cause oxidation of the membrane lipids and lethal to cell viability as shown previously. Oxidative stress can induce Stx prophages in *E. coli* (Łoś et al., 2009). To determine whether drying induces the expression of Stx prophages, we determined the induction of Stx prophage and reduction of cell counts of *E. coli* O104:H4 Δ*stx2a*:*gfp*:*amp*^{r} with a GFP fusion in the prophage to report the expression of Stx (Table 5.2). Drying with peptone only achieved a less than 1 $\log_{10}$ (CFU/mL) reduction of cells counts of *E. coli* O104:H4. The presence of trehalose in the drying matrix enhanced the cell survival by 0.5 $\log_{10}$ (CFU/ml). In both drying conditions, GFP was expressed in *E. coli* O104:H4 Δ*stx2*:*gfp*:*amp*^{r} in an approximately 5% of the population.

Table 5. 2. Flow cytometric quantification of the expression of GFP in *E. coli* 104: H4 Δ*stx2*:*gfp*: *amp* after drying with different matrices.

	Reduction of cell counts $\log_{10}(N_0/N)$	**GFP+ population (%)**	**GFP– population (%)**
Control	0	0.61 ± 0.75^b	99 ± 0.76^a
Peptone (0.1%)	0.87 ± 0.05^a	4.7 ± 2.0^a	95 ± 1.96^b
Trehalose (10%)	0.28 ± 0.15^b	4.8 ± 1.2^a	95 ± 1.2^b

Values in the same column that do not share a common superscript are significantly different (P<0.05). Data represent means ± standard deviation of the means for four independent experiments of treatments with peptone (0.1%) and trehalose (10%). Three independent experiments were conducted for control.

5.3.5. Effect of drying on the expression of *gfp, recA, oxyR, soxR*, and *rpoS*

Drying caused oxidation of membrane lipids in non-pathogenic *E. coli* and induced the prophage expression in STEC. To explore whether the expression of Stx prophage is related to oxidative stress response, the expression of genes coding for oxidative stress resistance and the SOS response in *E. coli* was quantified (Figure 5.7). The fluorescence assay of C_{11}-BODIPY$^{581/591}$ cannot be used with *E. coli* O104:H4

Δ*stx2a*:*gfp*:*amp*^{r} because the fluorescence of GFP interferes with quantification of oxidized C_{11}-BODIPY$^{581/591}$. After drying and rehydration of STEC, the SOS response regulator *recA* was overexpressed. SodR and OxyR, regulators of the oxidative stress response, and global regulator RpoS were not significantly overexpressed in STEC after drying.

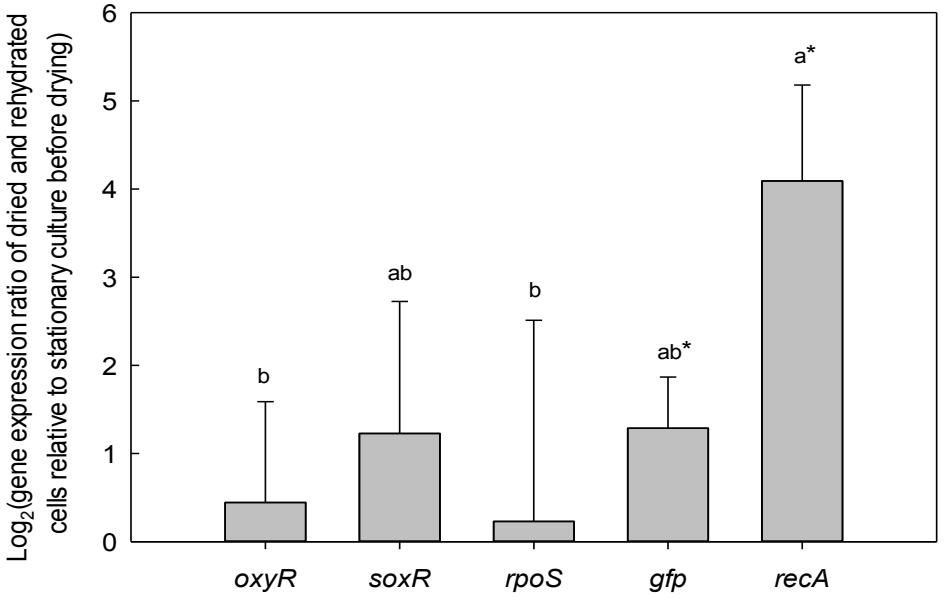

Figure 5. 7. Relative gene expression of *oxyR, soxR, rpoS, gfp* and *recA* in *E. coli* O104: H4 Δ*stx2a*:*amp*:*gfp* after drying and rehydration. Relative gene expression was quantified by RT-qPCR with *gapA* as a housekeeping gene and stationary cultures in LB broth as reference conditions. The significant difference between target genes is labeled with different superscript (P<0.05). The single asterisk indicated the relative gene expression of the target genes is significantly different from one. Data represent means ± standard deviation of the means from three independent experiments with duplicate RNA isolations from each culture.

5.4. Discussion

This study establishes the relationship of oxidative stress, SOS response, and expression of virulence genes of STEC after desiccation and rehydration. Moreover, this

study introduced a method based on C_{11}-BODIPY$^{581/591}$ as a novel tool to determine the oxidative stress and lipid peroxidation in Gram-negative bacteria.

Fluorescence probes used in bacteria to determine oxidative stress employed alkaline phosphatase (Aertsen et al., 2005b), redox-sensitive GFP2 (roGFP2) (Müller et al., 2017), H_2DCFDA (2',7'–dichloro-dihydro-fluorescein diacetate) (Marcén et al., 2017), MitoSOX (Mols et al., 2011), and C_{11}-BODIPY$^{581/591}$ (Johnson et al., 2012). These assays detect cellular oxidative stress by direct interaction with ROS. C_{11}-BODIPY$^{581/591}$ is lipophilic and sensitive to peroxide radicals but not hydroperoxides (Drummen et al., 2002). The oxidation of C_{11}-BODIPY$^{581/591}$ is thus caused by lipid peroxide radicals that are formed after the challenge with H_2O_2 (Laguerre et al., 2007). In the current study, C_{11}-BODIPY$^{581/591}$ was introduced after the removal of H_2O_2 to ensure that H_2O_2 or HO• did not directly oxidize the fluorescent probe. Lipid chain of C_{11}-BODIPY$^{581/591}$ mimics the membrane lipids, which is commonly used to determine the lipid peroxidation in eukaryotic cells (Ball and Vo, 2002). Quantification of C_{11}-BODIPY$^{581/591}$ fluorescence with spectrophotometry and fluorescence microscopy were used in *Pseudomonas* (Johnson et al., 2012) and *Lactobacillus* (Carlsen et al., 2009). Flow cytometric quantification of C_{11}-BODIPY$^{581/591}$ was previously applied only in eukaryotic cells and algae (Cheloni and Slaveykova, 2013; Makrigiorgos et al., 1997), but not in bacteria. This study quantified C_{11}-BODIPY$^{581/591}$ by flow cytometry to determine the level of lipid peroxidation in *E. coli.* Flow cytometry allows the quantification of the C_{11}-BODIPY$^{581/591}$ stained and non-stained cells, which shows that C_{11}-BODIPY$^{581/591}$ had a low penetration to the membrane of Gram-negative bacteria. The problem was undetected by the spectrophotometry or fluorescence microscopy in *Pseudomonas* and *Lactobacillus*

(Carlsen et al., 2009; Johnson et al., 2012). Treatment with lysozyme and EDTA are required to increase the permeability of cells to C_{11}-BODIPY$^{581/591,}$ which compromises viability of *E. coli.* This limits the application of this protocol to measure the oxidative stress in a real-time fashion.

C_{11}-BODIPY$^{581/591}$ is hydrophobic and mostly excluded from the cytoplasm and periplasm of Gram-negative bacteria (this study). The outer membrane has a low permeability to hydrophobic inhibitors because the outer leaflet consists of highly ordered and hydrophilic lipopolysaccharides (Vaara, 1992). Disruption of the outer membrane with EDTA and lysozyme (Vaara, 1992) allowed the staining with C_{11}-BODIPY$^{581/591}$ without affecting the oxidation of lipids in the cytoplasmic membrane (this study). Chelating agents, such as EDTA were used to enhance the membrane permeability of hydrophobic dye (Szivak et al., 2009). Using EDTA alone did not significantly increase the permeability of the outer membrane to C_{11}-BODIPY$^{581/591}$ in this study (data not shown). Cell treated with lysozymes appears spherical ensured that *E. coli* were not lysed after treatment with lysozyme. Flow cytometry ensured that the C_{11}-BODIPY$^{581/591}$ was incorporated in the majority of the cells.

The role of oxidative stress in desiccation tolerance was documented in *Lactobacillus* (Laguerre et al., 2007) and *E. coli* (this study). Trehalose increases the desiccation tolerance as it maintains the membrane structure (Leslie et al., 1995) and reduces the level of lipid peroxidation in yeast (Pereira et al., 2003) and *E. coli* (this study). Trehalose was suggested to prevent the lipid from oxidation by forming a stable complex between hydroxyl groups of trehalose and double bond of unsaturated fatty acids (Oku et al., 2003).

CFA in membrane lipids are synthesized from unsaturated fatty acids to protect bacterial cells against desiccation (this study) and *Pseudomonas putida* from freeze-drying (Muñoz-Rojas et al., 2006). This study indicated that desiccation tolerance is related to the lipid oxidation in the membrane. The relative oxidative stability of CFA and unsaturated fatty acids remains unknown. CFA and unsaturated fatty acids were oxidized during desiccated storage (Castro et al., 1995; Grogan and Cronan, 1986; Teixeira et al., 1996). The comparison of *E. coli* AW1.7 and AW1.7 Δ*cfa* demonstrated that conversion of unsaturated fatty acids to CFA reduced the lipid peroxidation in hydrated but not in dry membranes (this study). The role of CFA on the oxidative stability of bacterial membranes thus depends on other biophysical properties of the membrane. The packing density of phospholipids is altered when the membrane transition from the liquid to the gel phase upon dehydration (Crowe and Hoekstra, 1992). CFA maintain the membrane integrity because the cyclopropane ring of CFA is rigid and resistant to packing into acyl chain array of phospholipids, which maintains the lamellar structure of the phospholipid membrane (Poger and Mark, 2015). In contrast, *cis*-unsaturated fatty acids are flexible and can bend and promote a compact bilayer (Poger and Mark, 2015). This suggests that physical and chemical properties, particularly the oxidative stability of unsaturated and CFA were different in the hydrated or dry membrane; however, the mechanisms that cause this differential effect remain unexplored.

Desiccation induces oxidative stress; however, the transcriptional response to oxidative stress is poorly documented in desiccated bacteria. During desiccation, the Fe-protein and Fe-superoxide dismutase (SodF) were overexpressed in desiccated

Salmonella and *Cyanobacteria*, suggesting that Fe-proteins improve survival after desiccation (Gruzdev et al., 2012; Shirkey et al., 2000). In *E. coli* and other bacteria, SoxR and OxyR pathway are involved in preventing oxidative damage. SoxR and OxyR are transcriptional factors, which bind on the promoter region and control the transcription of proteins that prevent or repair oxidative damage. Oxidation of SoxR and OxyR reduces binding to the promoter region and activates transcription of genes for peroxide detoxification (Choi et al., 2001; Ding et al., 1996). This study found that the expression of SoxR and OxyR in dry and rehydrated STEC was not overexpressed different from stationary phase STEC without drying. OxyR was suggested as a repressor, which leads to overexpression of catalase genes including *kata, dps, ftn* and *cydA* in *Corynebacterium*, and mediates resistance to H_2O_2 (Kim and Holmes, 2012; Teramoto et al., 2013). In *E. coli,* the protein level of OxyR was not different after H_2O_2 treatment (Zheng et al., 1998). Both studies indicated that OxyR acts as transcriptional regulator mediated by changes in protein confirmation upon oxidation instead of involving significant changes in the mRNA level of *oxyR* in response to stressors. SoxR was is a positive regulator of *soxS,* whose transcriptional level indirectly indicates the oxidative or non-oxidative state of SoxR (Koo et al., 2003). Therefore, determination of expression of genes regulated by OxyR or SoxR is required to further understand the role of the regulatory proteins in the oxidative stress response.

Oxidative stress also relates to the SOS response and the RpoS-regulated general stress response (Farr and Kogoma, 1991). RpoS regulated gene expression is strictly related to the growth phase (Farr and Kogoma, 1991), which may limit the transcription of RpoS following a short re-hydration period as used in this study. RecA regulated by

the SOS response was significantly overexpressed in STEC after drying and rehydration, which indicates that the oxidative stress in dry cell leads to the DNA damage.

The mechanism of desiccation tolerance of pathogenic *E. coli* matches the response of commensal and generic *E. coli* (Louis et al., 1994); however, the expression of the virulence gene is not fully explored. STEC have similar resistance to desiccation compared with generic *E. coli* and additionally over-expressed the Stx prophage after desiccation and re-hydration (this study). Induction of lambda prophage during desiccation was reported previously (Webb and Dumasia, 1967). Stx phages belong to lambdoid phages but the regulation of prophage expression is different from lambda phage (Beutin et al., 2012). The induction of Stx prophage after desiccation corresponds to the overexpression of RecA (this study), which regulates the expression of prophage late region (Waldor and Friedman, 2005). The expression of Stx prophage after desiccation and rehydration might lead to the production of phage particles that potentially transduce non-virulent *E. coli* before or after ingestion and thus amplify the production of toxin (Bielaszewska et al., 2007). The outbreak in 2011 likely involved long-term survival of *E. coli* O104:H4 on dry fenugreek seeds, followed by rehydration during sprouting (Beutin and Martin, 2012). Survival and persistence of STEC in dry foods is a concern of the food industry and consumer safety (Beuchat et al., 2013).

5.5. Conclusions

The current study developed a methodology using the fluorescence dye C_{11}-BODIPY$^{581/591}$ to probe lipid oxidation of desiccated bacteria; and used GFP-labeled STEC to indicate that drying induced *stx* expression. Oxidative damage is lethal to desiccated bacteria and could enhance the production of Stx. This study improved the

knowledge on the impact of drying on oxidative stress and its effect on the regulation of genes for oxidative stress response and *stx* in STEC.

5.6. References

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CHAPTER 6. Virulence Gene Transfer Mediated by Shiga toxin Phage (Φ11-3088) from *Escherichia coli* O104:H4 *in vivo* and During Sprout Production

6.1. Introduction

Stx are major virulence factors of Shiga toxin producing *E. coli*, and encoded on temperate lambdoid prophages (Herold et al., 2004). Infection by STEC results in a range of diseases from mild diarrhea to severe symptoms associated with severe renal damage (Antonovskii and Shurukhin, 2005; O'Loughlin and Robins-Browne, 2001; Trachtman et al., 2012). The pathogenesis of STEC dependents on the adhesion of *E. coli* to the epithelial mucosa and absorption of Stx into the systemic circulation (O'Loughlin and Robins-Browne, 2001). The transcription of Stx prophage is repressed in the lysogenic cycle when the prophage DNA is replicated with the chromosome (Beuchat et al., 2013; Croxen et al., 2013; Ranieri et al., 2014). The prophage converts to the lytic cycle by induction when the bacterial SOS response is activated, commonly by DNA damage (Waldor and Friedman, 2005). Induction of Stx phage results in the production and release of toxin and phage (Chapter 4; De Sablet et al., 2008; Łoś et al., 2009).

A novel STEC, *E. coli* O104:H4, caused 4,000 cases and 50 deaths in Germany in 2011, making it one of the largest outbreaks of STEC (Frank et al., 2011). The outbreak strain *E. coli* O104:H4 encodes Stx2a and aggregative adherence fimbria; the virulence profile of which is different from *E. coli* O157:H7 and other enterohemorrhagic *E. coli* (EHEC) carrying adhesion factors for attaching/effacing lesions (A/E) (Beutin and Martin, 2012). *E. coli* O104:H4 share a high degree of nucleotide identity with the previously described enteroaggregative *E. coli* O104:H4, which are *stx2a* negative (Grad et al., 2013a; Mossoro et al., 2002; Smith et al., 2012b). The combination of virulence factors

suggests that the parental strain of *E. coli* O104:H4 has acquired Stx2 by transduction or lysogenic infection (Grad et al., 2013a; Mellmann et al., 2011; Smith et al., 2012b). The outbreaks of STEC O104:H4 in Germany and France were linked to the consumption of sprouts produced from contaminated fenugreek seeds (Beutin and Martin, 2012; Rasko et al., 2011). Seeds are stored dry and rehydrated during sprouts germination. Dry storage allows for a long term survival of enteric pathogens including *E. coli* on the surface of seeds (Beuchat and Scouten, 2002). During sprouting, seeds are rehydrated and kept in a warm and moist environment, which supports seed germination but also provides suitable conditions for bacterial recovery and growth.

Transduction of virulence genes was documented by Stx phages including Φ734, $\Phi 24_{B,}$ 933W, ΦA557 and Φ3538 derived from STEC O157:H7 (Gamage et al., 2004; Herold et al., 2004; Imamovic et al., 2009; McCarthy et al., 2002; Schmidt et al., 1999). Transduction of *stx2* by Φ933W, ΦA557 or Φ3538 was reported in liquid foods and solid foods including ground beef and lettuce (Imamovic et al., 2009; Nyambe et al., 2017). The regulation of prophage genes among Stx phages is diverse due to the mosaic and plastic phage genomes (Beutin et al., 2012; Herold et al., 2004; Ranieri et al., 2014). The Stx2 phage of STEC O104:H4 exhibits significant differences in the modules for DNA replication, super-infection immunity, and tail fibre proteins compared to the previously described Stx phages (Beutin et al., 2012). Phage infection is established through the recognition and binding between phage tail fibers and host membrane proteins (Chatterjee and Rothenberg, 2012; Letellier et al., 2004). The heterogeneity on the tail protein thus changes the specificity of the binding receptor to the host (Zhou et al., 1990).

This study aimed to investigate the host specificity of Stx phage Φ11-3088 from STEC O104:H4 11-3088, a strain isolated from a patient diagnosed with HUS in Canada. The transduction frequency of Φ11-3088 was investigated during seed germination using the derivative Φ11-3088 with *stx2a* replaced with a cassette of *gfp* and *amp*^{r} (Chapter 4). The in-frame fusion of the green fluorescence protein in the prophage allows for a single-cell quantification of *stx2a*-expressed cells, and was used to probe the effect of the host range on the effect on the *stx2a* expression.

6.2. Materials and methods

6.2.1. Bacterial strains and growth conditions

Bacterial strains used in this study are listed in Table 6.1 and Table 6.2. *E. coli* O104:H4 Δ*stx2a:gfp:amp*^{r} with *stx2a* replaced with *gfp* and *amp*^{r} was an outbreak strain (Chapter 4). *E. coli* O104:H4 strain 11-3088 (GenBank GCA_001309755.1) was provided by Health Canada (Liu et al., 2015). *E. coli* O104:H4 11-3088 carries the Stx prophage Φ11-3088. Pathogenic bacteria included three EHEC, two uropathogenic *E. coli* (UPEC), five ETEC, and two *Shigella* spp. The ETEC strains were provided by Reference laboratory for *E. coli* (ECL). Commensal *E. coli* including nine human isolated *E. coli*, fourteen *E. coli* from rectum or vagina of dairy cows raised in the University of Dairy Research and Technology Center (Dlusskaya et al., 2011), and ten beef processing isolates from cattle in a commercial slaughter plant (Aslam et al., 2004). All beef processing isolates were received from the Agriculture and Agri-Food Canada, Lacombe Research Centre, Alberta. *E. coli* DH5α was used as a positive control for phage lysogenic and lytic infection with phage Φ11-3088. *E. coli* DH5α carry the plasmid pJIR750ai, which encode a chloramphenicol resistance gene and was used as a selective

marker for the selection of *E. coli* DH5α after phage transduction. *E. coli* and *Shigella* strains were streak out from frozen stock on Luria-Bertani (LB) (Becton Dickinson, Mississauga, ON, CA) and Brain Heart Infusion (BHI) (Becton Dickinson) media at 37 °C aerobically, respectively. A final concentration of 100 mg/L ampicillin (Amp) and/or 30 mg/L chloramphenicol (Chlor) were added into the nutrient media to select for antibiotic-resistant strains.

Table 6. 1. Pathogenic *E. coli* strains used in this study

Strain	Virulence factors	Origin	Amp Resistance	Reference
E. coli O104:H4 Δ*stx2:gfp:amp*^{r}	*agg*	Human	+	Chapter 4
E. coli O145:NM 03-6430	*stx1, eae*	Human	-	Health Canada
E. coli O26:H11 05-6544	*stx1, eae*	Human	-	Health Canada
E. coli O45:H2 05-6545	*stx1, eae*	Human	-	Health Canada
E. coli O103:H2 PARC 444	*pap*	Chicken	-	Roger Johnson
E. coli O103:H2 PARC 445	*pap*	Turkey	+	Roger Johnson
E. coli O111:NM PARC 447	*stx1, stx2, eae*	Unknown	-	Burton Blais
E. coli O26:H11 PARC 448	*eae*	Unknown	-	Burton Blais
E. coli O145:NM PARC 449	*eae*	Unknown	-	Burton Blais
E.coli ATCC 31618	*estIa*	Calf	-	ATCC strain collection
E. coli ECL 13086	*estIa,estIb,astA,* F4	Pig	+	This study
E. coli ECL 13795	*estIb*, *astA,* K88	Pig	+	Chen et al., 2014
E. coli ECL13998	*estIa*, *estIb*,*astA,* F4	Pig	+	Chen et al., 2014
E. coli ECL 14408	*estIb*, *astA,* F4	Pig	+	This study
E. coli B3-62 FUA 1069	unknown	Grizzly bear	-	Stenhouse et al., 2009
Shigella sonnei ATCC 25391	unknown	Human	-	This study
Shigella flexneri A62 FUA 1148	unknown	Human	-	This study

Table 6. 2. Non-pathogenic strains used in this study

Origin	Strain number	Ampicillin Resistance	Reference
Human isolates	*E.coli* FUA 1170	-	Strain collection from Food Microbiology lab in the University of Alberta
	E.coli FUA 1171	-	
	E.coli FUA 1172	-	
	E.coli FUA 1415	-	
	E.coli FUA 1416	-	
	E.coli FUA 1409	-	
	E.coli FUA 1412	-	
	E.coli FUA 1413	-	
	E.coli FUA 1414	-	
	E.coli FUA 1405	+	
	E.coli FUA 1406	+	
	E.coli FUA 1407	+	
	E.coli FUA 1408	+	
	E.coli FUA 1173	+	
Cow vagina	*E.coli* FUA 1050	-	(Dlusskaya et al., 2011)
	E.coli FUA 1174	-	
	E.coli FUA 1176	-	
	E.coli FUA 1178	-	
	E.coli FUA 1059	-	
Cow rectum	*E.coli* FUA 1040	-	(Dlusskaya et al., 2011)
	E.coli FUA 1044	-	
	E.coli FUA 1045	-	
	E.coli FUA 1046	-	
	E.coli FUA 1047	-	
	E.coli FUA 1048	-	
	E.coli FUA 1049	-	
	E.coli FUA 1042	-	
	E.coli FUA 1043	-	
Beef processing	*E.coli* AW1.7	-	(Aslam et al., 2004)
	E.coli GM9-3	-	
	E.coli AW1.8	-	
	E.coli AW1.7	-	
	E.coli MB10-1	-	
	E.coli GM9-1	-	
	E.coli GM9-5	-	
	E.coli GM9-8	-	
	E.coli GM11-2	-	
	E.coli GM11-3	-	
	E.coli GM11-4	-	
Commercial cloning strains	*E. coli* DH5α, *recA*$^-$	-	(Phue et al., 2008)
	E. coli DH5α pJIR750ai	-	Sigma

6.2.2. Preparation of phage filtrates and plaque assay

E. coli O104:H4 Δ*stx2a:gfp:amp*^{r} were donors of Stx2 phage particles. Preparation of phage filtrate was described by Iversen et al. (2015). A log-phase culture (OD_{600} of 0.4-0.6) of *E. coli* O104:H4 Δ*stx2a:gfp:amp*^{r} grown in LB broth containing $CaCl_2$ (5 mM) was induced with 0.5 g/L mitomycin C followed by incubation at 37 °C for 18 h. Phage particles were collected by centrifugation at 5,311x g for 10 min, and the supernatant was filtered using 0.22 μm filters (Fisher Scientific, Ottawa, Ontario). Trypsin (Sigma-Aldrich, St Louis, MO, USA) with a final concentration of 0.1 g/L was added to the phage-filtrate, and the filtrates were incubated for 1 h at 37 °C to remove the colicin (Iversen et al., 2015). The phage concentration was determined by plaque assay using *E. coli* DH5α as a recipient strain. The mixture of 100 μL of log-phase *E. coli* DH5α culture and 900 μL of phage filtrate was incubated at 37 °C for 30 min without agitation. After incubation, the phage and bacterial cultures were mixed with 3 mL of 0.7% LB agar, and the mixture was transferred onto LB agar containing 10 mM $CaCl_2$ followed by incubation for 18 h at 37 °C. The phage concentration was presented as plaque forming units/mL (PFU/mL).

6.2.3. Lysogenic and lytic phage infection with recombinant Φ11-3088 (Δ*stx2a:gfp:amp*^{r})

The ability of *E. coli* to form lysogens and plaques was used to indicate the lysogenic and lytic infection by Φ11-3088 Δ*stx2a:gfp:amp*^{r}. Lysogenic infection was described by Schmidt et al., (1999). Recipient strains were tested for ampicillin resistance before the experiment. The ampicillin resistant strains were excluded from this study. The log-phase culture (100 μL) was mixed with 100 μL of Φ11-3088 (Δ*stx2:gfp:amp*^{r}) with a

concentration of 10^4 PFU/mL, followed by incubation for 4 h at 37 °C. The mixture was further incubated in LB broth containing ampicillin (100 mg/L) for 24-48 h at 37 °C and 200 rpm. Bacteria were collected by centrifugation at 5,311x g for 30 min and plated onto LB agar containing 100 mg/L ampicillin. Colonies on the LB plates containing ampicillin were suspended in 10 µL H_2O, treated at 95 °C for 5 min and tested by PCR amplification with *gfp* specific primers (F: TCCTGGTCGAGCTGGACG; R:TGGAGTTCGTGACCGCCG) to confirm the presence of *gfp*.

Fourteen strains were tested for lytic infection and performed by spot agar assay (Iversen et al., 2015). *E. coli* DH5α was used as recipient strains to lytic infection by Φ11-3088 Δ*stx2a:gfp:amp*^{r}. Stationary-phase *E. coli* DH5α (100 µL) was mixed with 3 mL of warm 0.6% LB agar and transferred onto standard LB agar. After the solidification of the top layer of the agar, 10 µL of phage filtrate were spotted on the top of agar. Clear zones formed on the plates following after the incubation at 37°C for 18 h indicate that the recipient strains were susceptible to lytic infection by phage.

6.2.4. Flow cytometric quantification of GFP fluorescence and forward light scatter (FSC)

The method to quantify GFP fluorescence and FSC was developed in Chapter 4 section 4.2.6. Exponential phase culture of six lysogenic strains including *E. coli* DH5α, *E.coli* O103:H2 PARC 444, ETEC ATCC 31618, cow isolated *E.coli* FUA 1043 and *Shigella sonnei* ATCC 25391 were induced with mitomycin C (0.5 g/L) for 3 h. Stationary-growing cultures without induction served as controls. Bacterial cultures with a volume of 200 µL were diluted with 1 mL of 0.85% NaCl (Fisher Scientific, Ottawa, ON, CA) and further diluted with FACS buffer (1% PBS, 2% fetal calf serum [FCS],

0.02% sodium azide) to maintain the running speeds to no more than 3000 events per second. Flow cytometry was performed using a BD LSR-Fortessa X20 (BD Biosciences, San Jose, CA, USA) equipped with a 488 nm excitation from a blue air laser at 50 mW to excite green fluorescence (530±30 nm). Sample injection and acquisition started simultaneously and stopped when about 10,000 events were collected. FCS files were extracted from FACSDiva 8 software and analyzed by FlowJo software (Tree Star, Ashland, OR, USA). FSC is proportionally increased with cell size and thus used to measure the cell volume (Koch et al., 1996). The gating of GFP fluorescence and FSC was manually set to include more than 97% of the cells in control samples as normal size and GFP negative. Four subpopulations including GFP positive and elongated cell (GFP+, FSC+), GFP positive and regular sized cell (GFP+, FSC-), GFP negative elongated cell (GFP-, FSC+), and GFP negative and regular sized cell (GFP-, FSC-) were divided by two reference lines.

6.2.5. Transduction of Stx phage during sprouts germination and validation of lysogens

E. coli O104:H4 Δ*stx2:gfp:amp*^{r} was grown in 5 mL LB broth at 200 rpm and 37 °C for 18-20 h. Bacterial culture (100 µL) was plated on the surface of LB agar and incubated overnight at 37 °C. Cells were removed from the surface of the agar by washing with 1 mL of 0.85% NaCl (Fisher Scientific, Ottawa, Ontario). The resulting cell suspension had a cell count of 10-11 $\log_{10}$ CFU/mL. This bacterial culture was used to inoculate the mung beans to investigate the transduction of *stx2* during seed germination.

Mung beans were purchased from the local grocery market and decontaminated with 0.2% (vol/vol) sodium hypochlorite in water prepared with 20% sodium

hypochlorite (Sigma-Aldrich, St. Louis, MO, USA) for 15 min. Mung beans were washed twice with sterilized water, dried on sterilized filter paper in the biosafety cabinet for 2 h, and then stored in a desiccator. Ten-grams of mung beans were inoculated with 1 mL stationary-phase culture of *E. coli* O104:H4 Δ*stx2a:gfp:amp*^{r} with a concentration of 10-11 $\log_{10}$ CFU/mL.

Transduction of Stx recombinant phage during the sprouting process was determined by the formation of Stx2a lysogenized *E.coli* DH5α. Mung beans (10 g) were transferred into a 0.22 μm Millipore® Stericup^{TM} filtration unit (Fisher scientific) and rehydrated with 10 mL sterilized water for 3-4 h. Stationary phase culture of *E. coli* DH5α carrying pJIR750ai (Sigma-Aldrich) was added into the filtration unit. The colonies from beans (2 kernels) or sprouts (2 pieces) were removed by vortex with 1 mL 0.1% peptone water, and bacterial suspensions were serially diluted with 1 mL 0.1% peptone water (BD, Fisher Scientific, Ottawa, ON, CA). Dilutions were plated onto LB agar, LB agar containing ampicillin (100 mg/L), LB agar containing chloramphenicol (30 mg/L), and LB agar containing both antibiotics to select for antibiotic-resistant cells after five days of sprouting. Colonies on the LB plates containing both ampicillin and chloramphenicol were possible lysogens and confirmed with PCR amplification using *gfp*-specific primers (section 6.2.3). Plasmids were extracted using the plasmid DNA isolation GeneJET kit (Thermo Fisher Scientific, Ottawa, ON, CA) from the strains that were positive for GFP. The size of the plasmid was compared with pJIR750ai from *E.coli* DH5α by gel electrophoresis (120 mV, 60 min).

6.2.6. Statistical analysis

The data from Table 6.5 and Figure 6.1 were analyzed by one-way and two-way analysis of variance (ANOVA), respectively. Statistical differences among treatments were determined by LSD test with $P<0.05$ using SPSS 21.0 (SPSS Inc., Chicago, IL, USA).

6.3. Results

6.3.1. Infection of Stx phage to *stx2*-negative *E. coli*

To investigate the bacterial spectra that are sensitive to the Φ11-3088 mediated phage infection, pathogenic *E. coli* (Table 6.1) and ampicillin sensitive commensal *E. coli* (Table 6.2) were infected with Φ11-3088 (Δ*stx2:gfp:amp*^{r}). Five out of forty-five strains were susceptible to phage transduction, including *E. coli* DH5α, *E. coli* O103:H2 PARC 444, ETEC ATCC 31618, *Shigella sonnei* ATCC 25391, and *E. coli* FUA1043 (Table 6.3). Lysogens were screened by ampicillin resistance, and confirmed by PCR amplification using primers targeting *gfp*. Strains of EHEC containing *stx1*, *eae,* or both were not lysogenized by the Stx2 phage from *E. coli* O104:H4 (Table 6.1).

Table 6. 3. Validation of transduced strains using Amp-resistance assay, PCR and spot agar assay

Pathgroup	Strain	Amp-resistance in/on broth and agar	PCR detection of *gfp*
Lab strain	*E. coli* DH5α	+	+
UPEC	*E. coli* O103:H2 PARC 444	+	+
ETEC	*E. coli* ATCC 31618	+	+
Cow isolates	*E. coli* FUA 1043	+	+
Enteroinvasive *E. coli*	*Shigella sonnei* ATCC 25391	+	+

To investigate the susceptibility of *E. coli* to Φ11-3088 (Δ*stx2:gfp:amp*^{r}) by lytic infection, strains were tested by spot agar on lawn assay. Only *E. coli* DH5α was susceptible to lytic infection, which lacks *recA* and *endA* for DNA repair and replication (Phue et al., 2008; Table 6.4).

Table 6. 4. Susceptibility of *E.coli* to lytic infection by Stx2 phage

Pathogroup/origin of isolates	Strain	Presence of lytic zones on the bacteria lawn
RecA deficient strain	*E. coli* DH5α	+
UPEC	*E. coli* O103:H2; PARC 445	-
	E. coli O103:H2; PARC 444	-
Shigella spp.	*Shigella sonnei* ATCC 25391	-
Cow rectum	*E.coli* FUA 1043	-
ETEC	ECL 14408	-
	ECL 13998	-
	ECL 13795	-
	ECL 13086	-
	ATCC 31618	-
Human	FUA 1405	-
	FUA 1406	-
	FUA 1407	-
	FUA 1408	-
	FUA 1173	-

6.3.2 Induction of Stx2 prophage in lysogens after treatment with mitomycin C

Mitomycin C induces the SOS response in *E. coli* and consequently results in the expression of lambdoid prophages and cell filamentation (Chapter 4; Justice et al., 2008). Cell filamentation and induction of Stx2 phage in lysogens were compared by the quantification of fluorescence of GFP and forward scatter light using flow cytometry (Figure 6.1). Lysogenic strains of *E. coli* O103:H2 PARC 444 and *Shigella sonnei* ATCC 25391 were induced by mitomycin C with an approximately 46% of the cells expressing GFP. ETEC ATCC 31615, *E. coli* DH5α and *E. coli* FUA1043 with less than 15% of the population expressing GFP. Filament cells were detected less than 10% in the population

of *E. coli* O104:H4 Δ*stx2a*:*gfp*:*amp*^{r}, *E. coli* O103:H2 PARC 444, ETEC ATCC 31615 and *Shigella sonnei* ATCC 25391 after the induction of mitomycin C; while no GFP expression and filaments were found in untreated cells (Figure 6.1).

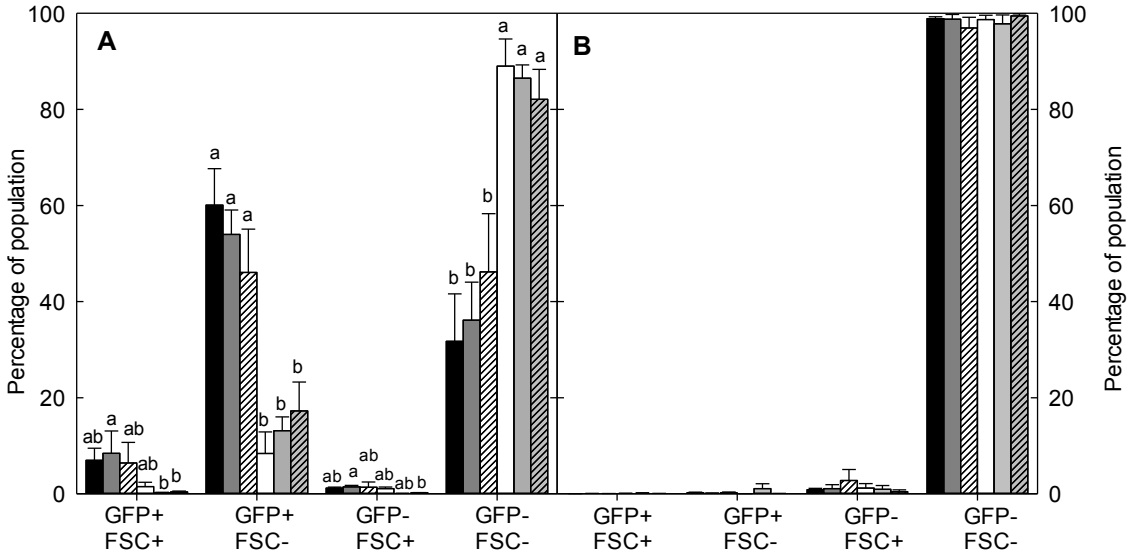

Figure 6. 1. Quantification of *gfp* expression in five Stx2-phage lysogenic strains after induction of exponentially growing cells with mitomycin C (panel A). Exponentially growing cells without induction served as control (panel B). *E. coli* O104:H4 Δ*stx2a*:*gfp* (black bar), *E.coli* O103:H2 PARC 444 (dark-grey bar), *Shigella sonnei* (hatched bar), ETEC ATCC 31615 (white bar), *E. coli* DH5α (light-grey bar), *E. coli* FUA1043 (hatched-grey bar) were incubated in LB broth with the addition of mitomycin C to a concentration of 0.5 mg/L for 3 h. Bacterial cultures were harvested and the proportion of GFP fluorescent and filament cells was quantified by flow cytometry. Data represent means ± standard deviation for four independent experiments. Significant differences among different strains in the same groups are indicated with different letters (P<0.05).

6.3.4. Transduction of Stx phage during sprouting

Sprouting of seeds provides a favourable condition for bacterial growth and may support phage transduction to the microbiota from seeds. To investigate the links between drying on the induction of the expression of Stx phage and the transduction of Shiga toxin phage during rehydration, *E. coli* O104:H4 Δ*stx2a*:*gfp*:*amp*^{r} inoculated and dried on mung beans were rehydrated and co-inoculated with *E. coli* DH5α. *E. coli* O104:H4

Δ*stx2a*:*gfp*:*amp*^{r} and *E. coli* DH5α were Stx phage donors and recipients, respectively. The Φ11-3088 lysogenic *E. coli* DH5α were detected during sprouting for 5 days (Table 6.5). Mung beans were treated with chlorine to remove the native microbiota. The cell counts on sprouts from chlorine-treated beans remained below the detection limit of $\log_{10}$ 2.3 CFU/g. Ampicillin resistance and chloramphenicol resistance were used to select *E. coli* O104:H4 Δ*stx2*:*gfp*:*amp*^{r} and *E.coli* DH5α, respectively. Both chloramphenicol and ampicillin resistant colonies were isolated as tentative phage lysogenic *E.coli* DH5α. A total of 92 colonies were selected and further confirmed with plasmid isolation (Table 6.6), in which 34/92 colonies were positive for *gfp*, and also encoded the plasmid pJIR750ai, confirming that they are derivatives of *E. coli* DH5α. The plasmid profile in *E. coli* O104:H4 Δ*stx2a*:*gfp*:*amp*^{r} was distinct from *E. coli* DH5α, which allowed differentiation of the Φ11-3088 lysogenic strains from *E. coli* O104:H4 Δ*stx2a*:*gfp*:*amp*^{r} (Figure 6.2). Both donor and recipient strains persisted during the seeds germination without a significant increase in cells counts during seeds germination. At the last two days of sprouting, over 50% of tentative lysogen was confirmed positive of *gfp* and pJIR750ai.

Table 6. 5. Cell counts of *E. coli* O104:H4 Δ*stx2a: gfp: amp*^{r} (ampicillin resistance), *E. coli* DH5α (chloramphenicol resistance), and Φ11-3088 lysogenic *E. coli* DH5α (ampicillin and chloramphenicol resistance) during sprouting. Cell counts of chlorine treated mung beans were below the detection limits of 2.3 $\log_{10}$ CFU/g.

Time (d)	Colony Counts ($\log_{10}$ CFU/g)			
	LB	LB (Amp)	LB (Chlor)	LB (Amp/Chlor)
1	8.53±0.48	8.46±0.52	7.10±0.038	1.18±1.67[b]
2	8.31±0.68	8.30±0.68	6.72±1.06	3.93±1.21[a]
3	8.62±0.34	8.71±0.45	7.47±0.58	3.17±0.95[a]
4	8.99±0.31	8.72±0.25	7.58±0.70	3.66±0.36[a]
5	8.69±0.38	8.68±0.44	7.71±0.36	3.88±0.76[a]

Values in the same column that do not share a common superscript are significantly difference (P<0.05). Data represent means ± standard deviation of the means for three independent experiments.

Table 6. 6. Validation of Φ11-3088 lysogenic strains using PCR amplification with GFP primers and plasmid preparation**.**

Time (d)	Total number of colonies positive of *gfp*	Total number of colonies positive for pJIR750ai
D2	6/28 (21%)	n.d.
D3	3/27 (11%)	3/3
D4	13/20 (65%)	8/8
D5	12/17 (71%)	8/8
Total	34/92	19/19

n.d. not determined

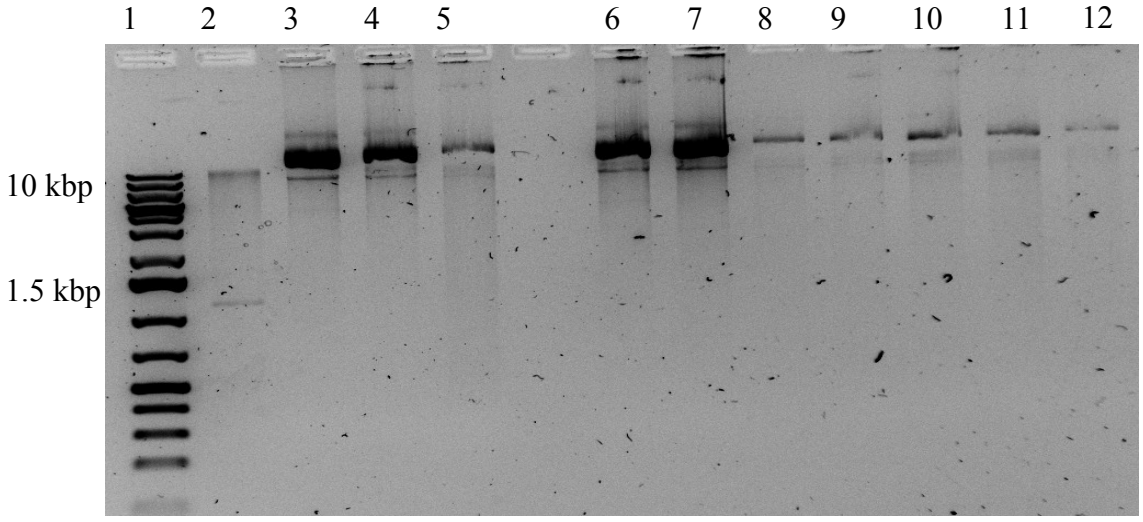

Figure 6. 2. Agarose gel electrophoresis of plasmid profile of *E. coli* O104:H4 Δ*stx2a*:*gfp:amp*^{r} (lane 2), *E. coli* DH5α (lane 3), and Φ11-3088 lysogenic strain (lane 5 to 12). Lane 1 is the 1 kb plus DNA ladder.

6.4. Discussion

Bacteriophage mediates the transmission of *stx* and generation of novel STEC in foods (Imamovic et al., 2009; Nyambe et al., 2017). Dry seeds were suggested as the primary source of contamination of STEC O104:H4 in the German outbreak (Beutin and Martin, 2012; Rasko et al., 2011). The survival and persistence of STEC in dry foods are important for the transmission of STEC in the food system. Solid foods with low fluidity,

therefore limit interaction between phage and bacteria (Imamovic et al., 2009). The concentration of donor and recipient strains required to generate lysogens in solid foods was above 10^5 CFU/g, indicating that the frequency of lysogenic infection by phage is restricted in solid foods (Imamovic et al., 2009). Bean sprouts contain high microbiological load with the concentration of 5 to 9 $\log_{10}$ CFU/g and *Enterobacteriaceae* are among the dominating bacterial groups (Abadias et al., 2008; Martínez-Villaluenga et al., 2008).

Transmission of *stx2a* during seeds germination involves the survival of STEC in dry seeds and induction of Stx prophage associated with drying and rehydration. Desiccation of microorganisms induces cellular oxidative stress (França et al., 2007), caused the oxidation of membrane lipids by the cellular reactive oxygen species (Garre et al., 2010), and accumulation of Fe-protein as part of the oxidative stress response (Gruzdev et al., 2012; Shirkey et al., 2000). Oxidation of DNA induces the RecA-dependent SOS response, and in turn induces the expression of Shiga toxin phage (Chapter 4; Waldor and Friedman, 2005). Stress associated with drying and rehydration of *E. coli* also induced the λ-prophage (Webb and Dumasia, 1967). The results in Chapter 5 indicated the up-regulation of *recA* and expression of *stx2a* in *E. coli* O104:H4, which provides the molecular mechanisms underlying the induction and transduction of Shiga toxin phage in dry foods following rehydration.

This study determined range of strains that are susceptible to Φ11-3088 derived from STEC O104:H4 in Germany. Stx phages Φ 933W, Φ PT32 derived from *E. coli* O157:H7 and Φ13374 derived from *E. coli* O104:H4 (Gamage et al., 2004, Beutin et al., 2012) are less likely to develop lytic infection during the interaction with bacteria, which

also confirmed in this study. Development of lytic cycle or production of Stx genetically links to the host recombinase system (Grzegorz et al., 2012). *E. coli* DH5α are deficient of major recombinase RecA, appeared to be more susceptible to lytic infection by Stx phages (this study, Muniesa et al., 2004a, 2004b). How cells switch between the lytic cycle and lysogenic cycle also depends on the competition of prophage repressor CI repressor and lytic repressor Cro, which was identified in Φ13374 derived from *E. coli* O104:H4 (Beutin et al., 2012). To further understand the molecular mechanisms that mediate the lytic or lysogenic cycle, the activity of CI and Cro during infection and genotype of the host are required to be investigated.

The expression of *stx2* was significantly lower in *E. coli* DH5α compared with other strains in this study. RecA interact with damaged DNA and forms a active form, which cause autoproteolysis of prophage repressor CI (Waldor and Friedman, 2005). The deficiency of RecA blocks the transcription activity of prophage encoded genes under the induction with mitomycin C, including pR' under the regulation of antiterminator Q protein, which controls the transcription of *stx* (Rozanov et al., 1998b; Trachtman et al., 2012). The interaction between phage late gene promoter pR' and antiterminator Q protein was suggested controlling the transcriptional level of phage late genes (Shimizu et al., 2009b). Expression of *stx* is regulated by the phage late gene promoter pR' located in the downstream of CI, which controls the transcription of *stx* and *lys* located in the downstream of *stx* (Waldor and Friedman, 2005). Induction of lytic cycle in Stx prophage results in the production of viable phage and Stx (De Sablet et al., 2008; Shimizu et al., 2009b), which is the only pathway to deliver toxin outside of the cells (Johannes and

Römer, 2010). Differential induction of Φ11-3088 in hosts with diverse genetic background affects the progress and severity during STEC infection.

The host spectrum of Φ11-3088 appeared to be narrower than the host spectrum of Φ933W (Gamage et al., 2004). The phage genome P13343 derived from *E. coli* O104:H4 is identical to Φ11-3088 (99.8% DNA homology, this study), but differs from Φ933W (65% DNA homology) (Ranieri et al., 2014). P13343 encodes unique tail gene cluster (Ranieri et al., 2014), which might contribute to this narrow host spectrum of Stx phage derived from *E. coli* O104:H4 when compared to STEC O157-derived phage Φ734, $\Phi 24_{B,}$ 933W, ΦA557 and Φ3538 (Gamage et al., 2004; Herold et al., 2004; Imamovic et al., 2009; McCarthy et al., 2002; Schmidt et al., 1999). Phage infection is mediated by the specific binding between membrane receptor and tail proteins (Chatterjee and Rothenberg, 2012; Wang et al., 2000). Genetic modification of the tail spike protein shifts the host range of phages (Holmes, 2009; Pepin et al., 2010). The membrane protein YaeT is required for the recognition of the tail protein located on the Stx phage (Smith et al., 2007). Of note, *Shigella sonnei* were susceptible to infection by Φ11-3088. Clinical isolates of *Shigella* spp. generally carry Stx 1 (Beutin et al., 1999; Strauch et al., 2001); however, integration of Stx 2 prophages was reported only after *in vitro* transduction (McCarthy et al., 2002; Schmidt et al., 1999; Tozzoli et al., 2014).

6.5. Conclusions

Induction of Stx phage during sprouting generated infectious phage particles and increased the Stx producers by phage transduction. The expression and production of Stx depends on the environmental stress, properties of the genetic components of phage with respect to regulation, and genetic background of host range. Induction of Stx phages after

drying and rehydration likely involves the oxidative stress after desiccation. Transmission of *stx* by phage infection occurs in the gastrointestinal tract of animals (Cornick et al., 2006) and phage lysogenic conversion of commensals in the intestine may contribute to the severity of disease (Gamage et al., 2006; Iversen et al., 2015). Phage particles produced during sprouting may transfer virulence factors to non-pathogenic strains that are present on sprouts, or to commensal *E. coli* after ingestion.

6.6. References

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CHAPTER 7. Conclusions and Future Direction

E. coli survive in the ever-changing environment and transfer between multiple vertebrate hosts through fecal to oral transmission. Robustness of stress resistance and adaptation strategies to cope with diverse environmental niches are enabled to the genome plasticity, which eases the genomic modification (Leimbach et al., 2013). This Ph.D research explored the effect of ecological factors on genetic structure and regulation of virulence factors in *E. coli.* The stressors that exist in the ecological system contribute to the phylogenetic relationship of *E. coli* and transduction of Stx. This study found that *E. coli* located in different phylogroups share different profiles of virulence and resistance genes. The acquisition of different stress resistance mechanisms by *E. coli* is attributed to natural selection. Different ecological niches are associated with specific stressors, which drive the evolution of *E. coli* towards host and niche-specialization. For example, desiccation and starvation survival in aqueous ecosystems may be linked to oxidative stress while the mammalian digestive tract exposes *E. coli* to acid stress. Accordingly, the LHR is frequently found in environmental isolates; while acid resistance is highly associated with gastrointestinal pathogens, particularly STEC (Chapter 3). Therefore, stressors contribute to shape the genetic structure of *E.coli* by selectively maintaining or acquiring the beneficial genes, and enhance their survival in the ecological systems.

7.1. Ecology of STEC in the animal gut

E. coli switch roles between a commensal and a pathogen depending on the host susceptibility (Leimbach et al., 2013). *E. coli* primarily inhabit the gut of warm-blooded animals (Carlos et al., 2010) but adapt to secondary reservoirs, including soil, water,

sediment, and foods (Fratamico et al., 2004; Leimbach et al., 2013; Savageau, 1983). The ecological, epidemiological, and genetics analysis of STEC serotype O157:H7 indicate that *E. coli* O157:H7 transit from commensalism in cattle to pathogenesis in humans (Karmali et al., 2010). Phylogenetic analysis reveals that Stx is arbitrarily distributed over the phylogeny; however, genotypes carrying EHEC virulence genes are mainly found in phylogroup B1 (Chapter 3). The uneven distribution of EHEC in the phylogeny infers that the evolution of EHEC is likely related to adaptation in the gut of ruminant animals (Zhi et al., 2015). The bovine rumen is rich in protozoa, which are either host or predator of STEC (Burow et al., 2005). As a host, STEC are able to persist in protozoa and survive in the bovine gut (Vaerewijck and Houf, 2014). As a predator, protozoa produce hydrogen peroxide to kill and digest bacteria (Fok and Allen, 1975; Matz and Kjelleberg, 2005). It was hypothesised that STEC produce Stx to defend against protozoa (Russell and Rychlik, 2001). Hydrogen peroxide that is produced by human neutrophils or by protozoa induces oxidative stress in bacteria, which in turn up-regulates the expression and production of Stx phage (Chapter 4; Fok and Allen, 1975; Łoś et al., 2009; Tozzoli et al., 2014; Wagner et al., 2001). The relationship between phage and STEC, or STEC and protozoa explain the mechanisms that mediate the survival of STEC in the vertebrate host and environmental niches. Phages infect bacteria and then use their host as machinery to replicate their genetic material, produce phage particles and continue the replication cycle by the switch between lytic and lysogenic infection (Feiner et al., 2015). Protozoa fed on bacteria or form cysts and protect bacteria from the stress of the environment (Kilvington and Price, 1990). Oxidative stress induces the expression of Stx prophage (Chapter 4),

which is an important factor that regulates the prey and predator relationship of STEC and protozoa in the ecological systems.

7.2. Effect of abiotic stress on the expression of STEC

Increased number of foodborne outbreaks associated with vegetables, fruits and water are due to contamination with STEC transmitted by bovine feces (Chalmers et al., 2000; Tauxe, 1997). Abiotic stressors are used in conventional and novel food interventions to reduce the concentration of microorganisms, and induce bacterial stress responses and expression of Stx (Bonanno et al., 2017; Imamovic and Muniesa, 2011; Chapter 4, 6 and 7). Results of this study showed that the expression of Stx occurs in a sub-population associated with specific stressors and genetic background of the hosts (De Sablet et al., 2008; Zhang et al., 2018; Chapter 4; Chapter 6). Most of the stressors other than acid induce the expression of Stx prophage regulated by the RecA-dependent SOS response (Chapter 6), which implies a unique prophage regulation in response to acid (Chapter 4).

This study demonstrated that drying induces the oxidation of the membrane lipids and the expression of *stx* through RecA-dependent SOS response (Chapter 5). The dehydration induced by drying has a low bactericidal effect but always causes oxidative damage and induces the expression of Stx in metabolically activated cells (Chapter 5). Remarkably, this study illustrated that STEC survival on dry seeds mediates virulence gene transfer by phage transduction during seeds germination (Chapter 6). Seeds contaminated with STEC serve as vehicle transferring pathogen into the food system. Use of STEC contaminated seeds for sprout production not only introduces STEC but also

results in the expression of Stx prophage and transduction of Stx phage to non-pathogenic strains of *E. coli* that may be present in food.

7.3. Effect of ecological factors on the host specialization

The selection pressures in environments and adaptation of organisms maintain their survival and drive the evolution towards host or niche specific differentiation. The host-specific adaptations of *Lactobacillus,* are clearly associated with phylogenetic differences (Duar et al., 2017). In *Salmonella,* host adaptation relates to the serotype and the phylogenetic position of the strain, and is associated with the acquisition of specific virulence factors (Tsolis et al., 1998). However, the host specificity of *E. coli* is still under debate.

This study illustrated that environmental pressure had a certain level of impact on the evolution of phylogeny (Chapter 3). Gastrointestinal pathogenic *E. coli* not only encode virulence genes such as *stx* and *eae,* but also carry urease as an acid-tolerance strategy. Virulence genes facilitate the pathogen to establish the infection, and urease activity contributes to enhancing cell survival through the passage in the digestive tract. LHR is mostly associated with *E. coli* adapted in the environment (Mercer et al., 2015). Noteworthy, LHR did not co-occur with Stx. The LHR was not identified in gastrointestinal *E. coli,* which indicated that the acquisition of stress resistance is specific to the ecological systems; whereas, *E. coli* that acquired LHR or urease might adapt to separate ecological systems.

The LHR confers heat and chlorine resistance (Mercer et al., 2015). Heat stress is often used in industrial application, including food processing but not often associated with an animal host. To survive and colonize in the gut of the animal, the development of

acid resistance is more beneficial than heat resistance. Overall, environmental pressure drives the host- and environmental adaptation and shapes the genotypes associated with the divergence of pathogenic strains of *E. coli*.

In conclusion, this study improved the understanding of the ecology, virulence and stress resistance of *E. coli.* The research provided insight and direction on the route of transmission and spread of virulent genes associated with pathogenic *E. coli* during food production and manufacture. Understand the ecology and transmission route of *E. coli* are remarkable to reduce the frequency of contamination in food systems.

7.4. Recommendation and future direction

This study developed two powerful methods based on a fluorescence probe to investigate the expression of *stx* and oxidation of membrane lipids. Combination of the fluorescence probe with flow cytometry allows a quantitative analysis of single-cell or sub-population to assess the genetic and physiological characteristics of the microorganisms. It also provides a future direction on the investigation of the correlation of genetic response between a single-cell and a microbial population. Fluorescence based methods to quantify gene expression and oxidation of macromolecules are powerful quantitative analysis assays. To further confirm the function of the cellular component, deactivate the target gene by mutation is necessary in the future studies.

This study investigated the ecological factors and related abiotic stressors that determine the distribution of virulence profiles of *E. coli*. The *in silico* phylogenetic analysis revealed comprehensive population genetics of *E. coli*; however, the origins of the strains are either unknown or isolated from their secondary habitats. The missing information of the natural habitats of each serotype restricts the in-depth interpretation of

the link between host- or niche-adaptation and phylogeny of pathogenic *E. coli*. The genes coding virulence and stress resistance that related to the ecology are required to be further confirmed by isolation of microbe from a representative environmental associated with different stressors.

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