Diarrheagenic *Escherichia coli* signaling and interactions with host innate immunity and intestinal microbiota

by

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B.S., China Agricultural University, 2007
M.S., The Ohio State University, 2012

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Diagnostic Medicine/Pathobiology
College of Veterinary Medicine

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Abstract

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In the first project, we identified an ETEC-secreted factor (ESF) that blocked TNF-induced NF-κB activation. One of the consequences of TNF-induced NF-κB activation is the production of pro-inflammatory cytokines that help to eliminate pathogens. Modulation of NF-κB signaling may promote ETEC colonization of the host small intestine. In this study, we fractionated ETEC supernatants and identified flagellin as necessary and sufficient for blocking the degradation of the NF-κB inhibitor IκBα in response to TNFα.

In the second project, we attempted to identify an ETEC cAMP importer. ETEC diarrhea leads to cAMP release into the lumen of the small intestine. cAMP is a key secondary messenger that regulates ETEC adhesin expression. We hypothesized that a cAMP importer is present in ETEC, accounting for its hypersensitivity to extracellular cAMP. We used Tn5 transposome-mediated mutagenesis to construct a mutant library and screen for cAMP-hyporesponsive mutants. However, none of the 17,956 mutants we screened were cAMP-hyporesponsive.

In the third project, we focused on gut microbiota and the T3SS effector NleH. We used the mouse-specific pathogen *C. rodentium* and transplanted performed microbiota between different mouse strains. We evaluated microbiota populations as a function of infection with WT and *ΔnleH*

C. rodentium strains before and after microbiota transplantation. Microbiota transfer altered the resistance to WT *C. rodentium* infection in C57BL/10ScNJ mice and the NleH effector promoted host resistance to *C. rodentium*.

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Major Professor:

Dr. Philip Hardwidge

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Chapter 1 - Literature Review

Escherichia coli

Escherichia coli (E. coli) is a common group of bacteria colonizing the intestinal tract of human and warm-blood animals. The bacterium was first described by Theodor Escherich in 1885 (Escherich, 1988). In taxonomy, *E. coli* belongs to the enteric bacterial family, *Enterobacteriaceae*. The bacteria in this family share the same physiologic and biochemical characteristics: facultative anaerobes, non-spore-forming, gram-negative straight rods (Croxen et al., 2013). They can move by peritrichous flagella and ferment glucose to acquire energy. *E. coli* have been categorized into two distinct groups: commensal strains (non-pathogenic) and pathogenic *E. coli* (Croxen et al., 2013; Kaper et al., 2004). Commensal *E. coli* and its hosts can coexist in good health and with mutual benefits for each other.

Pathogenic of *Escherichia coli*

Pathogenic *E. coli* consist of two groups: extra-intestinal pathogens and intestinal pathogens. Extra-intestinal pathogens include strains that are responsible for urinary tract infections and neonatal meningitis. Intestinal pathogenic *E. coli* strains cause intestinal diseases including abdominal pain, watery or bloody diarrhea. The diseases caused by pathogenic intestinal *E. coli* depend on distribution and expression of an array of virulence determinants including adhesins, invasins, and toxins, and abilities to withstand host defenses (Kaper et al., 2004).

It has been widely accepted that pathogenic *E. coli* acquire the virulence and fitness traits through gain and loss of genes at a number of hot spots throughout the genome (Croxen et al., 2013; Kaper et al., 2004). Given that size of *E. coli* genome differs by a million base pairs between commensal and pathogenic strains, pathogenic *E. coli* acquire new virulence traits and fitness advantages

through horizontal gene transfer by conjugation, transformation and transduction of mobile genetic elements such as transposons, insertion sequences, bacteriophages and plasmids. In addition, gene loss can also favor the fitness or adaptation of a pathogen in a particular habitat.

Classification of *Escherichia coli*

Serotypes of *Escherichia coli*

Serotyping of *E. coli* is a traditional method for identifying pathogenic *E. coli* on the basis of O (somatic), H (flagellar) and K (capsular) surface antigen profiles (Fratamico et al., 2016). Since few laboratories has capabilities to type capsular K antigens, serotyping based on O- and H-antigens has become the gold standard for *E. coli* typing (Fratamico et al., 2016; Joensen et al., 2015).

O-antigens

O-antigen is a heat-stable lipopolysaccharide (LPS) component found in the cell wall of *E. coli*. LPS is anchored in the outer membrane of Gram-negative bacterium (Chow et al., 1999; Joensen et al., 2015). It is composed of three parts: lipid A (the toxic component), the core region, and the O-antigen polysaccharides. Conventional serotyping is carried out based on agglutination reactions of the O-antigen with antisera that are generated in rabbits against each of the O-groups (Joensen et al., 2015). This method is easy to perform, but laborious and error-prone. Therefore, molecular methods now are better alternatives. Currently, there are a total of 186 O-groups except for O31, O47, O67, O72, O94, and O122 that have not been designated (Fratamico et al., 2016). Moreover, O-antigen is important virulence factor that plays a major role in the pathogen-host interactions. LPS is the ligand for Toll-like receptor 4 (Chow et al., 1999).

H-antigens

H-antigen is a major component of flagellar filament that is made up of flagellin (Haiko and Westerlund-Wikstrom, 2013). Flagellin is encoded by the *fliC* gene, which is responsible for *E. coli* movement (Haiko and Westerlund-Wikstrom, 2013). H typing is important because strains causing epidemic diarrheal diseases can be differentiated from the normal stool flora by their unique O:H antigenic combination (Fratamico et al., 2016). Molecular H typing is based on the sequence of *fliC* gene. The FliC consists of highly conserved N- and C-terminal and a variable central region that is the surface exposed antigenic part of the flagellar filament, so the H types are determined due to amino acid difference within the central region (Haiko and Westerlund-Wikstrom, 2013; Wang et al., 2003). Currently, a total of 53 H-antigens have been defined (Fratamico et al., 2016). Flagellin is the ligand for toll-like receptor 5 (Hayashi et al., 2001).

K-antigens

K-antigen refers to the envelope or capsule antigens (Whitfield, 2009). K-antigens weapon the *E. coli* with resistance to phagocytosis by the host leucocytes (Weinstein and Young, 1978). Most of K antigens are found in *E. coli* associated with virulence for urinary tract infection in clinical studies (Sarkar et al., 2014). Eighty K antigens were documented (Fratamico et al., 2016).

Phylotypes of *Escherichia coli*

Phylogenetic analysis reveals that *E. coli* consist of four main phylogenetic groups (A, B1, B2, and D) on basis of presence and absence of genetic markers *chuA*, *yjaA* and the DNA fragment TspE4.C2 (**Table 1**) (Carlos et al., 2010; Doumith et al., 2012). Compared to group A and B1, *E. coli* strains in B2 and D phylotypes contain more virulence factors and larger genomes. Environmental *E. coli* strains usually belong to B1 group. The commensal *E. coli* belongs to group

A and B1. Intestinal pathogenic *E. coli* are composed of A, B1, and D group, while extra-intestinal pathogenic strains belong to group B2 and D.

Table 1 Phylotypes of *Escherichia coli.*

Phylotypes	Genetic marker
A	*chuA*-, TspE4.C2-
B1	*chuA*-, TspE4.C2+
B2	*chuA*+, *yjaA*+
D	*chuA*+, *yjaA*-

Pathotypes of *Escherichia coli*

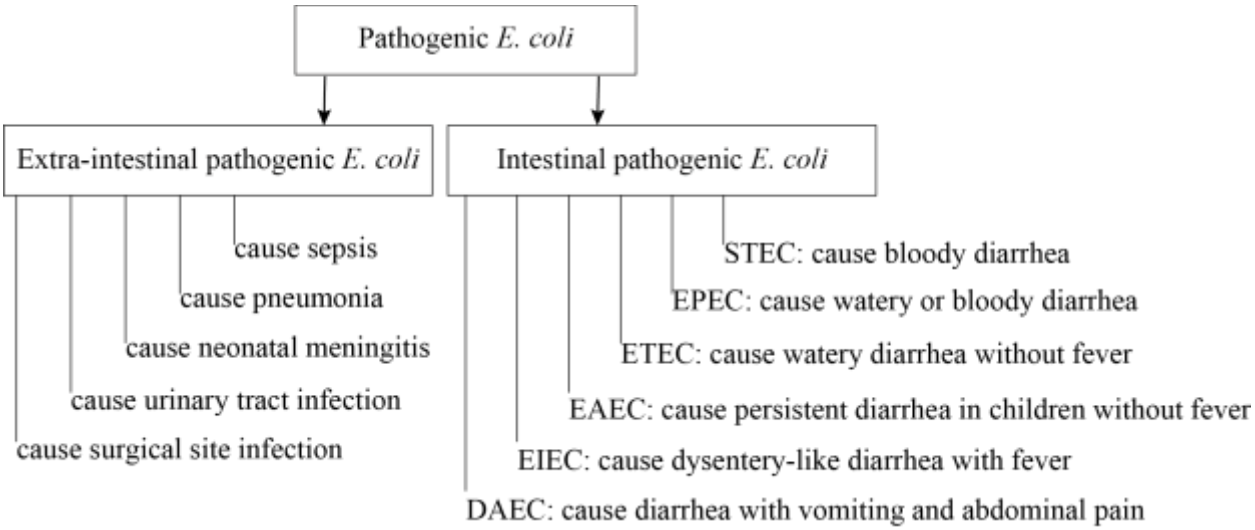
Pathogenic *E. coli* (intestinal and extra-intestinal pathogenic *E. coli*) are classified into pathotypes based on the production of diverse virulence factors and on the mechanism by which they cause diseases (Figure 1) (Kaper et al., 2004).

Extra-intestinal Pathogenic *E. coli* (ExPEC) is a group of *E. coli* strains that can cause extra-intestinal infections, which mainly consists of uropathogenic *E. coli*, septicemia-associated *E. coli*, meningitis-associated *E. coli* and other strains (Poolman and Wacker, 2016). ExPEC has been reported to account for a primary cause of urinary tract infections in young healthy women, a common cause of bacteremia in older adults, and a frequent cause of meningitis in neonates. ExPEC can be defined by encoding: 1) virulence factors that contribute to bind to human cells; 2) factors that contribute to its survival in human body; 3) factors that contribute to damage human cells and tissues; 4) production of bacteriocins. Recently, a study suggested that *E. coli* strains from phylogenetic B2 group encoding ExPEC genes were identified from the stools of patients with active ulcerative colitis (Mirsepasi-Lauridsen et al., 2016).

To date, 7 pathotypes (Clements et al., 2012; Croxen et al., 2013; Kaper et al., 2004) of intestinal pathogenic *E. coli* associated with diarrheal diseases have been documented: enterotoxigenic *E.*

coli (ETEC), enteroinvasive *E. coli* (EIEC), Shiga toxin-producing *E. coli* (STEC) (e.g. enterohemorrhagic *E. coli* [EHEC]), enteropathogenic *E. coli* (EPEC), and enteroaggregative *E. coli* (EAEC), diffuse-adherent *E. coli* (DAEC), and adherent invasive *E. coli* (AIEC). However, there are also hybrid pathotypes. For example, an EAEC O104:H4 strain causing a large outbreak with 54 deaths in Germany acquired the Shiga toxin gene of STEC (Frank et al., 2011; Navarro-Garcia, 2014).

Figure 1 Classification of pathogenic *E. coli*



Enterotoxigenic *Escherichia coli*

Enterotoxigenic *Escherichia coli* (ETEC) is an important cause of childhood diarrheal disease in developing countries and a common cause of diarrhea in travelers (Croxen et al., 2013; Fleckenstein et al., 2014). ETEC-induced diarrhea results in higher morbidity and mortality in children under 5 years of age (Croxen et al., 2013). A population-based study estimated that there were nearly 840 million annual cases of ETEC in developing countries, with approximately 280 million cases in children aged 0 – 4 (Wenneras and Erling, 2004). ETEC strains are characterized by production of heat-labile enterotoxin (LT), heat-stable enterotoxins (STs) or combination of LT

and STs (Fleckenstein et al., 2010). ETEC strains also encode a diverse set of colonization factors (CFs) contributing to adherence to intestinal epithelial cells (IECs) (Kharat et al., 2017; Madhavan and Sakellaris, 2015). Two proteins (the outer membrane protein Tia and the glycosylated autotransporter TibA) contribute to intimate cell attachment (Croxen et al., 2013; Lindenthal and Elsinghorst, 2001). Initially, ETEC employs EtpA, a TPS exoprotein adhesin located on the tip of ETEC flagella, to mediate a loose attachment to mammalian cells (Roy et al., 2009). Subsequently, ETEC CFs interacts with host cells and EtpA becomes degraded by EatA, a serine protease autotransporter of *Enterobacteriaceae*. Finally, Tia and TibA mediate the intimate attachment of ETEC to host cells. Upon intimate adherence to epithelial cells, ETEC produces two major enterotoxins (LT and ST) causing diarrhea. The mechanism of ETEC-induced watery diarrhea has been widely described (Clements et al., 2012; Fleckenstein et al., 2010). In general, LT and ST cause diarrhea by inducing imbalance of water and electrolytes in intoxicated IECs. LT is composed of a single copy of A subunit and 5 copies of B subunits, and functions similarly to cholera toxin. The B subunit of LT is responsible for internalization of LT into IECs by binding to its receptor, GM1 ganglioside receptor, on the surface of IECs. Upon entry into IECs, A subunit that possesses ADP-ribosylation activity enzymatically modifies α subunit of Gs GTP-binding protein, resulting activation of adenylate cyclase encoded by *cyaA* gene in *E. coli*. Activated adenylate cyclase subsequently promotes the generation of 3',5'- cyclic adenosine monophosphate (cAMP) from adenosine triphosphate (ATP). Increased intracellular concentration of cAMP activates protein kinase A (PKA) pathway leading to activation of cystic fibrosis transmembrane conductance regulator (CFTR), causing chloride and water secretion into the intestinal lumen resulting in watery diarrhea. In addition to LT-associated diarrhea, ST cause diarrhea by binding to and activate guanylate cyclase-C (GC-C) receptor resulting in increased concentration of

intracellular cyclic guanosine monophosphate (cGMP). Over-production of cGMP subsequently activates cGMP-dependent protein kinase II leading to the phosphorylation of CFTR and imbalance of ion absorption and secretion, ultimately resulting in diarrhea. It was found that ST-positive ETEC strains, not LT-only-positive strains, were commonly associated with diarrhea and a major contributor to infantile diarrhea resulting in increased risk of death (Croxen et al., 2013). The adhesin and toxin profiles of ETEC isolates vary and differ among geographical regions and populations, making it is complicated to development of effective vaccines for ETEC infections (Bourgeois et al., 2016). Currently, oral rehydration and maintaining fluid and electrolyte balance are demonstrated to be very effective treatment for ETEC infections (Croxen et al., 2013).

Shiga toxin-producing *Escherichia coli*

Shiga toxin-producing *Escherichia coli* (STEC) strains are defined by presence of the Shiga toxin gene acquired by a lambdoid bacteriophage, which can cause mildly to severely bloody diarrhea and hemorrhagic colitis syndrome (HUS) (Bryan et al., 2015). EHEC is a subset of STEC, which was originally described by its association with hemorrhagic colitis (HC), and carries LEE (the Locus for Enterocyte Effacement) pathogenicity island (PAI) (Croxen et al., 2013). Currently, EHEC has been used describe LEE-negative STEC strains. Several well-characterized fimbrial (pili) adhesins contribute to initial attachment of STEC to IECs (Croxen et al., 2013). The mechanism of STEC-induced bloody diarrhea has been intensively investigated (Bryan et al., 2015; Obata and Obrig, 2014). Shiga toxin (Stx) is produced in the colon and damages the tissues leading to bloody diarrhea. It can access to kidney through bloodstream and destroy renal endothelial cells resulting in renal inflammation. Stx binds to Gb3 receptor on the surface of endothelial cell and subsequently is subjected to internalized and trafficked to host cell cytoplasm where A subunit of Stx inhibits protein synthesis and cause cell death. Stx can be divided into two

types, Stx1 and Stx2, with Stx1 having 3 subtypes and Stx2 having 7 subtypes. STEC isolates can carry *stx*$_1$, *stx*$_2$, or both *stx*$_1$ and *stx*$_2$. Although both *stx*$_1$- and *stx*$_2$-containing STEC infections are able to cause HUS, *stx*$_2$-containing STEC is more often associated with severe diseases. STEC infection usually is self-limiting and resolves after a week (Croxen et al., 2013). However, it is difficult to prevent the HUS development following STEC infections. Intravenous fluids, renal function and platelet monitoring have been reported to treat STEC-induced HUS (Kavanagh et al., 2014). The use of antibiotics is highly discouraged, as antibiotics use increase the risk of developing HUS by stimulating the production of Stx (Skinner et al., 2015). Monoclonal antibodies and probiotics might provide an alternative to prevention and treatment of STEC infection (Rahal et al., 2015; Skinner et al., 2015). Currently, vaccines are being developed targeting on reducing carriage and shedding in cattle and on mechanism of delivery Stx into host cells (Smith, 2014).

Enteropathogenic *Escherichia coli*

Enteropathogenic *Escherichia coli* (EPEC) is a common cause of infantile diarrhea in the developing countries, which was first described in the 1940s and 1950s (Hu and Torres, 2015). EPEC now belongs to a group of bacteria known as attaching and effacing (A/E) pathogens that also include LEE-positive STEC strains as well as several animal pathogens such as rabbit EPEC, porcine EPEC, dog EPEC, and mouse-specific pathogen *Citrobacter rodentium* (*C. rodentium*) (Franzin and Sircili, 2015). The pathogenesis of EPEC is characterized by formation of A/E lesions on the surface of small IECs resulting in loss of absorptive microvilli (Gaytan et al., 2016). EPEC isolates carry a LEE pathogenicity island encoding a secretion system termed type three secretion system (T3SS) that is required for EPEC pathogenesis. The genome of EPEC contains several LEE-encoded effectors associated with intimate attachment of EPEC to IECs and several non-LEE

(Nle)-encoded effectors that all have been demonstrated to subvert host immune responses, of which NleB, NleC, NleD, NleE, and NleH have been reported to inhibit NF-κB activation through different mechanisms (Yen et al., 2016). Unlike ETEC, EPEC-associated diarrhea is not caused by toxins (Croxen et al., 2013). However, the exact mechanism of diarrhea production is not fully understood, which is a result of multiple factors (Croxen et al., 2013). First, A/E lesions lead to decrease of absorptive surface and disruption of proper absorption channels. Second, T3SS-associated effectors are responsible for inhibiting the sodium-D-glucose transporter resulting in failure of fluid uptake from the intestine, water transport and chloride transport. Third, T3SS-associated effectors also contribute to disruption of tight junction leading to decrease of intestinal permeability. In most cases, EPEC-induced diarrhea is self-limiting and can be effectively treated with oral rehydration. So far, there is no vaccine available to control EPEC infections (Croxen et al., 2013). Proper personal hygiene and clean water will contribute to prevent the transmission and spread EPEC infection.

Enteroaggregative *Escherichia coli*

Enteroaggregative *Escherichia coli* (EAEC) is a group of *E. coli* causing persistent diarrhea in children and adults, and is a causative agent for traveler's diarrhea (Okhuysen and Dupont, 2010). Based on the presence of *aggR* (aggregative regulator) gene that is a key virulence regulator, EAEC can be divided into typical (containing *aggR*) and atypical (lacking *aggR*) groups (Croxen et al., 2013). Further classification of EAEC is based on the colonization sites, as some strains infect only small intestine, while others colonize both small intestine and colon. The clinical symptoms of EAEC infection comprise watery diarrhea, fever, vomiting, abdominal pain, occasionally with blood and mucus and intestinal inflammation characterized by the presence of fecal lactoferrin and pro-inflammatory cytokines such as IL-8 (interleukin-8). The most concerned public health issue

is that persistent EAEC infection can cause malnourishment in children in developing countries (Gomes et al., 2016). In 2011, a STEC outbreak in Germany caused over 900 patients to develop HUS and 50 deaths (Grad et al., 2012), which was later demonstrated to be a hybrid strain of EAEC and STEC. Therefore, the pathogenesis of EAEC differs in Stx-positive and Stx-negative EAEC isolates. In non-Stx EAEC isolates, EAEC aggregative adherence to the intestinal mucosa is the first stage, which is mainly associated with both aggregative adhesion fimbria and afimbrial adhesins. The attachment of EAEC to IECs requires aggregative adherence fimbriae (AAF) and Tia and Hra1/2 (heat-resistant agglutinin 1 and 2) contribute to intimate attachment to IECs as well. Toxin production is the second stage of EAEC pathogenesis resulting in microvillus vesiculation, enlarged crypt openings and increased epithelial cell extrusion. These toxins consist of Pet (a cytoskeleton-altering toxin), EAST-1 (a heat-stable enterotoxin), ShET1 (a subunit toxin that can activate cAMP- and cGMP-mediated secretion) and Pic (a mucinase) (Clements et al., 2012). The stage of inflammatory diarrhea is initiated by adherence of EAEC to epithelial cells resulting in release of IL-8 and CCL20. Treatment of EAEC-induced diarrhea is dependent on causative strains. It is recommended to use antibiotics to treat EAEC-associated traveler's diarrhea. For diarrhea caused by Stx-containing EAEC strains, generally there is no standardized treatment (Croxen et al., 2013). Currently, there is no vaccine available for prevention of EAEC infections. However, it is possible to develop EAEC vaccines targeting EAEC virulence factors.

Enteroinvasive *Escherichia coli*

Enteroinvasive *Escherichia coli* (EIEC) is a group of facultative intracellular pathogens, which shares biochemical, genetic and pathogenetic characteristics with *Shigella (Kaper et al., 2004)*. Both EIEC and *Shigella* are highly invasive pathogens that use the intracellular milieu of IECs in the large intestine as their replicative niche (Croxen et al., 2013). Virulence factors such as T3SS-

dependent and T3SS-independent protein effectors that are encoded by chromosome and plasmids account for EIEC/*Shigella* pathogenesis (Clements et al., 2012). EIEC and *Shigella* infections result from multiple steps involving penetrating the epithelial barrier, induction of macrophage cell death, IEC invasion, subverting the immune responses, intra- and intercellular movement, and modulation of epithelial integrity (Croxen et al., 2013). In general, EIEC shows decreased virulence compared to that of *Shigella*, such as decreased expression of virulence genes and less scale of pro-inflammatory responses. Additional virulence factors such as *Shigella* enterotoxins 1 and 2 contributing to watery diarrhea in EIEC/*Shigella* infections have been described. The clinical presentation of EIEC/*Shigella*-induced diarrhea is commonly accompanied by abdominal cramp, tenesmus, scanty stools with blood and mucus, and dehydration. Other symptoms such as fatigue, malaise, fever and anorexia are also observed in the early stage of disease. Generally, EIEC-induced diarrhea is self-limiting. Currently, antibiotic treatment is the most effective treatment regardless of the presence of antibiotic resistance. Yet, no vaccine now is available for either EIEC or *Shigella*.

Diffuse-adherent *Escherichia coli*

Diffuse-adherent *Escherichia coli* (DAEC) is a unique group of pathogenic *E. coli* that has been classically defined by diffuse adherence (DA) to cultured epithelial HEp-2 cells in a scattered pattern over the entire surface of cells, which is different from other *E. coli* pathotypes such as the formation of A/E lesions in EPEC (Croxen et al., 2013; Kaper et al., 2004). DAEC can cause watery diarrhea and a severe persistence disease in young children from age of 18 months to 5 years. It has reported that asymptomatic DAEC infection may account for the chronic inflammatory intestinal diseases named Crohn's disease (CD) (Prorok-Hamon et al., 2014). A characteristic of DAEC is the production of Afa/Dr adhesins that have been reported to be

responsible for the release of pro-inflammatory cytokine IL-8 in DAEC infection, resulting in activation of mitogen-activated protein kinase, extracellular signal-regulated kinase 1 and 2, P38, and Jun-C kinase (Croxen et al., 2013; Gomes et al., 2016). Currently, the treatment of DAEC-associated watery diarrhea is rehydration. There is currently no vaccine available for controlling the spread of DAEC infections.

Adherent Invasive *Escherichia coli*

Adherent Invasive *Escherichia coli* (AIEC) has been recently implicated as the most likely causative agent for CD, which is defined by the ability to adhere to and invade IECs with unknown invasive determinants, and the ability to survive and replicate within macrophages (Martinez-Medina and Garcia-Gil, 2014). Adhesion to IECs is suggested to be mediated by type 1 pili that interferes with the glycoprotein CEACAM6 in a mannose-associated manner (Croxen et al., 2013; Shawki and McCole, 2017). Overexpression of CEACAM6 is observed in CD patients with ileal disease, which make them more susceptible to over-colonization by AIEC. Besides, AIEC strains are able of translocation through M cells (Chassaing et al., 2011) gaining the access to lamina propria and via alteration of intestinal permeability by inducing the expression of the pore-forming protein claudin-2 and by displacing ZO-1 and E-cadherin from apical tight junction, resulting in decreased trans-epithelial resistance and loss of barrier function (Shawki and McCole, 2017). AIEC infections are also reported to induce production of TNFα and IL-8. Furthermore, AIEC strains are able of escape of host immune responses via subversion of the interferon gamma (IFNγ) pathway in IECs (Ossa et al., 2013).

Innate immunity and pathogen recognition

Vertebrates have evolved immune systems to fight against the infective pathogens in the body, which is composed of innate immunity and adaptive immunity. The innate immune system (**Table 2**) is the first line of defense against pathogens and is mediated mainly by immune cells including macrophages, dendritic cells (DCs), and neutrophils through direct killing of invading pathogens by phagocytosis or inducing the production of pro-inflammatory cytokines contributing to elimination of pathogens (Akira et al., 2006). Furthermore, innate immunity is constitutively present and is initiated immediately following infection. Innate immunity is characterized by non-specific immune responses because its protective response is the same regardless of the stimuli. By contrast, the adaptive immune system is slower, responds specifically, and generates an immunological memory, which is mainly mediated by T and B lymphocytes (Clark and Kupper, 2005). One important function of the innate immune system is to stimulate the adaptive immune response via antigen presentation (Iwasaki and Medzhitov, 2015). **Table 2** briefly lists the difference between innate immunity and adaptive immunity.

Table 2 Comparison between innate immunity and adaptive immunity.

	Innate immunity	Adaptive immunity
Specificity	Common microbial structures (PAMP)	Specific to unique antigens
Cells	Macrophages, DCs, Neutrophils, NK cells	B cells and T cells
Onset time	Rapid (minutes - hours)	Slow (days - weeks)
Memory	No memory	Production of antibodies, lifetime
Evolution	Ancient (fungi, plants, insects)	Recent (fish, birds, mammals)
Effectiveness	Does not improve	Improve with exposure

Abbreviation: PAMP, pathogen-associated molecular pattern; DCs, dendritic cells; NK cells, natural killer cells.

Rapid activation of innate immune system is dependent on timely recognition of pathogenic microbes through pattern recognition receptors (PRRs) that are able to recognize conserved molecular components in microorganisms termed pathogen-associated molecular patterns

(PAMPs) (Akira et al., 2006). To date, five major PRRs (**Table 3**)(Brubaker et al., 2015) have been described based on protein domain homology, consisting of Toll-like receptors (TLRs), nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs), absent in melanoma 2 (AIM2)-like receptors (ALRs), RIG-I-like receptor (RLRs) and C-type lectin receptors (CLRs). These PRRs can be further classified into two main groups: membrane-bound PRRs and cytoplasmic PRRs. The former group is composed of TLRs and CLRs that are found at the cell surface or on endocytic compartments. The latter group includes NLRs, RLRs and ALRs that are located in cytoplasm. Upon sensing the PAMPs, these receptors not only activate the signaling pathways such as NF-κB and AP-1, which leads to production of inflammatory cytokines and interferons (IFNs) for elimination of viral and bacterial infections (Brubaker et al., 2015; Yin et al., 2015), but also contributes to induction of phagocytosis, autophagy, cell death and cytokine processing (Clark and Kupper, 2005).

Table 3 Pattern-recognition receptor families.

Family	Members	Shared domains	Cellular location
TLR	1-10 in humans, 1-9 and 11-13 in mice	LRR, TIR	Cell surface, endosomal compartments
CLR	Dectin-1, Dectin-2, …	C-type lectin	Cell surface
RLR	RIG-1, MDA5, LGP2	DExD/H helicase	Cytoplasm
ALR	AIM2, IFI16	PYRIN, HIN-200	Cytoplasm, nucleus
NLR	NOD1, NOD2, NLRC3-5, NLRP1-9 and 11-14, NAIPI1, -2, -5, -6	Nucleotide binding, LRR	Cytoplasm, plasm, and endosomal membrane associated

Abbreviation: AIM, absent in melanoma; CARD, caspase recruitment domain; IFI, interferon γ-inducible; LGP, laboratory genetics and physiology; LRR, leucine-rich repeat; MDA, melanoma differentiation gene; NAIP, NLR family, apoptosis inhibitory protein; NLRC, NLR family CARD domain containing; RIG-1, retinoic acid-inducible gene I; NLRP, NLR family PYD domain containing; NOD, nucleotide-binding oligomerization domain; TIR, Toll/IL-1 receptor.

Toll-like receptors

The Toll protein was first identified in fruit flies and was subsequently shown to play important roles in inducing immune responses against the fungal infection, which lead to the identification of homologues of Toll proteins in human termed Toll-like receptors (TLRs) (Akira et al., 2006). To date, 10 TLRs have been identified in human, whereas 12 TLRs have been found in mice (Takeuchi and Akira, 2010). The TLRs are expressed in various types of cell including B cells, NK cells, DCs, macrophages, fibroblast cells, epithelial cells and endothelial cells (Kumar et al., 2009). Based the cellular location, the TLRs are divided into two classes. TLR1, TLR2, TLR4, TLR5, and TLR6 are present on the cell surface, whereas TLR3, TLR7, TLR8, and TLR9 are expressed in the endosomes (Kawasaki and Kawai, 2014; Kumar et al., 2009).

Among the TLRs, TLR2 recognize lipoprotein from *E. coli* and other components from bacteria, mycoplasma, fungi, and viruses (Kumar et al., 2009). TLR1 and TLR6 are able to interact with TLR2 forming TLR1/ TLR2 and TLR2/ TLR6 heterodimers to sense triacyl lipoprotein in Gram-negative bacteria and diacyl lipoprotein derived from mycoplasma, respectively (Takeuchi and Akira, 2010). The well-known ligand of TLR4 is LPS (lipopolysaccharide) from Gram-negative bacteria (Kumar et al., 2009). In addition, TLR4 recognizes viral envelope proteins and modulates the pathogenesis of H5N1 avian influenza virus infection by detection of a DAMP (Damage-associated Molecular Patterns) rather than virus itself (Shinya et al., 2012). TLR5 is highly expressed on DCs of lamina propria (LPDCs) in small intestine as well as epithelial cells and detects flagellin from flagellated bacteria (Takeuchi and Akira, 2010). Flagellin from *Salmonella typhimurium* is composed of 494 amino acids and amino acid 89-96 are essential for TLR5 recognition (Andersen-Nissen et al., 2005). In response to flagellin, LPDCs are responsible for mediating induction of B cells into IgA-producing plasma cells and differentiation of antigen-

specific Th17 and Th1 cells from naïve T cells, which is a TLR5-dependent manner (Takeuchi and Akira, 2010). TLR11 is not found in human (Kumar et al., 2009). However, mice TLR11 is expressed on epithelial cells of bladder and can recognize uropathogenic bacteria and a profiling-like molecule in *Toxoplasma gondii* (Kumar et al., 2009).

Intracellular TLRs are able to sense nucleic acids from bacteria and viruses (Takeuchi and Akira, 2010). Activation of these TLRs leads to production of both pro-inflammatory cytokines and type I IFNs (Takeuchi and Akira, 2010). TLR3 can sense double-strand RNA (dsRNA) from viruses and a synthetic dsRNA analog, polyinosinic polycytidylic acid (poly I:C) (Kumar et al., 2009; Takeuchi and Akira, 2010). TLR3 is expressed on conventional DCs, macrophages, B cells, NK cells and epithelial cells, but not plasmacytoid DCs (Kumar et al., 2009). Mice TLR7 and human TLR7/8 recognize single-strand RNAs (ssRNA) derived from RNA viruses as well as RNAs from Group B Streptococcus (Takeuchi and Akira, 2010). TLR9 is able to sense unmethylated DNA with CpG motif from bacteria as well as viruses (Kumar et al., 2009). It is reported that TLR9 recognize hemozoin derived from malaria parasite (Coban et al., 2005). Unlike TLR3, TLR7 and TLR9 are highly expressed on plasmacytoid DCs (Takeuchi and Akira, 2010).

The TLR protein belongs to the family of type I membrane glycoproteins and is composed of an extracellular leucine-rich repeat (LRR) domain, a transmembrane domain and a cytoplasmic TIR (Toll-IL-1 receptor) domain (Brubaker et al., 2015). The extracellular domain is responsible for ligand recognition and ligand-induced TLR dimerization, and contains multiple LRR motifs. It is reported that the variation of LRR among different TLRs accounts for the ligand specificity. The TIR domain of TLRs is homologous to the cytoplasmic signaling domain of IL-1 (interleukin-1) receptor (IL-1R). Upon activation of TLRs by ligands, the TIR domain is subjected to oligomerization and recruitment of TIR-containing adaptor proteins, including MyD88 (myeloid

differentiation primary response gene 88), Mal (MyD88-adaptor-like), TRIF (TIR-domain-containing adaptor-inducing interferon-β) and TRAM (TRIF-related adaptor molecules) (Brubaker et al., 2015; Yin et al., 2015). In addition to TIR domain, MyD88 contains a death domain (DD) and is able to interact with IL-1R-associated kinases (IRAKs) such as IRAK1, IRAK2, IRAK4, and IRAK-M via DD-DD interaction forming the myddosome (Warner and Nunez, 2013). Myddosome subsequently activates TRAF6 (TNFR-associated factor 6) for poly-ubiquitination and finally results in activation of MAPKs, NF-κB and IRFs pathways to induce the production of pro-inflammatory cytokines and type I IFNs (Yin et al., 2015). In summary, MyD88 is essential for the TLR-mediated downstream signaling transduction with the exception of TLR3 (Takeuchi and Akira, 2010). In response to ligands, TLR3 recruits another adaptor protein, TRIF, for signal transduction. TRIF can interact with TRAF3 and TRAF6 through TRAF-binding domain as well as interacts with RIP1(receptor-interacting protein) and RIP3 via receptor-interacting protein homotypic interaction motif, finally leading to activation of MAPKs, NF-κB and IRFs pathways.

The mechanism by which TLR4 recognize LPS and initiate signaling pathways has been elucidated (Brubaker et al., 2015; Lu et al., 2008; Park and Lee, 2013; Yin et al., 2015). LPS is a primary component of outer membrane of Gram-negative bacteria and is composed of a hydrophobic lipid A, core region and O-antigen. Lipid A is responsible for the immunologic activities of LPS. Recognition of LPS by TLR4 requires cooperation with MD-2 (myeloid differentiation protein-2), CD14 and LBP (LPS-binding protein). LBP is a soluble molecule and can bind LPS contributing to transfer LPS monomer to CD14. Subsequently, CD14 transfers LPS to TLR4-bound MD-2, which causes TLR4 dimerization and promotes signal transduction. Upon activation of TLR4, adaptor protein MyD88 is recruited and is subjected to oligomerization

forming a large signaling platform named myddosome. IRAK4 is first recruited to the myddosome and a major contributor to activate IRAK1 and IRAK2. In turn, activated IRAK1 and IRAK2 interact with MyD88 and TRAF6 to regulate NF-κB activation. TRAF6 is a E3 ubiquitin ligase that can contribute to form a complex containing TAK1, TAB1, TAB2 and TAB3 through generation of K63-linked poly-ubiquitination chains to control transcriptional activities of NF-κB and AP-1.

NOD-like receptors

NOD-like receptors (NLRs) are a group of pattern recognition receptors that are located in the cell cytosol and are responsible for detection of intracellular PAMPs and induce inflammatory immune responses contributing to clear infections (Franchi et al., 2009; Saxena and Yeretssian, 2014). The NLRs are found in immune cells as well as epithelial cells and other cell types. So far, 23 NLRS in human and 34 NLRs in mice have been identified (Kumar et al., 2009).

The NLRs are composed of a N-terminal domain, a central NOD domain and a C-terminal domain (Franchi et al., 2009; Kumar et al., 2009). Of the three domains, the C-terminal domain contains leucine-rich repeats that are responsible for sensing PAMPs consisting of LPS from Gram-negative bacteria, peptidoglycan (PGN) from Gram-positive bacteria, flagellin and microbial nucleic acids. The central NOD domain is critical for activation of NLRs. The N-terminal domain is composed of several effector regions such as caspase recruitment domain (CARD), pyrin domain (PYD), acidic domain, and baculovirus inhibitor repeats (BIRs), which mediates the signaling through interaction with downstream factors that containing CARD and/or PYD domains. Based on the presence of sub-regions in N-terminal domain, NLRs are divided into 5 subfamilies: four well-characterized groups (CARD, PYD, BIR and acidic domain) and one undefined group (NLRX).

NLRs containing CARD such as NOD1 and NOD2 mediates NF-κB and MAPK activation resulting in transcription of genes associated with both innate immunity and adaptive immunity, whereas NLRs containing PYD or BIR including NLRC4, NLRP3 and NLRP1 contribute to caspase-1 activation through forming inflammasome (Brubaker et al., 2015). NOD1 and NOD2 are two well-documented members of NLRs, which contain one or two CARD domains. NOD1 senses *meso*-diaminopimelic acid-containing PGN, whereas NOD2 recognizes muramyl dipeptide-containing PGN. Upon stimulation of NOD1 and NOD2, the serine-threonine kinase RICK (receptor-interacting protein 2) is directly recruited to NOD1 or NOD2 via CARD-CARD interactions. RICK binds to NEMO, which stimulates the ubiquitination and activation of IKKα and IKKβ leading to activation of NF-κB signaling. Moreover, RICK mediates recruitment of TAK1 in a ubiquitin-dependent manner, which requires NOD1- and NOD2-induced K63-linked polyubiquitination of RICK. TAK1 plays an essential role in IKK complex activation through interaction with TAB1, TAB2, and/or TAB3. In addition to NF-κB activation, NOD1 and NOD2 are reported to activate MAPK signaling, which requires the involvement of TAK1.

The NLRs (NLRC4, NLRP3 and NLRP1) as well as an adaptor protein ASC are also involved in activation of inflammasome in response to PAMPs, which leads to activation of caspases associated with inflammation responses and apoptosis (Brubaker et al., 2015; Franchi et al., 2009). Activated inflammasome recruits pro-caspase-1 and induces the formation of active caspase-1 through auto-proteolytic cleavage of pro-caspase-1. Subsequently, active caspase-1 promotes the generation of active IL-1β and IL-18 via cleavage of precursor cytokines pro-IL-1β and pro-IL-18. Active caspase-1 is also involved in inflammatory cell death termed pyroptosis. For example, it was documented that NLRP3 and NLRC4 were required for protection from *C. rodentium* in mice models.

NF-κB signaling pathway

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is transcription regulator that was first identified in 1986 (Zhang et al., 2017). So far, NF-κB has been reported to play vital roles in various cellular processes such as cell proliferation, death, survival, inflammation and innate and adaptive immune responses (Ghosh and Hayden, 2012; Zhang et al., 2017). Canonical NF-κB pathway (or classical pathway) and non-canonical NF-κB pathway (or alternative pathway) have been documented (Hoesel and Schmid, 2013). Canonical NF-κB pathway depends on inducible degradation of inhibitors of NF-κB (IκBs), particularly IκBα, resulting in nuclear translocation of various NF-κB complexes. However, non-canonical NF-κB pathway relies on the inducible processing of p100 and/or p105. The non-canonical NF-κB pathway is involved in lymphoid organogenesis, B-cell survival and maturation, dendritic cell activation, and bone metabolism (Sun, 2012).

The mammalian NF-κB family is composed of 5 well-known subunits, RelA (also named p65), RelB, c-Rel, p50 (NF-κB1), and p52 (NF-κB2), which can form homo- and hetero-dimerization through their Rel homology domain (RHD) (Zhang et al., 2017). The five Rel proteins are classified into two groups. First, RelA, RelB, and c-Rel are synthesized as mature proteins carrying transcription transactivation domain (TAD). Second, p50 and p52 are produced from their precursor proteins, p105 and p100, respectively. So far, 13 of the 15 potential NF-κB dimers have been described, and RelA:p50 and RelB:p52 represent the two major dimers (Zhang et al., 2017). TAD-containing hetero-dimers are transcriptional activators, whereas p50 and p52 homo-dimers function as transcriptional repressors. These NF-κB proteins share a conserved N-terminal RHD domain containing 300 amino acids (Ghosh and Hayden, 2012; Hoesel and Schmid, 2013). The

RHD is responsible for sequence-specific DNA binding, dimerization, and interaction with IκBs. Biochemistry studies reveal that the RHD consists of a N-terminal subdomain and a C-terminal subdomain. Both of two subdomains possess the beta-barrel immunoglobulin-like folds and the two subdomains are connected together by a hinge that bind to both sides of the cognate NF-κB site across the major groove of the DNA. The N-terminal subdomain is responsible for p53 DNA-binding and specific DNA recognition, whereas the C-terminal subdomain contains hydrophobic residues for dimerization and interaction with IκB.

In unstimulated cells, the activities of NF-κB are inhibited by IκBs that are composed of IκBα, IκBβ, IκBε, BCL3, IκBζ, IκBNS, IκBη and the precursor proteins p100 (IκBχ) and p105 (IκBδ) (Ghosh and Hayden, 2012; Zhang et al., 2017). All of the IκBs contain five to seven tandem Ankyrin repeats (AnkRs) that can bind to NF-κB, masking the nuclear localization sequence. In canonical NF-κB pathway, IκBα is the predominant regulator that restrains p50/p65 heterodimer in cytoplasm, whereas NIK (NF-κB-inducing kinase) and IKKα are the central components that mediates the nuclear translocation of RelB/p50 heterodimer in non-canonical pathway (Zhang et al., 2017). Upon stimulation, activation of NF-κB pathway differs in canonical and non-canonical pathway. In the canonical pathway, proteolytic degradation of IκBs is controlled by the IKK (IκB kinase) complex that is composed of three components, IKKα, IKKβ, and a non-catalytic subunit NEMO (NF-κB essential modolator or IKKγ) (Israel, 2010). NEMO is responsible for formation of the IKK complex and recruitment of the IKK complex to upstream activator. Phosphorylation of IKKβ and poly-ubiquitination of NEMO is required for activation of the IKK complex. Activated IKK complex in turn directly phosphorylate IκBα on Ser32 and Ser36 and IκBβ on Ser19 and Ser23 for subsequent ubiquitination by $SCF^{\beta\text{-}T\gamma CP}$ E3 ubiquitin ligase and proteasome-

dependent degradation, which leads to release and nuclear localization of NF-κB (RelA/p50) (Zhang et al., 2017). Binding of NF-κB to the specific site in promoter region initiate the NF-κB-induced gene expression launching rapid responses to pathogens or inflammatory stimuli. In non-canonical pathway, NF-κB subunit p100 associates with RelB with its AnkR domain and exist in cytoplasm under unstimulated condition. The C-terminal domain of p100 carries a NIK-responsive domain and two phosphorylation sites (Ser866 and Ser870) that resemble the phosphorylation site of IκBs. In absence of stimuli, NIK binds to TRAF3 (TNF receptor-associated factor 3). Under this situation, TRAF3 functions as adaptor protein to recruit TRAF2 and cIAP1 or cIAP2 (cIAP1/2, E3 ubiquitin ligases) forming T3-T2-cIAP E3 complex resulting in constant ubiquitination and degradation of NIK, which blocks the activation of non-canonical NF-κB pathway (Sun, 2012). In response to signals from activated receptors, the T3-T2-cIAP E3 complex is recruited to activated receptor leading to degradation of TRAF3 and/or TRAF2 as well as accumulation and auto-phosphorylation of NIK in cellular cytoplasm, which in turn activates the IKK complex only containing IKKα homo-dimer. Activated IKKα phosphorylates p100 on Ser776 and Ser780 causing ubiquitination and subsequent proteolysis of C-terminal AnkR domain of p100 to produce p52 forming nuclear co-localization of RelB:p52, which activates non-canonical NF-κB pathway (Zhang et al., 2017).

Following nuclear translocation, the NF-κB is able to bind to the promoter and enhancer region containing κB sites with the consensus sequence 5'-GGGRNNYYCC-3' (N – any base, R – purine, and Y - pyrimidine) initiating transcription of NF-κB-induced genes (Zhang et al., 2017). Previous study revealed that the N-terminal subdomain of RHD is responsible for specific DNA recognition, whereas the C-terminal subdomain contains hydrophobic residues for dimerization formation (Hayden and Ghosh, 2012). RelA, RelB, and c-Rel have TAD contributing to transcription

activator, whereas homo-dimers of p50 and p52 function as transcription repressors due to lack of TAD. More and more publications suggest that a full induction of NF-κB-induced genes requires co-activators in nucleus (Wan and Lenardo, 2010). A recent study identified another essential subunit of native NF-κB complex, ribosomal protein S3 (RPS3), that can associate with Rel dimers to achieve full binding and transcriptional activity through determining the DNA binding affinity and regulatory specificity of NF-κB (Stanborough et al., 2014; Wan et al., 2007). A list of novel molecules has been reported to regulate the NF-κB transcriptional activity in nucleus, which was reviewed by Fengyi Wan and Michael J. Lenardo (Wan and Lenardo, 2010).

TNF-NF-κB signaling pathway

Tumor necrosis factor alpha (TNFα) as well as interleukin-1β (IL-1β), interleukin-6 (IL-6) and chemokines are pro-inflammatory cytokines that are able to elicit inflammatory reactions such as fever, inflammation and cell death (Dinarello, 2000). TNFα is mainly produced by macrophages but also other cells such as lymphoid cells, mast cells, endothelial cells, and fibroblasts (Wajant et al., 2003). TNFα plays important roles in regulating cell proliferation, differentiation, apoptosis and production of other cytokines and immune responses (Brenner et al., 2015). TNFα-induced cellular responses are primarily mediated through two TNF receptors (TNFRs), TNF receptors 1 (TNFR1) and TNF receptors 2 (TNFR2) (Brenner et al., 2015). TNFR1 has been found in most types of cells, whereas TNFR2 is identified primarily from cells of immune system (Faustman and Davis, 2010). A major difference between TNFR1 and TNFR2 is the absence of a death domain in TNFR2 (Naude et al., 2011). Upon activation by TNF, TNF-TNFR complex can downregulate the activation of TNF-induced apoptosis, canonical NF-κB and MAPK (ERK, p38 and JNK) signaling pathways (Li and Lin, 2008). Previous study suggests that TNFR1 is the key mediator

of TNF signaling in most of cells and TNR2 plays a major role in lymphoid system (Li and Lin, 2008).

The mechanism of TNF-mediated cellular responses has been widely studied in past decades (Brenner et al., 2015; Cendrowski et al., 2016). A model was proposed, which TNFR1 sequentially activate NF-κB and apoptosis (Micheau and Tschopp, 2003). Upon binding of TNF to TNFR1, TRADD (TNF Receptor-Associated Death Domain) is recruited to receptor, and TRADD subsequently recruits RIP1 (TNF Receptor Interacting Protein) and TRAF2 (TNF Receptor-Associated Factor 2) to form a signaling complex at the cell surface that activates NF-κB by recruitment of TAK1 (TGF-β activated kinases 1) and the inhibitor of κB (IκB) kinase (IKK) leading to phosphorylation, ubiquitination and degradation of IκBα and subsequently liberating NF-κB. After binding to DNA, NF-κB regulates the expression of various genes that are involved in cell survival, proliferation, invasion, angiogenesis, metastasis, chemo-resistance, radio-resistance and inflammation. Subsequently, TNFR1 is internalized and RIP1, TRAF2, and TRADD are modified and dissociated from the receptor, and then TRADD and/or RIP1 bind to FADD resulting in recruitment of caspase-8 and activation of apoptosis. Recent study suggested that internalization of TNFR1 is dependent on caveolae or lipid rafts, but independent of clathrin-mediated endocytosis, in response to TNFα stimulation. Another study described that lipid disruption was able to block IκBα phosphorylation and to sensitize cells to TNFα-induced apoptosis (Legler et al., 2003). In summary, TNF-mediated apoptotic and cytotoxic responses are dependent of TNFR internalization, whereas NF-κB-mediated anti-apoptotic and pro-inflammatory responses are independent of TNFR internalization. In response to TNFα stimulation, canonical NF-κB pathway is activated and induces the transcriptions of TNFα-regulated genes (Li and Lin, 2008). So far, the signaling components identified in the TNF-NF-κB

activation pathway include the IKK complex, RIP, TRAF2 and MAP3K (Mitogen-Activated Protein Kinase Kinase Kinase) (Brenner et al., 2015). RIP is a serine/threonine kinase containing an N-terminal kinase domain, an intermediate domain and a C-terminal death domain required for association with the upstream signaling component TRADD (Li and Lin, 2008). The intermediate domain is responsible for RIP-mediated NF-κB activation and also plays an essential role in interaction with downstream signaling components. TRAF2 is an adaptor protein that contains a N domain, a conserved C domain, a RING domain, and several zinc fingers (Vince et al., 2009). The RING domain has been reported to associate with E3 ligase activities and the clAPs recruited to TNF- NF-κB compartment by TRAF2 are critical E3 ligase required in TNF-induced canonical NF-κB signaling pathway (Brenner et al., 2015). MAP3K is a serine/threonine-specific protein kinase. It was reported that a MAP3K, TAK1, targeted for IKKβ phosphorylation (Shinohara et al., 2005).

Constitutive activation of NF-κB is associated with chronic inflammation and immune system dysfunction promoting the development of cancers (Zhang et al., 2017). Therefore, negative regulators of NF-κB activity is required to attenuate NF-κB signaling. Of those negative regulators, IκBα is a key component (Li and Lin, 2008). Upon TNF stimulation, activated NF-κB induces expression of IκBα and the newly synthesized IκBα binds to NF-κB and inhibit its activities. A20 (TNFα-induced protein 3) containing a cytoplasmic zinc finger domain is another regulator that can inhibit NF-κB activity (Shembade et al., 2010; Zhang et al., 2017), which was reported to block recruitment of RIP and NEMO to TNFR1 complex and A20/ABIN1 was able to disrupt the interaction of RIP with NEMO to disrupt NF-κB activation. Further study identifies that A20 possesses de-ubiquitination activity, suggesting that A20 is able to remove K63-linked

poly-ubiquitination chain off RIP and add K48-linked ubiquitin to RIP (Wertz et al., 2004; Zhou et al., 2016). The tumor suppressor protein, CYLD, is the third described regulator that can modulate NF-κB activation through interference with TRAF2 ubiquitination or TAK1 activation (Li and Lin, 2008; Zhang et al., 2017). The roles of phosphatases in negative regulation of TNF-NF-κB signaling is poorly studied. Previous studies identified a distinct protein phosphatase 2A (PP2A) that can dephosphorylate IKK, NF-κB, and TRAF2 complex leading to down-regulation of NF-κB transcriptional activities (Kray et al., 2005; Li et al., 2006). However, further studies are required to determine whether there is a phosphatase that regulates specifically TNFα-induced NF-κB activation. Internalization of activated receptors is also considered as a means of signal attenuation. Recently, cell penetrating peptides (CPPs) that are short peptide (< 40 amino acids) and are able to enter cells through various mechanism including endocytosis were reported to interfere with TNF-induced NF-κB activation by induction of TNFR internalization, which is a clathrin-dependent manner (Fotin-Mleczek et al., 2005a). In summary, the duration of NF-κB activities is positively and negatively regulated by multiple factors.

Gut microbiota and enteric pathogens

Gut microbiota refers to the complex community of microorganism that reside in digestive tract of human and other animals, which is composed of bacteria, eukaryotes, viruses, and archaea (Clemente et al., 2012; Lozupone et al., 2012). To date, only a minority of gut microbiota can be cultured (Eckburg et al., 2005). However, the composition of gut microbiota has been greatly advanced by employing culture-independent high-through sequencing technologies such as 16S rRNA sequencing (Jovel et al., 2016). Large-scale projects such as the Human Microbiome Project and MetaHIT have been carried out to study the composition of gut microbiota (Human

Microbiome Project, 2012; Qin et al., 2010). These studies have shown that the gut microbiota is dominated by bacteria, especially by two major phyla: *Bacteroidetes* and *Firmicutes*, and demonstrated a large variability in the composition of the gut microbiota in healthy individuals (Clemente et al., 2012). Genetic factors play a critical role in the development of gut microbiota and diet (Dabrowska and Witkiewicz, 2016). The composition of gut microbiota is stable in healthy adult individuals over time. However, age, diet, disease, antibiotics use and environmental factor also contribute to the temporal dynamics of gut microbiota (Lozupone et al., 2012).

It is now well established that gut microbiota plays an important role in health and disease (Harmsen and de Goffau, 2016). The gut microbiota is associated with energy harvest and storage such as fermentation and utilization of undigested carbohydrates. The gut microbiota contributes to promote the maturation of immune cells and normal development of immune systems (Clemente et al., 2012). Germ-free mice have been characterized by reduction of secretory IgA, defects in development of gut-associated lymphoid tissues and smaller Peyer's patches and mesenteric lymph nodes (Round and Mazmanian, 2009). The gut microbiota accounts for host resistance/susceptibility to colonization by enteric pathogens. Germ-free mice are demonstrated to be more susceptible to enteric pathogens such as *Shigella flexneri*, *C. rodentium*, *Listeria monocytogenes* and *Salmonella enterica* serovar Typhimurium (Baumler and Sperandio, 2016). Another notable example is that transplantation of gut microbiota from strains of mice that are susceptible to *C. rodentium* infection induces similar susceptibility in mice that are previously resistance to *C. rodentium* infection (Willing et al., 2011). Gut microbiota-mediated control of enteric pathogens attribute to direct inhibition, barrier maintenance, immune modulation and metabolism.

Antimicrobial peptides (AMPs) are small molecular weight proteins with broad spectrum antimicrobial activities against bacteria, yeast, viruses and fungi (Dutta and Das, 2016). In host digestive tract, AMPs are produced by not only Paneth cells and neutrophils but also bacteria (Bahar and Ren, 2013). So far, more than 2,800 AMPs have been deposited into the Antimicrobial Peptide Database (APD, http://aps.unmc.edu/AP/) (Wang et al., 2016). AMPs-mediated killing of bacteria is associated with their ability to interact with bacterial membrane or cell wall (Zhang and Gallo, 2016). Some AMPs can bind to bacterial membrane and destroy the membrane integrity via non-enzymatic and enzymatic manners resulting in lysis of target microbes. Some AMPs are able to cross the lipid bilayer and kill bacteria through inhibiting enzyme activities and the synthesis of protein and nucleic acid. Bacteriocins are a type of AMPs produced by commensal bacteria to limit the growth of the same or similar bacterial species (Hammami et al., 2013; Wang et al., 2016). For example, *E. coli* produces bacteriocins leading to inhibiting the growth of EHEC strains (Schamberger and Diez-Gonzalez, 2002). Bile acid is produced in liver and secreted into small intestine. Gut microbiota can metabolize bile acid to produce secondary bile acid with unique activities (Devlin and Fischbach, 2015). Transplantation of *C. scindens* strain that can modify bile acid into antibiotic-treated mice was reported to promote the resistance to *C. difficile* infection (Buffie et al., 2015).

The IECs are covered by a mucus layer that is composed of mucins produced by goblet cells (Birchenough et al., 2015). This layer of mucus functions as a protective barrier that separate microbiota as well as invading pathogens from IECs, and suppress pro-inflammatory responses. Reduced thickness of mucus layer was observed in antibiotic-treated mice and exposure of germ-free mice to gut microbiota induced the synthesis and secretion of mucin (Johansson et al., 2015). In addition to mucin, the mucus lay contains digestive enzymes, AMPs and immunoglobulin (IgA)

(Ribet and Cossart, 2015). These molecules also play important roles in limiting the contact of invading pathogens with IECs.

Over the past several decades, it has been well-accepted that gut microbiota plays key roles in development and maturation of immune systems. Previous studies have demonstrated that *Bacteroides fragilis* contributes to development of CD4 T lymphocytes and gut microbiota promotes the differentiation of Th17 cells, a contributor to modulate resistance against to pathogens and development of autoimmune pathology, from CD4 T cells (Ivanov et al., 2009; Johnson et al., 2015). Several other studies suggested that *Bacteroides* species and *Clostridium* belonging to XIVa cluster were involved in development of anti-inflammatory T regulatory cells (Hayashi et al., 2013; Johnson et al., 2015; Omenetti and Pizarro, 2015). Short-chain fatty acids (SCFAs) that were derived from indigestible plant fiber by gut microbiota were reported to maintain the balance of inflammatory and anti-inflammatory T cells. (Clemente et al., 2012; Kabat et al., 2014; McKenney and Pamer, 2015). In addition to adaptive immune system, gut microbiota also contributes to innate immune signaling. In mouse model, antibiotic worsened dextran sodium sulfate (DSS)-induced colitis (Hernandez-Chirlaque et al., 2016). Administration of Toll-like receptor ligands can rescue mice suggesting gut microbiota-mediated pattern recognition receptor (PRR) signaling is necessary for protection against epithelial damage. The gut microbiota also induces the expression of bactericidal C-type lectins via PRR signaling resulting in inhibiting bacterial growth.

Preferential consumption of nutrients is another strategy employed by gut microbiota to compete pathogenic bacteria (Kamada et al., 2013). For example, commensal *E. coli* competes EHEC for organic acids, amino acids, and other nutrients. Furthermore, gut microbiota-mediated production of secondary metabolites is involved in inhibiting the expression of virulence genes. The SCFAs

are reported to modulate the function of type 3 secretion system (T3SS) in *Salmonella enterica* serovar Enteritidis and Typhimurium. Moreover, *Bateroides thetaiotaomicron* is able to produce mucin-derived fucose to regulate the expression of the locus of enterocyte effacement (LEE) genes in EHEC (Pacheco et al., 2012).

Cyclic adenosine monophosphate (cAMP) and cAMP-receptor protein (CRP) in *Escherichia coli*

Cyclic adenosine monophosphate (cAMP or cyclic AMP) is a universal second messenger that is used by diverse forms of life including bacteria (McDonough and Rodriguez, 2011). Cyclic AMP is generated from ATP by adenylate cyclase (AC) and its degradation is catalyzed by phosphodiesterase (PDE) (McDonough and Rodriguez, 2011). Cyclic AMP activates cAMP-receptor protein (CRP) resulting in formation of active CRP-cAMP complex, a global regulator (Green et al., 2014). Activated CRP is subjected to allosteric conformational changes and binds to DNA initiating transcription of CRP-cAMP-induced genes (Green et al., 2014).

In *E. coli*, the AC is encoded by a *cyaA* gene (Bachmann, 1990). Molecular genetics and biochemical studies demonstrated that *E. coli* AC is composed of two domains (Roy et al., 1983). The catalytic domain is N-terminus, whereas the glucose sensitive regulatory domain is C-terminal domain. It was proposed that the activity of AC is transcriptionally and posttranslational regulated (Botsford and Harman, 1992). A previous study suggested that *cyaA* expression was negatively regulated by cAMP-CRP complex (Aiba, 1985). However, it was reported that cAMP-CRP played little role in regulation of *cyaA* expression *in vivo* by using gene fusion technique (Bankaitis and Bassford, 1982). Many studies now demonstrate that the majority of the effect on regulation of *cyaA* expression attributes to posttranscriptional regulation via reducing the level of phosphorylated $EIIA^{Glc}$ (glucose-specific enzyme IIA) (Goerke and Stulke, 2008). This

modulation involves phosphoenolpyruvate (PEP) – sugar phosphotransferase (PTS). In the absence of glucose, $EIIA^{Glc}$ is phosphorylated and activates AC resulting in an elevated level of cAMP. By contrast, in the presence of glucose, $EIIA^{Glc}$ is dephosphorylated and inactivate AC leading to a reduced level of cAMP.

CRP is encoded by the *crp* gene in *E. coli* (Green et al., 2014) (Botsford and Harman, 1992). CRP is a homodimer and consists of two domains connected by a short hinge region. The crystal structure of apo-CRP was published in 2009 (Sharma et al., 2009). The large N-terminal domain (residues 1-137) contains a cAMP-binding domain. It mainly consists of β-sheets responsible for CRP dimerization and carries a hydrophobic pocket for binding of cAMP resulting in functional activation of CRP. The small C-terminal domain (residues 138-209) is DNA-binding domain containing a characteristic helix-turn-helix motif. It dimerizes in apo-CRP, with the DNA recognition helix F buried within its core. Activated CRP recognizes specific DNA sites (upstream of promoter) via the C-terminal F-helix, which forms a typical helix-turn-helix motif, together with the neighboring E-helix. Two regions of CRP, activation region 1 (located in C-terminal domain) and activation region 2 (located in N-terminal domain), are known to interact with RNA polymerase (RNAP). Binding of CRP to DNA bends the DNA by about 87° and subsequent recruitment of RNAP initiates transcription of many catabolite genes. Depending on the location of the binding site(s) within a target promoter, cAMP-CRP may function as a repressor or activator. In addition, CRP is also involved in many other processes, such as osmoregulation (Balsalobre et al., 2006), stringent responses (Johansson et al., 2000), biofilm formation (Jackson et al., 2002), virulence (Balsalobre et al., 2006), nitrogen assimilation (Mao et al., 2007), iron uptake (Zhang et al., 2005), competence (Sinha et al., 2009), multidrug resistance to antibiotics (Nishino et al., 2008), and quorum sensing (Xavier and Bassler, 2005). The cAMP-CRP machinery is now

presumed to regulate transcription at nearly 200 different promoters (Hollands et al., 2007). By using comparative genome approach, a previous study predicated 161 strong and 285 weak candidates CRP-binding site in *E. coli* (Tan et al., 2001). In 2003, a study on identifying global regulators in transcriptional regulatory networks in bacteria discovered that 197 genes were under regulation by CRP (Martinez-Antonio and Collado-Vides, 2003). In 2004, one study by employing the ROMA (run-off transcription/microarray analysis) to study the CRP-regulated genes found that 280 genes present in 188 different operons had significantly high transcription and 20 genes present in 16 operons had reduced transcription in response to CRP (Zheng et al., 2004).

Adherence to biotic and abiotic surfaces is one of important traits in pathogenic *E. co*li. Several studies have described that many types of adhesive pili are mediated by CRP. CRP positively regulates the expression of ETEC K99 and 987P pili through direct and indirect pathways, respectively (Edwards and Schifferli, 1997; LoTseng et al., 1997). CRP also regulates the expression of other ETEC pili such as CFA/I, CS1, CS2, and CS3 because their expressions are under control of catabolite repression (Karjalainen et al., 1991). A previous found that the synthesis of CFA/I in ETEC was affected by glucose that inactivate AC (Evans et al., 1991). Addition of 0.5 % glucose to the growth medium diminish the promoter (cfaA) activity more than 70 %. And this repression can be reversed by addition of cAMP to the medium. This study also found the similar effect of glucose on CFA/II expression in ETEC. Another study demonstrates that the expression of the *tib* adherence locus in ETEC is dependent on CRP (Espert et al., 2011). The *tib* locus, *tibDBCA*, encodes a glycosylated autotransporter (TibA) that contributes to attachment to mammalian cells, autoagregation, and biofilm formation. Another study described that CRP negatively regulates the expression of type I fimbriae in UPEC contributing to mediate adhesion

to mannose-containing receptor in epithelial cells and promote the formation of intracellular community (Muller et al., 2009).

ETEC encodes a heat-stable toxin (ST) and/or a heat-labile toxin (LT). The A subunit of LT ADP-ribosylates Gsα, a protein that activates AC in eukaryotic cells, resulting in the overproduction of cAMP, which causes increased chloride secretion and disrupt sodium absorption (Fleckenstein et al., 2010). Cyclic AMP has been reported to play roles in regulating the expression of both LT and ST (Bodero and Munson, 2009; Munson, 2013). Cyclic AMP-CRP was implicated as the transcriptional regulator of *eltAB*, the gene encodes LT, and the expression of the *eltAB* was inhibited by glucose. One previous study shown that cAMP-CRP repressed the expression of LT by binding of CRP to an operator that was centered over the -35 hexamers of the *eltAB* promoter, resulting in the exclusion of RNAP from the promoter (Bodero and Munson, 2009). In contrast, they also found that the CRP was able to positively regulate the expression of ST by activating ST promoter through occupancy of the sites that facilitate productive interactions between the transcription factor and RNAP. Another study suggested that cAMP-CRP is negatively regulate the production of LT, but positively modulate the secretion of LT (Gonzales et al., 2013).

Cyclic AMP is essentially ubiquitous. It is found in animal cells, fungi, plants, and bacteria. Therefore, it is hypothesized that cAMP may be used as signal molecule for interspecies communication. Cyclic AMP-CRP has been demonstrated to regulate the expression of virulence genes in *E. coli*. Regulation of cAMP-CRP binding to specific region in DNA promoter may represent a potential invention for controlling bacterial infection. Several other studies demonstrated that *E. coli cyaA* mutants are able to utilize exogenous cAMP. Since cAMP has to bind CRP directly to activate CRP, it might suggest the presence of a transport system that allows the cAMP to be transported through cell membrane.

Modulation of inflammatory responses

Inflammation is a protective response that contributes to eliminate the harmful stimuli such as microbial pathogens, as well as damaged self-tissues (Takeuchi and Akira, 2010). Generally, inflammation presents 5 clinical symptoms: redness, swelling, heat, pain and loss of tissue function (Takeuchi and Akira, 2010). The innate immunity system plays a critical role in acute inflammation induced by microbial infection or tissue damages. Although immune cells such as macrophages and DCs play important roles, non-immune cells including epithelial cells, endothelial cells and fibroblasts also contribute to the induction of inflammation.

Enteric pathogenic *E. coli* express different types of virulence factors and fitness traits contributing to establish and sustain a robust colonization in host gut (Baumler and Sperandio, 2016; Reddick and Alto, 2014; Ribet and Cossart, 2015). These virulence traits such as toxins and flagellin also induce inflammatory responses. Given that most of intestinal pathogenic *E. coli* are extracellular pathogens, the inflammatory responses are mainly initiated by Toll-like receptors (TLRs), which is involved NF-κB and MAPK signal transduction pathways (Sanchez-Villamil and Navarro-Garcia, 2015). Here we briefly summarize the factors that initiate inflammatory responses and factors that block inflammatory responses.

EPEC and EHEC (LEE-containing STEC) strains share a pathogenicity island termed the locus of enterocyte effacement (LEE) that encodes effectors (intimin and Tir) responsible for formation of attaching and effacing (A/E) lesion and a type 3 secretion system (T3SS) that facilitate injection of bacterial proteins directly into host cells. During EPEC infection, TLR5 recognize flagellin and activates NF-κB and induce the production of TNFα (tumor necrosis factor α). H7 flagellin, hemorrhagic coli pili (HCP) and long polar fimbriae activate pro-inflammatory responses in

human colon epithelial cell in response to EHEC O157:H7 infection (Berin et al., 2002; Farfan et al., 2013). Outer membrane protein A (OmpA) of EHEC was reported to stimulate murine DCs to produce IL-1α, IL-1β, IL-10 and IL-12p70 (Jeannin et al., 2002). Another recent study found that hemolysin derived from EHEC O157:H7 is able to induce cytotoxicity and production of IL-1β via NLRP3-mediated pathway in human macrophages rather than C57BL/6 mouse macrophages (Cheng et al., 2015).

Both EPEC and EHEC strains express T3SS-dependent effectors such as NleB, NleC, NleE, NleH and Tir. These effectors subvert inflammatory response by targeting NF-κB signaling pathway. NleB is N-acetyl-D-glucosamine (GlcNAc)-transferase that can regulate GlcNAcylation of GAPDH to suppress TRAF2-GAPDH interaction resulting in inhibition of TRAF2 polyubiquitination and NF-κB activation in HeLa cells (Gao et al., 2013). NleB were reported to inactivate death domain of TRADD, FADD, RIPK1, and TNFR1 by arginine GlcNAcylation to block NF-κB activation in HEK293T cells (Li et al., 2013). Moreover, NleB was described to assist NleE-mediated inhibition of IκB degradation (Newton et al., 2010). NleC is zinc-dependent endopeptidase that specifically cleaves and inactivates p65 subunit of NF-κB leading to blockage of translocating p65/p50 complex in nucleus (Baruch et al., 2011; Yen et al., 2010). In addition, NleC was documented to inhibit p38 phosphorylation but not degradation in Caco-2 cells (Sham et al., 2011). NleD is a zinc-metalloprotease that was described to cleave JNK and p38 to block MAPK signaling but not NF-κB activation (Baruch et al., 2011). NleE is able to inhibit the phosphorylation of IκB resulting in inactivation of NF-κB signaling in HeLa cells even in the presence of TNFα and IL-1β (Nadler et al., 2010; Newton et al., 2010). EHEC NleH1, but not NleH2, bind the human ribosomal protein S3 (RPS3) to block NF-κB activation (Gao et al., 2009).

Another study demonstrated that EHEC NleH1 can specifically inhibit the phosphorylation of RPS3 resulting in blocking NF-κB activation (Wan et al., 2011). Tir was documented to interact with TRAF adaptor proteins and promoted their degradation in a proteasome-independent manner leading to inhibition of NF-κB pathway in HeLa cells (Ruchaud-Sparagano et al., 2011).

ETEC and EAEC cause diarrhea through production of toxins. In addition to cause watery diarrhea, LT can activate NF-κB pathway through the cAMP-dependent activation of Ras-like GTPase (Wang et al., 2012). Compared to soluble LT, LT-associated with outer membrane vesicles (OMVs) induced stronger inflammatory response by high production of IL-6 and TNFα through p38, ERK1/2, PKA and NF-κB pathways in T84 cells (Chutkan and Kuehn, 2011). There are few studies about inhibiting host inflammatory responses in ETEC infections. Recently, an ETEC secreted factor (ESF) was reported to prevent IκBα from degradation in response to TNFα stimulation in HCT-8 cells (Wang and Hardwidge, 2012).

Colonization of pathogenic *Escherichia coli* in hosts

Intestinal pathogenic *E. coli* accounts for one of common causative agents associated with diarrhea in human worldwide (Gomes et al., 2016). Human usually acquire *E. coli* infection through ingestion of contaminated food and water. When *E. coli* enters host, it employs several strategies such as utilization of nutrient in gut and expression of virulence factors contributing to colonize in small or larger intestine to cause diseases, regardless of the presence of host multiple defense mechanisms (Gomes et al., 2016).

Upon entry into human, *E. coli* is first exposed to acidic environment in stomach followed bile and bicarbonate influx in duodenum and increasingly anaerobic condition. In order to colonize in appropriate sites in gut tract, *E. coli* has to adapt to and survive in this acidic condition by

expression of certain genes that confer the protection against acidic environment (Foster, 2004; Lund et al., 2014). Four acid resistance (AR) systems in *E. coli* have been described. Induction of the first AR system requires the alternative sigma factor RpoS and cAMP receptor protein (CRP). *E. coli* O157:H7 *rpoS* mutant was reported to colonize poorly in experimental mice and calves. The second AR system is arginine-dependent, which requires arginine decarboxylase (AdiA) and the regulator of *adiA* (CysB). The third AR system is glutamate-dependent, and two glutamate decarboxylases (GadA and GadB) and a putative glutamate/g-aminobutyric acid (GABA) antiporter (GadC) are required to induce protection at acidic condition. The fourth AR system is a lysine-dependent system and requires the involvement of lysine decarboxylase (CadA) and a lysine/cadaverine antiporter (CadB). STEC O157:H7 isolate was reported to show increased acid resistance compared to other pathotypes of *E. coli* and commensal *E. coli*.

Upon entry into digestive tract, *E. coli* encounters an alkaline environment in duodenum with the presence of bile and bicarbonate (Begley et al., 2005). *E. coli* responds to the high pH stress by increasing production of metabolic acid and sugar fermentation to reduce the cytosolic pH (Padan et al., 2005). Bacteria are able to increase the ATP synthase expression and couples H^+ entry to promote the generation of adenosine triphosphates (ATP), and increase the activity of monovalent cation/proton antiporter. So far, four monovalent cation/proton antiporters have been documented to play critical roles in alkaline hemostasis in *E. coli*. By contrast, alkaline condition is a signal for inducing toxin expression (Gonzales et al., 2013). External alkaline condition plays a positive role in production and secretion of ETEC LT. Previous study suggested that LT secretion was inhibited under acidic condition and was significantly stimulated at neutral or alkaline conditions (Gonzales et al., 2013). Given the fact that ETEC colonize at human small intestine, it is possible that ETEC uses pH gradient as a signal to modulate its toxin production.

E. coli has evolved several strategies to out-compete for nutrients with gut microbiota, penetrate the mucus layer and escape the host defense contributing to adherence to and proliferation at the surface of IECs in digestive tract. The first line of defense is mucus layer. In host digestive tracts, the IECs are covered by a mucus layer serving a physical barrier to limit the contact of invading pathogens and normal gut microbiota with IECs. The mucus is composed of mucins produced by goblet cells, digestive enzymes, antimicrobial peptides produced by Paneth cells and secretory IgA produced by B cells in lamina propria. In order cross this mucus layer, bacteria produce proteases to directly degrade host mucins such as Pic from EAEC (Harrington et al., 2009) and metalloproteinase from EHEC (Hews et al., 2017). Bacteria develop resistance to AMPs through electrostatic repulsion escaping the AMPs physical contact and production of protease to eliminate soluble AMPs (Kraus and Peschel, 2006). *E. coli* expresses flagellum to travel in mucus layer. Gut microbiota also mediates host defense against pathogenic *E. coli*. Conversely, pathogenic bacteria develop strategies to outcompete with gut microbiota for survival in host. For example, to colonize a unique niche, EHEC strains have to compete with microbiota for nutrients (Baumler and Sperandio, 2016). EHEC can use monosaccharides or disaccharides as a source of carbon. Commensal *E. coli* strains utilize fucose. By contrast, EHEC preferentially use other source of sugar such as galactose, the hexuranates, mannose and ribose that are not fermented by commensal *E. coli*. EHEC also use fucose as signal to modulate the expression of its virulence genes and promote intestinal colonization (Pacheco et al., 2012).

Glucose is the preferred source of carbon for *E. coli* and the availability of glucose decreases from the upper part of small intestine to large intestine. Glucose inhibits the synthesis of cAMP in *E. coli*. By contrast, absence of glucose activates the production of cAMP and subsequently activates cAMP receptor protein (CRP). CRP-cAMP modulates the expression of over 200 genes

in *E. coli*, which may be involved in regulation of virulence factors in pathogenic *E. coli*. For example, CRP-cAMP was reported to modulate the expression of LT and ST in ETEC strains. CRP activates transcription of estA (ST), but repress the transcription of *eltAB* (LT). However, a recent study suggested that CRP-mediated repression of *eltAB* was indirect and was not dependent on the *crp* binding sites upstream of the *eltAB* promoter. Therefore, glucose in environment induces *eltAB* expression and inhibits *estA* expression. Given that ETEC-induced diarrhea results in high concentration of cAMP in lumen and possible lower availability of glucose, it is possible that ETEC may utilizes exogenous source of cAMP to stimulate expression of virulence factors. It was reported that CRP-cAMP was responsible for inducing adhesins expressions in ETEC (Johnson et al., 2009).

Bile is secreted into the small intestine from gallbladder. In addition to increase intestinal pH gradient, bile secondary molecules were reported to inhibit the growth of enteric pathogens. Bile and its specific components are another inducer of virulence factors in *E. coli*. For example, bile acid glycocholate hydrate and sodium deoxycholate were reported to induce ETEC colonization factor CS5 expression in a dose-dependent manner (Nicklasson et al., 2012). In EHEC O157:H7 strain, bile was reported to inhibit the transcription of LEE and genes associated with adhesins (Hamner et al., 2013). By contract, bile stress promote attachment of EPEC to IECs (de Jesus et al., 2005), suggesting that pathogenic *E. coli* utilize bile as an environmental signal to modulate its virulence expression.

Adherence to IECs is a crucial step for colonization of enteric pathogens in host. Pathogenic *E. coli* presents a diversity of surface structures contributing to bacterial adhesion to host cells (Kline et al., 2009; Ribet and Cossart, 2015). Pili or fimbriae are adhesive hair-like organelles that function to attach bacteria to a surface. The structure of pili consists of scaffold-like rod located

on the bacterial outer membrane and a bacterial adherence factor locating on the tip of scaffold. The first defined fimbriae are the P pili derived from UPEC, which is the chaperone/usher pathway-dependent fimbriae. Type I pili are another example of pili found in UPEC and DAEC. Type I pili are encoded by the *fim* gene cluster and exported by the chaperone/usher pathway. Type IV pili are the third category of fimbriae expressed in EHEC and EPEC. Type IV pili are able to form bundle structure. For example, EPEC type IV bundle-forming pili are required for initial bacterial attachment to brush border cells prior to the following formation of attaching and effacing lesions. Auto-transporters are non-polymeric adhesins expressed in DAEC and ETEC. ETEC-expressed TibA is responsible for intimate attachment on the surface of IECs. Tir-mediated attachment presents a unique adhesion system in EPEC and EHEC.

Host innate immune system aims to eliminate invading pathogens through phagocytosis, direct killing, and inflammation. Conversely, pathogens have evolved strategies to abolish host innate immune responses resulting survival and proliferation in host. First, pathogens modify the structure of their PAMPs to avoid detection by PRRs. For example, some bacterial strains modify the fatty acid side chain of lipid A of LPS to augment its detection by TLR4 (Miller et al., 2005). Some bacterial species modify recognition site in flagellin to escape TLR5 detection (Andersen-Nissen et al., 2005). Moreover, T3SS-dependent effectors from EPEC and EHEC can subvert host pro-inflammatory responses by targeting signal pathway such as NF-κB and MAPK and dampen the inflammation responses, which contributes to colonization of *E. coli* in host.

In summary, intestinal pathogenic *E. coli* encounters several obstacles during the process of colonizing host digestive tract. In order to survive and replicate in host, intestinal pathogenic *E. coli* has evolved a diversity of strategies to overcome the obstacles. For example, intestinal pathogenic *E. coli* is able to regulate the gene expressions to adapt to hostile environment,

modulate the immune responses, and out-compete with gut microbiota for nutrients. In the second chapter, our study focused on identification of a ETEC-secreted factor (ESF) that blocked TNF-induced NF-κB activation. One of the consequence of TNF-induced NF-κB activation is the production of pro-inflammatory cytokines that are responsible for orchestrating the inflammation responses, regulating the cell death of inflammatory tissues, and recruitment of white blood cells (neutrophils) to inflamed tissues resulting eliminating invading pathogens and repairing the damaged tissues. Through subverting TNF-induced NF-κB activation, ETEC strains are able to colonize and replicate in host small intestine. In the third chapter, our study focuses on identification of cAMP importer in ETEC. ETEC-associated watery diarrhea leads to not only flushing gut microbiota from the intestine but also release of cAMP into the lumen of small intestine. The cAMP is a key secondary messenger that can regulate the expression of over 200 genes including the genes encoding adhesins in *E. coli* promoting the adherence of pathogenic *E. coli* strains to target cells. Compared to nonpathogenic *E. coli* strains such as K-12, ETEC H10407, a human origin pathogen, is hyper-sensitive to extracellular cAMP. Given the cAMP's role in regulating the expression of adhesins, we hypothesize that a cAMP importer is present in ETEC strains accounting for their hypersensitivity to extracellular cAMP. In this study, we employed Tn5 transposome-mediated mutagenesis technique to construct mutant library and screen for the mutants with hyposensitivity to extracellular cAMP. In the fourth chapter, our study focused on gut microbiota and T3SS-dependent NleH effector. Gut microbiota refers to the collection of microbes colonizing the digestive tracts, which contribute to control and eliminate invading enteric pathogens. Pathogenic *E. coli* (EPEC and EHEC) strains developed strategies to out-compete with gut microbiota and modulate host immune response. For example, NleH effector in *C. rodentium* (NleH1 in EHEC) can subvert NF-κB signaling pathway. In this study, we used mouse-specific

pathogen, *C. rodentium*, to study the pathogenesis of EHEC and EPEC. We performed microbiota transplantation between C57BL/6J mice and C57BL/10ScNJ mice, or C3H/HeJ mice and C3H/HeOuJ mice and challenged the mice with WT and Δ*nleH C. rodentium* strains aiming to investigate the impact of gut microbiota and T3SS-dependent NleH effector on regulating the colonization of *C. rodentium* in mouse models.

Chapter 2 - Enterotoxigenic *Escherichia coli* flagellin blocks TNF-induced NF-κB activation

Introduction

Enterotoxigenic *Escherichia coli* (ETEC) causes travelers' diarrhea and diarrheal disease in children living in developing countries (Croxen et al., 2013). ETEC strains are characterized by encoding two main types of virulence factors: heat-labile and/or heat-stable toxins (LT and ST) that cause watery diarrhea (Fleckenstein et al., 2010) and colonization factors (CFs) that mediate ETEC adherence to intestinal enterocytes (Kharat et al., 2017). In addition to causing diarrhea, LT enhances ETEC adherence to host cells by activating host signaling pathways, and inhibits antimicrobial peptide and cytokine (e.g. IL-8) production by disrupting nuclear factor-κB (NF-κB) signaling pathway activation (Huang et al., 2004; O'Neil et al., 1999; Roy et al., 2010).

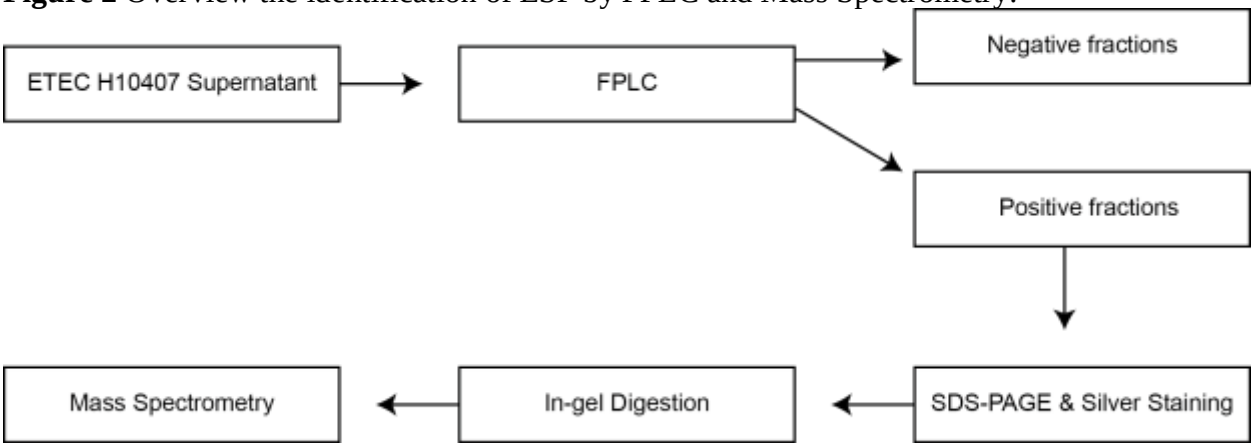
Flagellin, a primary component of flagella, plays critical roles in ETEC pathogenesis and modulation of intestinal innate immune response (Haiko and Westerlund-Wikstrom, 2013). In addition to flagella-mediated bacterial motility, flagella up-regulates the adhesion of ETEC strains to host cells. Previous study suggested that flagella associated with fimbriae promoted the attachment of ETEC C83902 strain to porcine small intestinal epithelial cells (Zhou et al., 2013). Moreover, flagella were reported to positively regulate the biofilm formation and activity of quorum sensing in ETEC C83902 strain (Zhou et al., 2014). Koushik Roy et al. described that EtpA, a TPS exoprotein adhesion, promoted the flagella-mediated attachment of ETEC H10407 strain to host cells (Roy et al., 2009)

NF-κB plays an important role in regulating inflammation and innate immune responses to microbial infections (Lawrence, 2009). NF-κB is normally sequestered in the cytoplasm by the

NF-κB inhibitor, IκBα. Upon TNF stimulation or microbial infection, the IκB kinase (IKK) complex is activated, which subsequently promotes phosphorylation, poly-ubiquitination and degradation IκBα, resulting in nuclear translocation of NF-κB subunits (Hacker and Karin, 2006; Karin and Ben-Neriah, 2000; Skaug et al., 2009). The innate immune system recognizes and responds to pathogens through a diverse set of cellular pattern recognition receptors (Akira et al., 2006; Yin et al., 2015). Pathogens have accordingly evolved mechanisms to subvert innate signaling pathways to promote their survival and transmission (Reddick and Alto, 2014).

We previously reported that pre-incubation of HCT-8 cells with ETEC H10407 supernatants prevented TNF stimulation from inducing IκBα degradation and NF-κB activation (Wang and Hardwidge, 2012). We attributed this phenomenon to a heat-stable protein we designated as ETEC Secreted Factor (ESF). Here we fractionated ETEC supernatants and identified flagellin as necessary and sufficient for this phenomenon. Our current data suggested that TLR5 was not involved into this phenomenon. Our finding might provide a potential pharmaceutically target for controlling intestinal inflammation in response to enteric pathogens infection.

Figure 2 Overview the identification of ESF by FPLC and Mass Spectrometry.



Materials and Methods

Reagents and Antibodies. Antibiotics used in this study were obtained from Fisher Scientific. The restriction enzymes were purchased from New England BioLabs. Human TNFα was obtained from Cell Signaling. TLR5 antagonist, hTLR5-Fc, was from InvivoGen. Antibodies used in this study were obtained from the following sources: HA, FLAG, and c-Myc (Sigma), His and Tubulin (Santa Cruz), IκBα (Cell Signaling).

Bacterial strains and plasmids. Bacterial strains and plasmids are described in Table 4. All ETEC strains used in this study were derived from wildtype ETEC H10407 (Evans et al., 1975). *E. coli* DH5α was used for routine molecular biological manipulations, while *E. coli* BL21 (DE3) and ClearColi BL21 (DE3) were used to express recombinant ETEC H10407 FliC protein. Plasmids pKD3 and pKD46 were used to construct ETEC H10407 mutants. Plasmids pET28a and pT7HMT (Geisbrecht et al., 2006) were used to construct recombinant protein. All bacterial strains were grown aerobically in Luria-Bertani (LB) broth or on LB plates with antibiotics at 37 °C.

Cell line and culture conditions. Intestinal epithelial cell line HCT-8 cells were maintained at 37 °C, 5 % CO2 in RPMI 1640 medium supplemented with 10 % (v/v) fetal bovine serum (FBS) and Penicillin-Streptomycin (100 U/ml). HCT-8 cells were seeded into 6-well plates at a concentration of 5 x 10^5 cells/well. The media was replaced by fresh RPMI 1640 medium lacking both FBS and Penicillin-Streptomycin prior to all experiments.

Table 4 Strains and plasmids used in Chapter 2.

Strain or Plasmid	Description	Source or Reference
Strains		
ETEC H10407	O78:H11, CFA/I, LT^+ and ST^+	(Evans et al., 1975)
E. coli DH5α	Cloning strain	New England BioLabs
E. coli BL21 (DE3)	Protein overexpression strain	Novagen
ClearColi BL21 (DE3)	Protein overexpression	Lucigen
ETEC *ΔfliC*	ETEC H10407 *fliC* mutant	This study
ETEC *ΔfliD*	ETEC H10407 *fliD* mutant	This study
ETEC *ΔfliC*/pFliC-FLAG	ETEC H10407 *ΔfliC* complemented with *fliC*	This study
Plasmids		
pFLAG-CTC	FLAG-tagged protein expression	Sigma
pET28a	His_6 fusion protein expression	Novagen
pT7HMT	His_6 fusion protein expression with TEV site	(Geisbrecht et al., 2006)
pKD3	Template for mutagenic PCR products	(Datsenko and Wanner, 2000)
PKD46	Lambda Red mediated mutagenesis	(Datsenko and Wanner, 2000)
pCMV	Mammalian expression vector with HA-tag	(Gao et al., 2009)
pET28a-FliC	FliC in pET28a	This study
pT7HMT-FliC	FliC in pT7HMT	This study
pT7HMT-FliC (176-395)	FliC (176-395) in pT7HMT	This study
pT7HMT-FliC (1-395)	FliC (1-395) in pT7HMT	This study
pT7HMT-FliC (176-488)	FliC (176-488) in pT7HMT	This study
pCMV-FliC (1-487)	FliC (1-487) in pCMV	This study
pCMV-FliC (176-395)	FliC (176-395) in pCMV	This study
pCMV-FliC (1-395)	FliC (1-395) in pCMV	This study
pCMV-FliC (176-487)	FliC (176-487) in pCMV	This study
pCMV-FliC (2-395)	FliC (2-395) in pCMV	This study
pCMV-FliC (2-487)	FliC (2-487) in pCMV	This study

Preparation of ETEC H10407 supernatant. ETEC supernatants were prepared by using either M9 minimal media (1 x M9 salts, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.4 % glucose) or RPMI 1640

medium described previously. The cell-free supernatant was concentrated by using Centricon Plus-70 Centrifugal Filter (EMD Millipore) with a membrane NMWL of 3 kDa and was subsequently subjected to acetone precipitation at -20 °C overnight. Protein pellets were collected by centrifugation at 15 000 x g, 4 °C for 10 min, and re-suspended into 0.15 M NaCl for further fast protein liquid chromatography analysis.

Fast Protein Liquid Chromatography and Mass Spectrometry. ETEC H10407 supernatants were fractionated using a GE Life Science AKTA Fast Protein Liquid Chromatography (FPLC) system to facilitate molecular characterization of ESF. Briefly, acetone-precipitated supernatant from M9-grown bacterial culture was resuspended in 10 ml 0.15 M NaCl, clarified by 0.22 µm filtration, and applied at 4 ml/min to a Superdex S200 26/60 column (GE Life Sciences) that had previously been equilibrated in 20 mM tris (pH 8.0), 200 mM NaCl. The ESF-containing eluent was collected from the column between 110-130 ml, and dialyzed against 4 l of 20 mM tris (pH 8.0) in preparation for further chromatography. The ESF-containing sample was applied to a 1 ml Resource Q anion exchange column (GE Life Sciences). The column was washed with a buffer of 20 mM tris (pH 8.0) until the OD_{280} value reach baseline, then the bound proteins were eluted with a gradient to 1 M NaCl in the same buffer. Fractions of 1 ml were collected, evaluated for their ability to prevent IκBα degradation in response to TNF, separated by SDS-PAGE, and detected by Silver Staining (Thermo Scientific). Proteins from active fractions were excised and digested in-gel with trypsin (Promega). Proteins were identified using mass spectrometry at the Mass Spectrometry & Analytical Proteomics Laboratory, University of Kansas.

Construction of ETEC H10407 mutants. ETEC were generated using the Lambda Red Recombinase system (Datsenko and Wanner, 2000). PCR products containing chloramphenicol resistance cassettes were amplified from plasmid pKD3 using primers (**Table 5**) flanked with

homologous upstream and downstream gene sequences. PCR products were transformed into ETEC H10407 carrying the pKD46 plasmid by using electroporation. Potential mutants were screened on LB plates containing chloramphenicol. All mutants were confirmed using DNA sequencing. ETEC H10407 *fliC* was also expressed from pFLAG-CTC for complementation studies.

Recombinant protein expression and purification. Recombinant protein expression was induced with 1 mM IPTG, 37 °C for 5 h. Recombinant proteins were purified by using nickel-affinity chromatography and subsequently dialyzed into PBS. Purified proteins were analyzed on 10 % SDS-PAGE and concentrations were quantified using the Bradford method.

Transfection. HCT-8 cells were seeded in 6-well plates at a concentration of 2 x 10^5 cells/well, grown to 70-80 % confluence, and transfected with 2.5 µg plasmid DNA using Lipofectamine 3000 (Life Technology). Transfected cells were stimulated with TNFα (20 ng/ml) at 12-48 h post-transfection.

Immunoblotting. HCT-8 cell pellets were resuspended in 10.0 mM HEPES, 1.5 mM $MgCl_2$, 10.0 mM KCl, 0.5 mM DTT, 0.05 % (v/v) NP-40 containing protease inhibitor cocktails (Thermo Scientific) and incubated on ice for 30 min. Lysates were centrifuged (10,000 x g, 4 °C, 10 min) and the supernatant was collected. Immunoblotting was carried out as previously described (Wang and Hardwidge, 2012) by separating proteins using 10 % SDS-PAGE and then transferring the proteins to nitrocellulose blotting membranes (GE Healthcare Life Sciences). Membranes were blocked in Odyssey blocking buffer (Li-Cor) at room temperature for 1 h, and then incubated with appropriate primary and secondary primary antibodies. Immunoblots were developed using the Odyssey infrared imaging system (Li-Cor). Tubulin abundance was used to normalize IκBα abundance.

Statistical analysis. For all quantitative data, tubulin immunoblotting was used to normalize IκBα abundance. The data represent at least 3 independent experiments and were analyzed using one-way ANOVA with the Dunnett's multiple comparisons test. Asterisks indicate significantly different ($p < 0.05$) IκBα abundance as compared with the 'TNF only' lane.

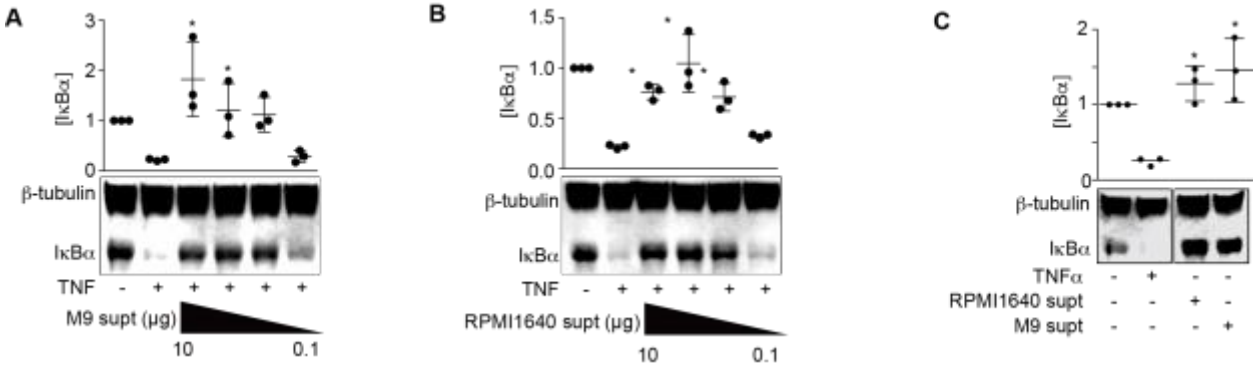
Table 5 Oligonucleotides used in Chapter 2.

Primer	Purpose	Sequence (5' - 3')
PRH-3427	Delete ETEC H10407 *fliD*	$A_2T_2GC_2GATA_2C_3GCT_2ATCTACTGT_3GCA_2TC$ $A_4G_2A_2T_2AG_2TGTGTA2CTG_2AGCTGCT_2C$
PRH-3428	Delete ETEC H10407 *fliD*	$T_2GTGCATAG_2CT_4GAGC_2GCTCGCG_2TATAC$ $ATGCTGAC_2TC_2GTGA_2TG_3A_2T_2AGC_2ATG_2TC_2$
PRH-3429	Verify *fliD* deletion	$TCTCTC_2TGT_6CT_2A_2CG_2CT$
PRH-3430	Verify *fliD* deletion	$GCTGAT_2GT_2GTC_2TGCATA_3CA$
PRH-3431	Delete ETEC H10407 *fliC*	$CGTG_3CA_2CAGC_3A_2TA_2CATCA_2GT_2GTA_2T_2GA$ $TA_2G_2A_4GATCGTGTAG_2CTG_2AGCTGCT_2C$
PRH-3432	Delete ETEC H10407 *fliC*	$GCG_3CAGA_6C_4GC_2G_2TG2CG_5T_2GAGCGA$ $TA_2GTGTA_4TG_3A_2T_2AGC_2ATG_2TC_2$
PRH-3433	Verify *fliC* deletion	$ATGATGCGCAGAGTAGAGT_2GTAT$
PRH-3434	Verify *fliC* deletion	$ATGAT_2ATC_2GT_3CTGCAG_3T_2$
PRH-3619	Clone *fliC* pCMV-XhoI	$TAC_2GCTCGAGATG_2CACA_2GTCAT_2A_2TA$
PRH-3620	Clone *fliC* pCMV-NotI	$ATA_2GA_2TGCG_2C_2GCACGCAGCA(GA)_2CAGTA$
PRH-3681	Clone *fliC* pET28a-Nde I	$GGA_2T_2C_2ATATG_2CACA_2GTCAT_2A_2TACA$
PRH-3682	Clone *fliC* pET28a-XhoI	$TAC_2GCTCGAGACGCAGCAGAGACAGTA$
PRH-3684	Clone *fliC* pFLAG-CTC-XhoI	$TAC_2GCTCGAG_2CACA_2GTCAT_2A_2TA$
PRH-3685	Clone *fliC* pFLAG-CTC-BglII	$G_2A_2GATCTACGCAGCAGAGACAGTA$
PRH-3788	Clone *fliC* 176-395 pCMV-XhoI	$TATAT_2ACTCGAG_2ATG_2CGCGCAGA_3GCA_2$
PRH-3789	Clone *fliC* 176-395 pCMV-NotI	$ATA_2GA_2TGCG_2C_2GCT_2GCA_2CGAT_4$
PRH-3543	Clone *fliC* pT7HMT-BamHI	$TACGCG_2ATC_2ATG_2CACA_2GTCAT_2A_2TACA_2$
PRH-3844	Clone *fliC* pT7HMT-NotI	$ATA_2GAT_2GCG_2C_2GCT_2A_2CGCAGCAGAGA$
PRH-3845	Clone *fliC* 176-395 pT7HMT-BamHI	$TACGCG_2ATC_2GATG_2CGCGCAGA_3$
PRH-3851	Clone *fliC* 176-395 pT7HMT-NotI	$ATA_2GAT_2GCGGC_2GCTCAT_2GCA_2CGAT_4$
PRH-3971	Clone *fliC* pCMV-XhoI	$TAC_2GCTCGAG_2CACA_2GTCAT_2A_2TACA_3CAGC_2$

Results

ETEC H10407 secretes ESF into M9 minimal media. We previously found that ETEC H10407 secretes a heat-stable protein (ESF; ETEC Secreted Factor) into RPMI 1640 medium that subsequently inhibits the ability of TNF to activate NF-κB signaling (Wang and Hardwidge, 2012). To facilitate identification of this protein using biochemical fractionation, we first determined whether this factor was also secreted into ETEC supernatants when ETEC was grown in M9 minimal medium. We incubated HCT-8 cells with either cell-free ETEC-M9 or ETEC-RPMI 1640 supernatants for 1.5 h and then treated the cells with TNFα (20 ng/ml, 20 min). Pre-incubating HCT-8 cells with ETEC-M9 supernatant significantly inhibited the degradation of IκBα in response to TNFα stimulation (**Figure 3A**), similar to the results obtained from pretreating HCT-8 cells with ETEC-RPMI 1640 supernatant (**Figure 3B**). Degradation of IκBα was not observed in cells pre-incubated with only ETEC-RPMI 1640 or ETEC-M9 supernatants for 1.5 h in the absence of TNF (**Figure 3C**). These data indicated that ETEC H10407 also secretes *ESF* into M9 minimal media.

Figure 3 ETEC H10407 secretes ESF into M9 minimal media.



Identification of ESF by using FPLC and Mass Spectrometry. We next used FPLC (Robinson et al., 2017) to fractionate ETEC-M9 supernatants and then assayed the fractions for their ability to block TNF-induced NF-κB activation. Two fractions (**Figure 4A**, fractions E and F) inhibited TNF-induced NF-κB activation in HCT-8 cells and silver staining showed that these two fractions had similar protein composition (**Figure 4B**). We excised these bands and identified the proteins using mass spectrometry (**Table 6**). We identified a major outer membrane lipoprotein, outer membrane protein A, the flagellar hook-associated protein FliD, and flagellin (FliC). *E. coli* K-12 strains do not encode the ESF (Wang and Hardwidge, 2012). The two outer membrane proteins are highly conserved between ETEC and *E. coli* K-12, but FliC and FliD are not (~50 % identity). We therefore focused on FliC and FliD for subsequent biochemical assays.

Figure 4 Preparation and visualization of ESF-containing fractions by using FPLC.

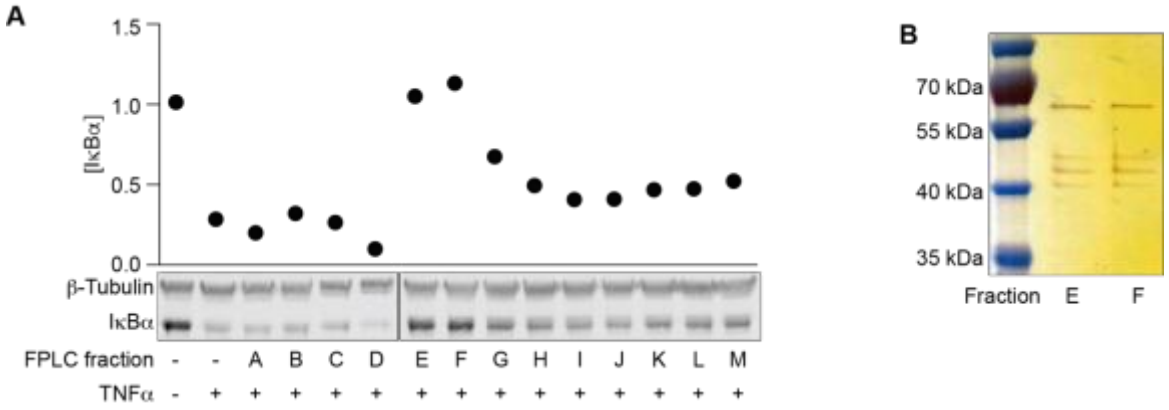
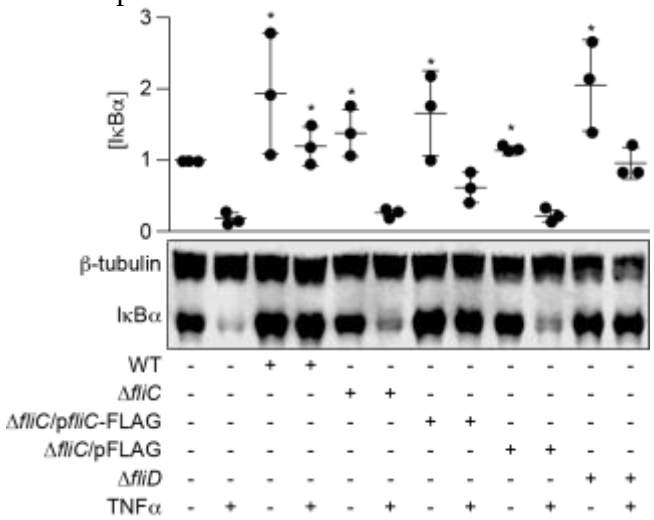


Table 6 Mass spectrometry results.

ESF Candidates	Identity to E. coli MG1655	GenBank Accession #
Major outer membrane lipoprotein	100 %	CBJ00536.1
Outer membrane protein A	99 %	CBJ01214.1
Flagellar hook-associated protein 2 (FliD)	50 %	CBJ01536.1
Flagellin (FliC)	52 %	CBJ01535.1

ETEC flagellin blocks TNF-induced IκBα degradation. To examine whether FliC and/or FliD prevent HCT-8 cells from TNF-induced NF-κB activation, we generated ETEC H10407 *fliC* and *fliD* mutants and prepared cell-free supernatants from these mutant strains in RPMI 1640. ETEC Δ*fliC* supernatants failed to block TNF-induced IκB degradation (**Figure 5**). Complementing ETEC Δ*fliC* with a *fliC* expression plasmid restored the protective phenotype (**Figure 5**) By contrast, the ETEC Δ*fliD* supernatant behaved similarly to the WT ETEC supernatant (**Figure 5**). We therefore concluded that *fliC* expression was necessary for the ESF phenotype.

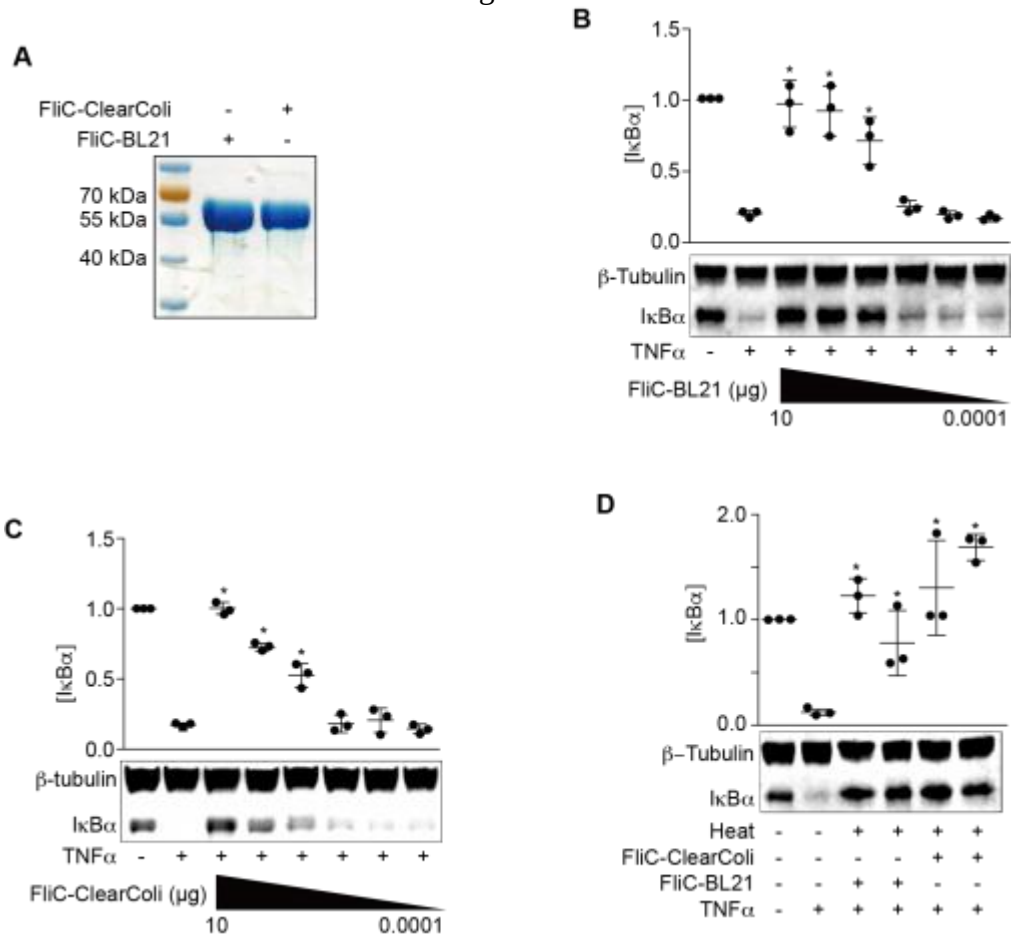
Figure 5 ETEC *ΔfliC* mutant supernatant failed to inhibit TNF-induced IκBα degradation.



We next determined whether recombinant FliC is sufficient to account for the ESF phenotype, we expressed ETEC FliC in *E. coli* BL21(DE3) and purified the recombinant protein using Ni-NTA chromatography (**Figure 6A**). Addition of FliC blocked IκBα degradation in response to TNF (**Figure 6B**). To eliminate the potential impact of LPS contamination, we also expressed and purified FliC from *E. coli* ClearColi 21(DE3). This strain produces a modified form of LPS that does not trigger endotoxic responses in human cells (Planesse et al., 2015). Pre-incubating HCT-

8 cells with FliC purified from ClearColi also protected IκBα from TNF-induced degradation (**Figure 6C**). The protective phenotype mediated by FliC was also heat-stable (**Figure 6D**), consistent with our previous data (Wang and Hardwidge, 2012).

Figure 6 ETEC FliC blocked TNF-induced degradation of IκBα.



FliC domain mapping. The function of flagellin is broadly conserved among all flagellated bacteria. Nevertheless, outside the N- and C-terminal regions that comprise its intramolecular coiled-coil, the flagellin (Fli) protein itself exhibits localized sequence/structure variability between otherwise closely related bacteria. For example, whereas the central region of *Salmonell*a FliC contains two distinct ~100 residue domains (Maki-Yonekura et al., 2010), sequence analyses

suggest that ETEC FliC contains only a single, fused central domain consisting of residues 176-395. Importantly, residues within this central region of *E. coli* flagellin comprise variable H serotype-specific epitopes (Haiko and Westerlund-Wikstrom, 2013), suggesting that these sequences could impart strain-specific activities to the FliC protein. To investigate if a specific FliC subdomain might account for the IκBα protective phenotype, we overexpressed and purified three truncated FliC proteins designated as FliC(1-395), FliC(176-395), and FliC(176-488) (**Figure 7A**). These proteins were expressed from pT7HMT to facilitate removal of the N-terminal His-tag using TEV protease. While the presence of a His-tag did not affect the activity of full-length FliC in blocking IκBα degradation in response to TNF (**Figure 7B**), none of the truncated FliC proteins were active. As a control, both tagged- and untagged-FliC were heat-stable, consistent with the heat-stability of the ESF we described previously [**Figure 7C**, (Wang and Hardwidge, 2012)].

Transfecting fliC expression plasmids does not block TNF-induced IκBα degradation. We next sought to determine whether mammalian expression of FliC would be sufficient to block TNF-induced IκBα degradation. We expressed FliC fragments from a mammalian cell expression vector and transfected these plasmids into HCT-8 cells (**Figure 8A**). These constructs were not toxic to HCT-8 cells (**Figure 8B**), but they were unable to block TNF-induced IκBα degradation (**Figure 8C**).

Figure 7 Only full length of ETEC FliC inhibited TNF-induced degradation of IκBα.

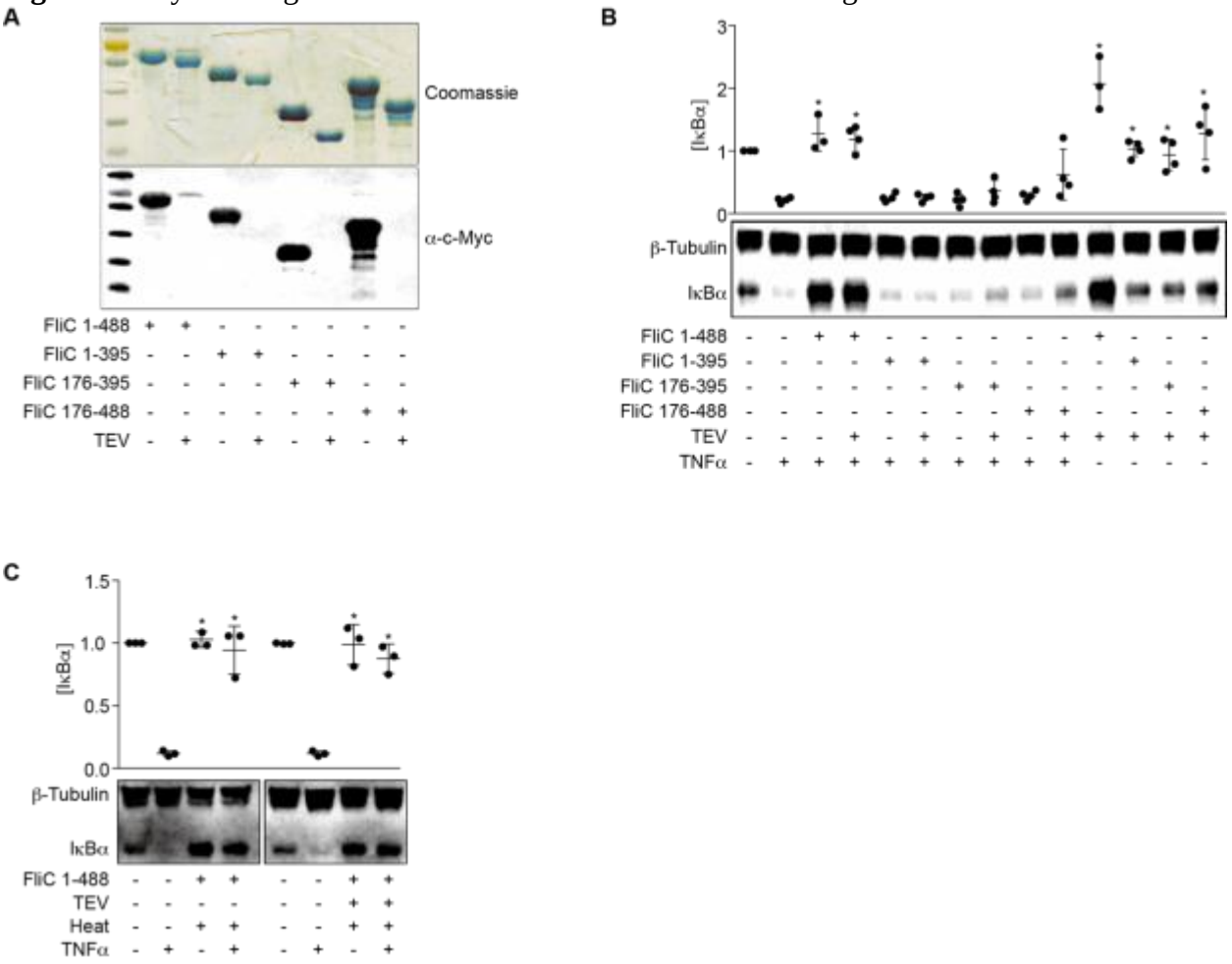
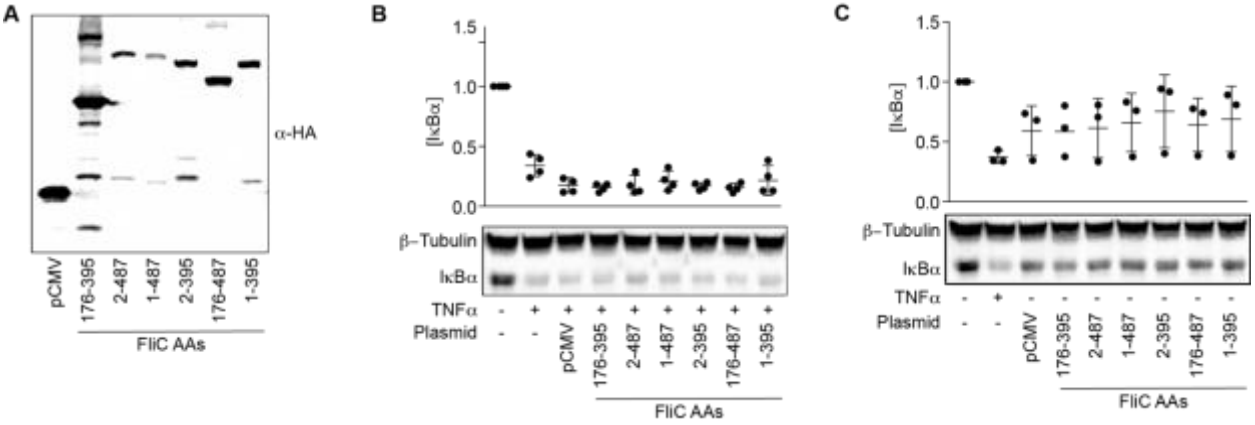


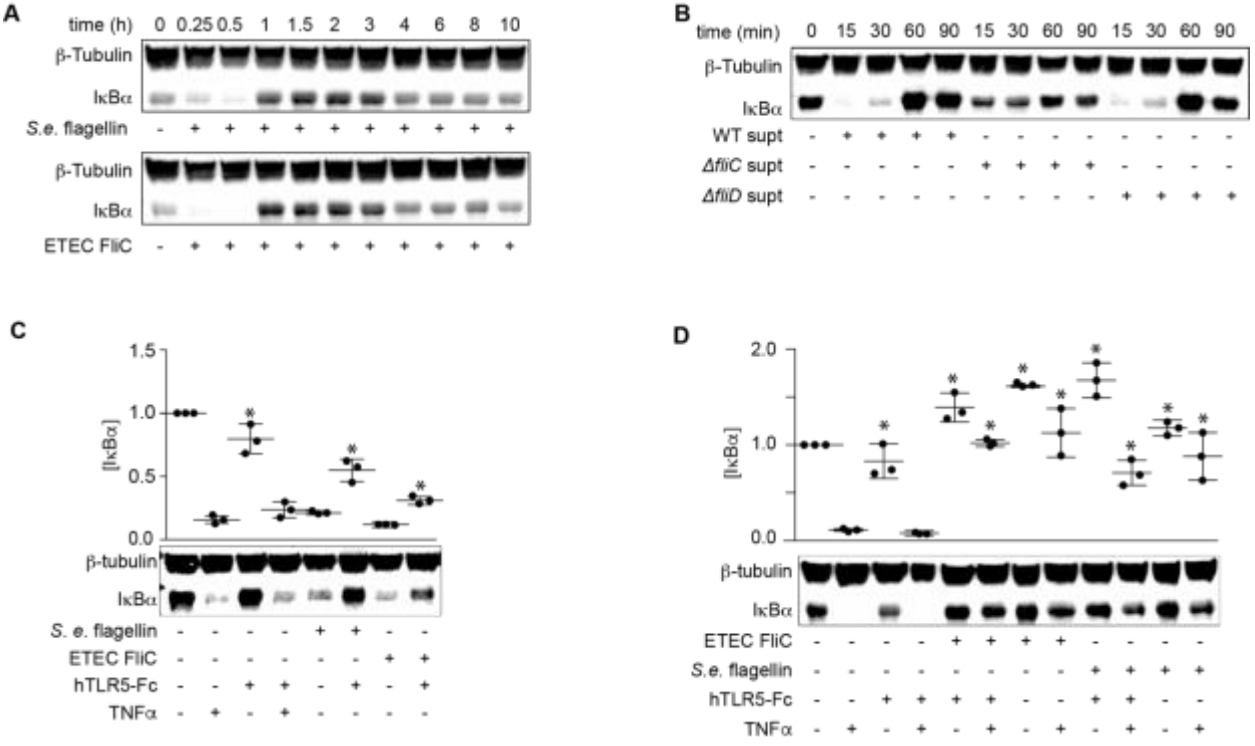
Figure 8 Expression of ETEC FliC in mammalian cells failed to block TNF-induced degradation of IκBα.



ETEC FliC inhibits IκBα degradation independently of TLR5. Toll-like receptor 5 (TLR5) recognizes bacterial flagellin (Hayashi et al., 2001), resulting NF-κB pathway activation. Because ETEC FliC subverts TNF-induced IκBα degradation, we speculated that ETEC FliC might somehow escape TLR5 recognition. To test our hypothesis, we first incubated HCT-8 cells with recombinant ETEC FliC protein (100 ng/ml) for up to 10 h. Immunoblotting results revealed that IκBα degradation was observed at the early phase of incubation (15 and 30 min) and the total IκBα returned to normal level after 1 h incubation (**Figure 9A**), indicating that ETEC H10407 FliC was unable to abrogate TLR5 recognition. Meanwhile, we incubated HCT-8 cells with ETEC H10407 wildtype and mutants (*ΔfliC* and *ΔfliD*) supernatants and found that ETEC H10407 *ΔfliC* supernatant failed to induce IκBα degradation (**Figure 9B**). Since ETEC FliC was confirmed to be a ligand for TLR5, we speculated whether TLR5 was involved in FliC-mediated inhibition of IκBα degradation in response to TNF treatment. We used TLR5 antagonist, hTLR5-Fc, to block the activity of TLR5. We first pre-incubated HCT-8 cells with hTLR5-Fc (1.5μg/ml, 60 min), followed by addition of recombinant ETEC FliC (100 ng/ml, 20 min). Results suggested that hTLR5-Fc was able to block IκBα degradation in response to ETEC FliC stimulation by inhibiting

TLR5 function (**Figure 9C**). Next, HCT-8 cells were first subjected to incubation with hTLR5-Fc (1.5μg/ml, 60 min) and subsequently addition of ETEC FliC (100 ng/ml, 90 min) followed by TNF stimulation (20 ng/ml, 20 min). Immunoblotting results suggested that the degradation of IκBα was inhibited in both presence of and absence of hTLR5-Fc (**Figure 9D**). Taken together, ETEC H10407 is able to subvert TNF-induced NF-κB activation in a TLR5-independent manner.

Figure 9 ETEC FliC-mediated blockage of TNF-induced degradation of IκBα was independent of TLR5.



Discussion

Here we identified ETEC FliC as necessary and sufficient to inhibit IκBα degradation in response to TNF stimulation. Surprisingly, blocking TLR5 recognition of flagellin did not eliminate this phenotype, suggesting a potential TLR5-independent mechanism of action. We were unable to define the FliC domain responsible for this activity, despite truncating the protein into soluble fragments that comprised the variable central domain. We also observed that endogenous

expression of FliC in mammalian cells did not reconstitute this phenotype, suggesting a potential TLR5-independent interaction partner for FliC.

TNF-TNFR1-mediated NF-κB activation occurs on the cell surface and is associated with lipid rafts (Li and Lin, 2008). Cationic cell-penetrating peptides can disrupt TNF-mediated NF-κB signaling by inducing TNF receptor internalization in a clathrin-dependent manner (Fotin-Mleczek et al., 2005b). The mechanism of FliC-mediated inhibition of IκBα degradation in response to TNF might be associated with TNFR internalization, as we had previously observed that blocking clathrin-dependent endocytosis affected the activity of the ETEC secreted factor (ESF; (Wang and Hardwidge, 2012)). Future characterization of the phenotype we report here has the potential to provide insight in the development of anti-inflammatory compounds that target NF-κB, an approach that might prove efficacious in prevention on development of cancers (Zhang et al., 2017) or other inflammatory diseases.

Chapter 3 - Identification of cyclic AMP importer in Enterotoxigenic *Escherichia coli*

Introduction

Enterotoxigenic *Escherichia coli* (ETEC) is the most common causative agent of childhood diarrhea in developing countries and also accounts for the principal etiology of travelers' diarrhea (Croxen et al., 2013). The pathogenesis of ETEC is associated with production of colonization factors (Kharat et al., 2017) and with heat-labile enterotoxin (LT) and/or heat-stable enterotoxin (ST) (Fleckenstein et al., 2010).

Attachment to IECs is a crucial step to colonize small intestine for ETEC infection, which is mainly mediated by production of colonization factors (Kharat et al., 2017). Currently, at least 23 CFs have been described and most of the CFs are encoded on plasmids (Kharat et al., 2017). Attachment of ETEC strains to IECs is achieved through binding of CFs to different receptors on host cells. For example, coli surface antigen 6 (CS6) has been reported to bind fibronectin and sulfatide, and colonization factor antigen I (CFA/I) was documented to bind to glycoproteins and glycosphingolipids located on the surface of host cells in the small intestine (Pereira and Giugliano, 2013). Upon binding to IECs, ETEC strains produce LT and ST causing watery diarrhea (Fleckenstein et al., 2010). The mechanism of LT- and ST-induced diarrhea has been described in Chapter 1.

Cyclic AMP (cAMP) is an important secondary messenger. In *E. coli* and other bacteria, cAMP controls gene expression, which occurs through cAMP receptor protein (CRP), which has been reviewed in Chapter 1. In ETEC strains, LT-positive ETEC strains are more adherent to mammalian cells *in vitro* study than LT-negative STEC strains (Allen et al., 2006; Johnson et al.,

2009). Our preliminary data suggested ETEC strains can utilize exogenous cAMP and stimulated the adherence of LT-negative ETEC strains to mammalian cells. Taken together, these studies suggested that cAMP functions as a soluble, adherence-promoting factor upon its secretion from intoxicated epithelial cells. Given that LT-induced release of cAMP into intestinal lumen, we reasonably hypothesize that there is a higher affinity importer for extracellular cAMP.

In this study, we employed a Tn5-mediated mutagenesis approach that was a powerful tool to manipulate bacteria genome by randomly inserting Tn5 transposon into chromosome of living bacteria (Reznikoff, 2008) to study the cAMP importer system in ETEC. Through construction of mutant library by using ETEC H10407 *ΔcyaA* mutant as a parent strain, we aimed to screen for the phenotype that ETEC H10407 *ΔcyaA* mutant carrying a Tn5 insertion in the genome was unable to utilize maltose on maltose MacConkey indicator agar supplemented cAMP.

Materials and Methods

Reagents. Antibiotics, maltose monohydrate and MacConkey agar base used in this study were obtained from Fisher Scientific. The restriction enzymes and T4 ligase were purchased from New England BioLabs. Adenosine 3',5'-cyclic monophosphate tris salt was from Sigma-Aldrich. Gel/PCR DNA Fragment Extraction Kit, Hi-Speed Mini Plasmid Kit, Super optimal broth (SOB) and Luria-Bertani (LB) broth were purchased from MidSci. EZ-Tn5™ <KAN-2>Tnp Transposome™ Kit and EZ-Tn5™ <R6K*γori*/KAN-2>Tnp Transposome™ Kit were obtained from Lucigen. Masterpure™ Complete DNA and RNA Purification Kit was obtained from Epicentre.

Bacterial strains, oligonucleotides and plasmids. Bacterial strains and plasmids were described in Table 1. ETEC H10407 *ΔcyaA* mutant derived from ETEC H10407 strain was used to prepare Tn-5-mediated mutant library. TransforMax EC100D pir-116 electrocompetent *E. coli* (Lucigen) was used to identify the disrupted gene in Tn-5-mediated mutants. Plasmids pKD3 and pKD46 were

used to prepare ETEC H10407 mutants. All bacterial strains except pKD46 were aerobically maintained in LB broth or on LB agar plate with antibiotics at 37 °C. Bacterial strain harboring pCP20and pKD46 was grown at 30 °C.

Table 7 Bacterial strains and plasmids used in Chapter 3.

Strains and Plasmids	Description	References
ETEC H10407 *ΔcyaA*	ETEC H10407 lacks AC	This study
ETEC H10407 *Δcrp*	ETEC H10407 lacks CRP	This study
E. coli K-12 *ΔcyaA*	*E. coli* K-12 lacks AC	(Brickman et al., 1973)
E. coli K-12 *Δcrp*	*E. coli* K-12 lacks CRP	(Brickman et al., 1973)
E. coli pir-116	*E. coli* strain contains *pir* gene	Lucigen
pKD3	Template for mutagenic PCR products	(Datsenko and Wanner, 2000)
pKD46	Lambda Red mediated mutagenesis	(Datsenko and Wanner, 2000)
pCP20	plasmid encodes FLP recombinase	(Datsenko and Wanner, 2000)

Preparation of maltose MacConkey indicator agar. Maltose MacConkey indicator agar was MacConkey agar base supplemented with 1 % (w/w) maltose monohydrate, 50 µg/ml kanamycin, and 50 µM adenosine 3',5'-cyclic monophosphate tris salt. ETEC mutants that cannot ferment maltose form white/colorless colonies on maltose MacConkey indicator agar.

Genomic DNA extraction. Extraction of genomic DNA was performed by using Masterpure™ Complete DNA Purification Kit following the manufacturer's instructions. The concentration of genomic DNA was determined using a NanoDrop 2000 Spectrophotometer (Fisher Scientific).

Transposon mutagenesis. Tn5 transposon was randomly introduced into the chromosome of ETEC H10407 *ΔcyaA* mutant by using EZ-Tn5™ <KAN-2>Tnp Transposome Kit and EZ-Tn5™ <R6K*γori*/KAN-2>Tnp Transposome Kit (Lucigen) following the manufacturer's instructions. Both Tn5 transposons harbor a broad host-range kanamycin resistance marker contributing to

selectively isolate Tn5-associated mutants. The only difference is that the EZ-Tn5™ <R6Kγ*ori*/KAN-2>Tnp Transposome carries an *E. coli* conditional origin of replication termed R6Kγ*ori*, which contributes to map the disrupted genes.

Electroporation competent cells preparation. A single colony of ETEC H10407 *ΔcyaA* mutant without antibiotic resistance gene grown on LB agar was inoculated into 5 ml LB broth and aerobically cultured with shaking (200 rpm/min) overnight at 37 °C. The cultures were diluted (1:100) into 100 ml LB broth and grown to an OD_{600} of 0.6 with shaking. Cells were harvested by centrifugation at 3,000 x g for 10 min at 4 °C and washed 3 times with 20 ml of ice-cold 10 % glycerol. Cells were finally resuspended into 1 ml of ice-cold 10 % glycerol and 100 µl aliquots were used for electroporation.

Electroporation conditions. Transposome (1 µl) was mixed gently with 100 µl competent cells in a 0.2 cm pre-chilled electroporation cuvette and incubated on ice for 5 min. Electroporation was carried out using a BioRad Gene Pulser (200 Ohms, 25 µF and 2.5 kV). After electroporation, 900 µl of pre-warmed SOC broth (SOB broth containing 20 % glucose) was immediately added and mixed gently. The electroporated cells were transferred to a sterile tube and incubated at 37 °C with shaking for 60 min.

Mutants harboring Tn5 transposon. The entire electroporated cells were diluted 1:10 and 50 microliters (50 µl) aliquots were plated on maltose-MacConkey agar plate supplemented with 50 µg/ml kanamycin and 50 µM adenosine 3',5'-cyclic monophosphate tris salt and incubated aerobically for 24 h at 37 °C. The mutants that were unable to utilize maltose were selected for mapping the disrupted genes.

Identification of transposon-disrupted genes. Among of the mutants carrying Tn5 transposon, colorless colonies (inability to ferment maltose) on maltose-MacConkey agar plate complemented with 50 μg/ml kanamycin and 50 μM adenosine 3',5'-cyclic monophosphate tris salt were selected. For identification of the disrupted genes from the mutants, two approaches were employed based on the commercial transposome kits used in this study.

(i). EZ-Tn5™ <KAN-2>Tnp Transposome Kit. Identification of the Tn5-associated disrupted gene was carried out by using RE-mediated inverse PCR. Briefly, five micrograms (5 μg) genomic DNA were digested using two restriction enzymes overnight at 37 °C, respectively. Subsequently, the digested genomic DNA fragments were self-ligated by using T4 ligase in a large reaction volume at 16 °C overnight to avoid cross-ligation. Next, we performed PCR in a 50 μl reaction volume by using self-ligated DNA as template, a forward primer (5'-ATGGCTCATAACACCCCTTGTATTA-3') and a reverse primer (5'-GAACTTTTGCTGAGTTGAAGGATCA-3') under the following conditions: 95 °C for 3 min, followed by 35 cycles of 30 s at 95 °C, 30 s at 53 °C, and 3 min at 72 °C and by a final extension at 72 °C for 10 min. PCR products were analyzed on 1.2 % agarose gels and gel-purified. Purified PCR products were submitted to Molecular Cloning Laboratories (CA, United States) for sequencing by using a forward primer (5'-ACCTACAACAAAGCTCTCATCAACC-3') and a reverse primer (5'-GCAATGTAACATCAGAGATTTTGAG-3').

(ii). EZ-Tn5™ <R6K*γori*/KAN-2>Tnp Transposome Kit. Identification of the Tn5-associated disrupted gene was carried out following the manufacturer's instructions. Briefly, five micrograms (5 μg) of genomic DNA were digested using restriction enzymes overnight at 37 °C. Subsequently, the digested genomic DNA fragments were subjected to self-ligation by using T4 ligase in a large reaction volume in order to avoid cross-ligation. Next, the self-ligated DNA fragments were

transformed into TransforMax EC100D pir-116 electrocompetent *E. coli* using electroporation followed by screening for the Tn5 transposon on LB agar containing kanamycin (50 µg/ml) overnight at 37 °C. Plasmids were extracted from the positive colony (defined by presence of kanamycin resistance cassettes) and were submitted to Molecular Cloning Laboratories (CA, United States) for sequencing by using a forward primer (5'-ACCTACAACAAAGCTCTCATCAACC-3') and a reverse primer (5'-CTACCCTGTGGAACACCTACATCT-3')

Preparation of electrocompetent cells containing pKD46. Plasmid pKD46 was transformed into ETEC H10407 *ΔcyaA* mutant by using electroporation and pKD46-containing colonies were identified on LB plate supplemented with ampicillin (100 µg/ml) at 30 °C. When ETEC H10407 *ΔcyaA* mutant containing pKD46 was grown to OD_{600} of 0.1 at 30 °C with shaking, L-arabinose was added to a concentration of 10 mM to induce pKD46 lambda-red expression. When OD_{600} reached to 0.4, the bacterial cells were collected (4,000 x *g*, 10 min, 4 °C), washed 3 times with 10 % ice-cold glycerol, and resuspended in 10 % ice-cold glycerol for subsequent electroporation.

Construction of mutants. Through bioinformatics analysis of the genome of ETEC H10407 strain, 10 potential genes (**Table 8**) that might serve as high affinity transporter for extracellular cAMP were selected for preparing mutants by using ETEC H10407 *ΔcyaA* mutant as parent strain. Mutants were generated using the Lambda Red Recombinase system (Datsenko and Wanner, 2000). Briefly, PCR products containing chloramphenicol resistance cassettes were amplified from plasmid pKD3 using primers **(Table 9)** flanked with homologous upstream and downstream gene sequences. PCR products were transformed into ETEC H10407 *ΔcyaA* mutant carrying the pKD46 plasmid by using electroporation. Potential mutants were screened on LB plates supplemented

with chloramphenicol. All mutants were verified using DNA sequencing and the primers used were listed in **Table 9** and **Table 10**.

Sequence analysis. Sequence data was analyzed by using DNASTAR software. BLAST searching of disrupted genes in ETEC H10407 were carried out at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST).

Table 8 Genes for mutation in Chapter 3.

Protein	Gene
CBJ01581, ABC-transporter	ETEC-2078
CBJ01738, putative ribitol transporter	ETEC-2235
CBJ02675, putative AMP binding protein	ETEC-3175
CBJ02680, putative membrane protein	ETEC-3180
CBJ02696, major facilitator superfamily protein	ETEC-3196
CBJ02754, putative permease	ETEC-3253
CBJ03427, putative transmembrane HD family hydrolase	ETEC-3920
CBJ03653, UPF721 transmembrane protein	ETEC-4148
CBJ03956, inner membrane protein	ETEC-4447
CBJ04059, predicted transporter	ETEC-4550

Table 9 Oligonucleotides used for preparation of mutants in Chapter 3.

Primer	Purpose	Sequence (5' – 3')
PRH3307	ETEC_2078	GC$_2$GT$_2$GATACAG$_2$C$_2$GC$_3$TGCGC$_2$A$_2$CAGT$_2$GCGT
	mutation F	ATG$_2$T$_2$C$_4$AT$_3$GTGTAG$_2$CTG$_2$AGCTGCT$_2$CG
PRH3308	ETEC_2078	TC$_2$T$_5$CTG$_2$CGTG$_2$AGC$_2$TGCGTGACAT$_2$CGCGCG
	mutation R	ACGC$_2$TGACGCATATGA$_2$TATC$_2$TC$_2$T$_2$AG
PRH3309	ETEC_2235	T$_3$CGA$_2$CTCGCAT$_2$CT$_2$ATCGCACTATAT$_2$A$_3$TCG
	mutation F	T$_2$AG$_2$CT$_2$CACACTGTGTAG$_2$CTG$_2$AGCTGCT$_2$CG
PRH3310	ETEC_2235	ACG$_2$TATA$_2$CAC$_2$ACG$_2$CA$_2$T$_2$GC$_2$G$_2$AGC$_2$AG$_2$A$_2$G
	mutation R	T$_2$AGACATAC$_2$ATATGA$_2$TATC$_2$TC$_2$T$_2$AG
PRH3311	ETEC_2078	TCCACCACCTCTTCCTGTG
	verification F	
PRH3312	ETEC_2078	CAATACTGAATCGCTGTCCC
	verification R	
PRH3313	ETEC_2235	GTTGGGTTTGCCACTGCA
	verification F	
PRH3314	ETEC_2235	GCTGCTCAACGCGAATGA
	verification R	
PRH3339	ETEC_3175	AG$_2$T$_3$C$_2$TGACTGCAG$_4$A$_2$TG$_2$CTGATACGTCGTA$_2$GA
	mutation F	TGATCA$_3$CG$_2$A$_4$CAC$_2$T(GT)$_2$AG$_2$CTG$_2$AGCTGCT$_2$CG
PRH3340	ETEC_3175	ATCG3TCA2G2(TA)$_2$GA$_2$CGAT$_3$C$_2$ATCTC$_2$TGCG$_2$T$_2$G
	mutation R	A$_2$C$_2$AGATG$_2$CGCTCA$_2$T$_2$CATATGA$_2$TATC$_2$TC$_2$T$_2$AG
PRH3341	ETEC_3180	C$_2$AC$_3$AGTA$_3$CGC$_4$GCGCTGT$_2$ATG$_5$CTAGTCTGC$_2$TG$_2$
	mutation F	TCATG$_2$CG$_2$CG$_2$CGTGTGTAG$_2$CTG$_2$AGCTGCT$_2$CG
PRH3342	ETEC_3180	TACAGC$_2$G$_2$TGAG$_2$ATA$_2$GCGTAGCG$_2$TCAG$_3$CGA$_2$CG
	mutation R	CTCGC$_2$AGA$_2$TGTG$_2$AT$_3$GAC(AT)$_2$GA$_2$TATC$_2$TC$_2$T$_2$AG
PRH3343	ETEC_3196	GAG$_2$T$_3$CTCGC$_2$A$_5$GCGC$_3$GC$_3$ACA$_3$T$_2$A$_2$TACGCGTACT
	mutation F	CATA$_2$CG$_2$T$_2$CTC$_2$TGTGTAG$_2$CTG$_2$AGCTGCT$_2$CG
PRH3344	ETEC_3196	CA$_2$T$_3$A$_2$TGA$_2$T$_5$A$_2$C$_2$T$_2$A$_3$CGA$_2$TA$_2$TCATC$_2$TGCACAG
	mutation R	T$_2$A$_3$G$_2$TAGCACTCATATGA$_2$TATC$_2$TC$_2$T$_2$AG
PRH3345	ETEC_3253	AG$_2$CAT$_3$GCTA(CT)2GA2GCAGTA2(TG)$_2$AGC3GC2GTG$_2$
	mutation F	TGT2GCGC2ACG2CG3T2(GT)$_2$AG$_2$CTG$_2$AGCTGCT$_2$CG
PRH3346	ETEC_3253	TG$_2$CG$_2$TGACGTCTGC$_2$GTACGCTG$_2$TG$_2$CTA$_2$CG$_2$CG
	mutation R	CGAT$_2$C$_4$TCAT$_2$C$_2$TG$_2$T$_3$CATATGA$_2$TATC$_2$TC$_2$T$_2$AG
PRH3347	ETEC_3175	CCTTGTGGACAGCCTGGAA
	verification F	
PEH3348	ETEC_3175	AAGCAGCGTGGTAGCATAATG
	verification R	
PRH3349	ETEC_3180	AACCTATCTGGAGAGCATTC
	verification F	
PRH3350	ETEC_3180	CGGCGTTAAGCATCACTA
	verification R	
PRH3351	ETEC_3196	TCCAGCAAGATACGAGGTT
	verification F	
PRH3352	ETEC_3196	AAGCGGTGATGTTGAAGC
	verification R	

Table 10 Oligonucleotides used for preparation of mutants in Chapter 3 (continued).

PRH3353	ETEC_3253 verification F	TTACCGCCTCTCCTTCTC
PRH3354	ETEC_3253 verification R	CCTGACCGCATTGTTGAT
PRH3373	ETEC_3920 mutation F	$ATCA_2TGTCT_2G_2C_2GCT_2AT_2ACAC_2ACTGCT_5A_2T_3C$ $T_2AGTCATA_3CGC_2AT_2GTGTAG_2CTG_2AGCTGCT_2CG$
PRH3374	ETEC_3920 mutation R	$GCTG_2TAC_2ATA_3CA_2GA_2TAGAGAG_2ATAT_2GCATG_3C$ $AGAC_2TA_2TGAG_2TAG_3ACATATGA_2TATC_2TC_2T_2AG$
PRH3375	ETEC_4148 mutation F	$T_2ACAG_2T_3CAT_2A_2T_2A_3GA_2TGTG_2TGCTG_2TATA_3T_2$ $A_4C_3GCATA_2GTGAT(GT)_2AG_2CTG_2AGCTGCT_2CG$
PRH3376	ETEC_4148 mutation R	$T_4AT_2A_2GA_4GA_2CGAGATCAT_2GT_3AG_3T_2GTAT_3ACA$ $CA_2CAG_2A_3TATC_2ATATGA_2TATC_2TC_2T_2AG$
PRH3377	ETEC_4447 mutation F	$C_2G_2A_3T_2G_3AGATGAG_2TGA_4G_5ACGCATATC_2TG_2$ $TC_2AT_3CAGTAT(GT)_4AG_2CTG_2AGCTGCT_2CG$
PRH3378	ETEC_4447 mutation R	$AGACTCAGTACAT(AC)_2A_5CAT_2CTGATA_3TCTG_3$ $CGTCAT_3CAGAGCA_6C_3(AT)_2GA_2TATC_2TC_2T_2AG$
PRH3379	ETEC_4550 mutation F	$AT_4CATAGATGT_2C2T_4CTA_2TA_3TATG_2CG_2CAG_2TG$ $C_2GC_2AGTGA_2T(GC)_2AT(GT)_2AG_2CTG_2AGCTGCT_2CG$
PRH3380	ETEC_4550 mutation R	$CT_2A_3CA_2C_2A(CT)_2A_3GATA_2CA_3CA_2CA_2C_2A_2C_2AT$ $AGTAG_2A_2TG_2AGA_3T_2CATATGA_2TATC_2TC_2T_2AG$
PRH3381	ETEC_3920 verification F	GCGATGAGGATGGTGTAA
PRH3382	ETEC_3920 verification R	GACTCTGCGTGGATTGAA
PRH3383	ETEC_4148 verification F	CCGCAATACCTGAACCAAT
PRH3384	ETEC_4148 verification R	GGCAGTTGAACCTATGAGTT
PRH3385	ETEC_4447 verification F	GGAAATTGGGAGATGAGGTGAAAA
PRH3386	ETEC_4447 verification R	ACTGGTTGACAGGCTGAATGAT
PRH3387	ETEC_4550 verification F	ACATAAGAACAGCACCGATA
PRH3388	ETEC_4550 verification R	GCAGCATTGAACATTACCAT

Note: 'mutation' represents the primers that were used to prepare mutants; 'verification' represents the primers that were used to verify the mutants by using DNA sequencing; 'F' means forward primer; 'R' means reverse primer.

Results

ETEC H10407 ΔcyaA mutant was more sensitive to extracellular cAMP. In *E. coli* strains, adenylate cyclase encoded by *cyaA* gene regulates the generation of intracellular cAMP from ATP. Our preliminary data suggested that ETEC strains can sense and utilize extracellular cAMP to promote adhesin expression. In this study, we first determined the sensitivity of ETEC H10407 *ΔcyaA* mutant to exogenous cAMP on maltose MacConkey agar supplemented with adenosine 3',5'-cyclic monophosphate tris salt. ETEC H10407 *Δcrp* mutant and *E. coli* K-12 *Δcrp* mutant formed colorless colonies on maltose MacConkey agar regardless of the concentration of exogenous cAMP added into maltose MacConkey agar (data not shown). Our results also demonstrated that ETEC H10407 *ΔcyaA* mutant can form red/pink colonies on maltose MacConkey agar supplemented with 25 μM cAMP (data not shown). Conversely, *E. coli* K-12 *ΔcyaA* mutant cannot produce red/pink colonies until the concentration of exogenous cAMP reached to 1 mM (data not shown). Here our evidence suggested that ETEC strains were more sensitive to exogenous cAMP than *E. coli* K-12 strains and CRP-cAMP complex regulated the utilization of maltose as a carbon source in *E. coli* strains.

Transposon library identified ETEC mutants that cannot utilize maltose. We generated a transposon library by using EZ-Tn5™ <KAN-2>Tnp Transposome Kit and EZ-Tn5™ <R6Kγ*ori*/KAN-2>Tnp Transposome Kit. Of 17,956 mutants (**Table 10**), we identified in total of 24 mutants that formed colorless colonies on maltose MacConkey indicator agar plate.

Table 11 Results of mutant library.

Transposome Kit	Mutant population (n=)	Suspect mutant (n=)
EZ-Tn5™ <R6Kγori/KAN-2>Tnp	6,192	7
EZ-Tn5™ <KAN-2>Tnp	11,764	17

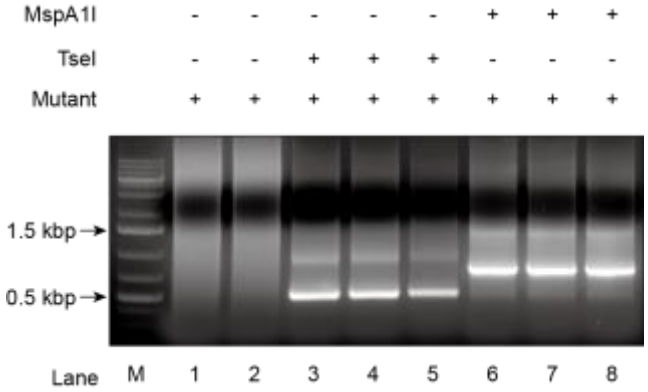
None of transposon-disrupted genes were associated with importer for cAMP. For mutants constructed by using EZ-Tn5™ <R6K*γori*/KAN-2>Tnp transposome kit, identification of disrupted genes was carried out following the manufacturer's instructions. Briefly, genomic DNA extracted from one of the mutants was digested using BanI restriction enzyme. There are 4 136 recognition sites throughout the chromosomal genome of ETEC H10407 (5,153,435 bp). Next, the DNA fragments were self-ligated and transformed into *E. coli pir*-116 cells and colonies were rescued on LB plates containing kanamycin (25 mg/ml). the rescued plasmids were submitted for sequencing. The disrupted genes were identified and listed in **Table 11**.

For mutants constructed by using EZ-Tn5™ <KAN-2>Tnp transposome kit, mapping the disrupted genes was performed by using inverse PCR technology. Genomic DNA from one mutant was digested by using restriction enzyme MspA (11,9061 recognition sites) and TseI (19,974 recognition sites), respectively. The PCR products were analyzed on 1.5 % agarose gels (**Figure 10**) and was extracted. The PCR products were sequenced and the disrupted genes were identified and summarized in **Table 11**.

In summary, of 24 potential suspected mutants, 6 genes were identified. However, none of the disrupted genes associated with cAMP importer. Instead, those genes were involved in utilization of maltose.

None of mutated genes were associated with importer for cAMP. Here, mutation of the gene (ETEC_4447) encoding inner membrane protein (CBJ03956) in ETEC H10407 *ΔcyaA* mutant was described. First, the sequence (1,034 bp) responsible for chloramphenicol resistance gene was successfully amplified by using PCR (**Figure 11**).

Figure 10 Inverse PCR results.

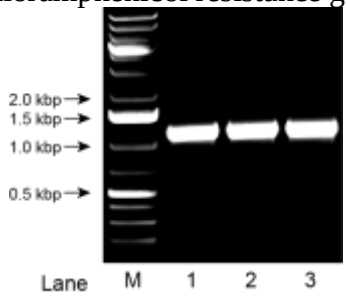


Note: Identification of ETEC_4289 (CBJ03795) by using inverse PCR. M, molecular weight; Lane 1-2, negative control, 2 replicates; Lane 3 – 5, TseI treatment, 3 replicates; Lane 6 – 8, MspA1I treatment, 3 replicates.

Table 12 Results of mapping the disrupted genes by Tn5 transposome.

Disrupted gene	Function of disrupted gene
CBJ03109	conserved hypothetical protein
CBJ03167	4-alpha-glucanotransferase
CBJ03169	regulatory protein
CBJ03794	maltose transport system, permease protein
CBJ03795	maltose transport system, substrate-binding protein
CBJ03796	maltose/maltodextrin transport system, ATP-binding protein

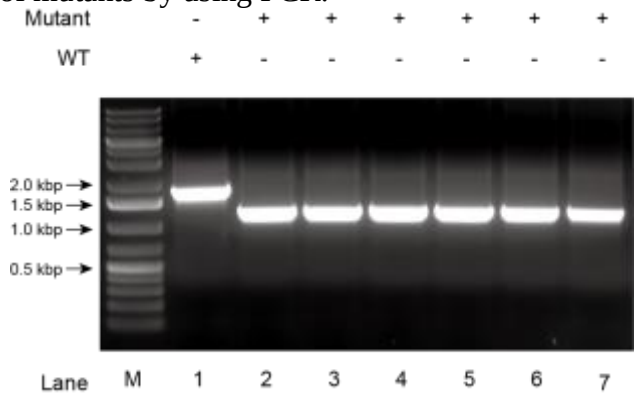
Figure 11 PCR amplification of chloramphenicol resistance gene from pKD3.



Note: Amplification of chloramphenicol resistance gene from pKD3 for mutation of ETEC_4447 (CBJ03956). M, molecular weight; Lane 1-3, PCR products of chloramphenicol resistance gene, 3 replicates.

Subsequently, the PCR product was transformed in electro-competent cells containing pKD46, and the suspected colonies were rescued on LB plate supplemented with chloramphenicol (25 µg/ml). A PCR reaction was carried out for detecting the presence of chloramphenicol resistance cassette in the mutant. The PCR product was analyzed on 1 % agarose gel (**Figure 12**). A negative control (1,728 bp) was included.

Figure 12 Verification of mutants by using PCR.



Note: Verification of mutated ETEC_4447 gene by using PCR. M, molecular weight; Lane 1, negative control; Lane 2 – 7, 6 suspect mutants were confirmed by PCR.

All of the 10 genes were successfully mutated and were streaked onto Maltose MacConkey agar plate supplemented without and with 25 μM cAMP, respectively. All of the mutants produced red colonies in presence of cAMP, and colorless colonies in absence of cAMP, suggesting that none of 10 genes are responsible for hypothetical importer for exogenous cAMP.

Discussion

ETEC is the main cause of traveler's diarrhea and childhood diarrhea worldwide. ETEC strains carry one or two enterotoxins (LT and ST), which are the attributors to watery diarrhea in human. Of two toxins, LT is able to activate the production of cAMP from intoxicated IECs and overproduction of cAMP is secreted into intestinal lumen through activated CFTR channel. Cyclic AMP is a secondary messenger that is able to interact with CRP. CRP-cAMP complex is a master transcription regulator that modulate the expression of over 200 genes such as LT and pili in *E. coli*. Previous study has suggested that ETEC H10407 strain is able to utilize exogenous cAMP and is more sensitive to extracellular cAMP. It is possible that a cAMP import pathway with high affinity to cAMP is present in ETEC strains. In this present study, we employed Tn5-mediated transposome technology to construct mutant library by using ETEC H10407 *ΔcyaA* mutant (inability to produce endogenous cAMP). By introducing randomly into the chromosomal genome

of ETEC H10407 *ΔcyaA* mutant, we successfully constructed a mutant library consisting of 17,956 mutants and mapping the disrupted genes was completed, suggesting that none of the genes were associated with cAMP importer. In addition, 10 genes encoding transmembrane-bound transporter were mutated in ETEC H10407 *ΔcyaA* mutant. None of the genes were involved in cAMP importer either.

Transposons are mobile DNA elements that can insert into target DNA molecules, which have been applied onto mutagenesis and gene tagging. In addition to Tn5, there are other transposons such as Tn7 and Mu are documented and commercially available. EZ-Tn5™ Transposome Kits have been widely used in Gram-negative and Gram-positive bacteria, especially *E. coli*. For transposon-mediated mutagenesis, uniform distribution of transposon insertion is a key factor for their application in molecular biology. A previous study suggested that Tn5 transposon, compared to Tn7 transposon, prefers to inserting into a sequence with high composition of G/C. In this study, we successfully used Tn5 transposon generating a mutant library by introducing Tn5 transposon into ETEC strain H10407 (3.5 insertions /1 Kbp on average). Given the Tn5's preference for G/C rich sequence, it is possible there is a bias on preparing the mutant library by using Tn5 transposon.

Maltose is a disaccharide containing two glucose molecules. Unlike glucose, *E. coli* cannot utilize maltose directly. In addition to a maltose transport system, fermentation of maltose requires the catabolite activator protein or cAMP receptor protein. CRP-cAMP binds to DNA and initiate the transcription of gene encoding enzyme for utilization of maltose. In this study, we identified the genes required for utilization of maltose rather than cAMP. However, our study might benefit to future study on identification of cAMP importer system in ETEC.

Chapter 4 - Influence of microbiota and NleH effector on colonization of *C. rodentium* in mouse models

Introduction

Enteropathogenic *Escherichia coli* (EPEC) and enterohemorrhagic *Escherichia coli* (EHEC) are important pathogens causing diarrheal disease in both developing and developed countries. These two gastrointestinal bacterial pathogens together with *C. rodentium* belong to a group of pathogens termed attaching and effacing (A/E) pathogens (Franzin and Sircili, 2015). The A/E pathogens are characterized by their ability to form distinctive lesions termed A/E lesions on the surface of IECs resulting in intimate attachment of A/E pathogen to host cell. The formation of A/E lesions is closely regulated by T3SS (type 3 secretion system) (Gaytan et al., 2016). Moreover, T3SS is responsible for translocation of several Nle (non-LEE-encoded) effectors into target cells contributing to manipulation of host cell functions such as host inflammatory responses (Galan et al., 2014). It has been documented that NleB and NleH subvert host pro-inflammation responses by targeting NF-κB signaling pathway contributing to bacterial colonization in host (Yen et al., 2016).

Human gut microbiota refers to the microorganisms that colonize the human digestive tract, which is mainly compose of bacteria, as well as viruses, yeast and archaea. The gut microbiota plays an important role in fermentation and absorption of undigested carbohydrates and promoting maturation of immune cells and development of immune system, as well as in regulating the host resistance to colonization of enteric pathogens (reviewed in Chapter 1).

C. rodentium is a Gram-negative bacterium, which is a natural pathogen of mouse (Bhinder et al., 2013). *C. rodentium* is closely related to human pathogens EHEC and EPEC. Infection of

mouse with *C. rodentium* is now an evaluable model to study the pathogenesis of EHEC and EPEC (Crepin et al., 2016). *E. coli* O157:H7 strain EDL933 encodes two NleH effectors, NleH1 and NleH2 (Gao et al., 2009). NleH1 and NleH2 are closely related T3SS effectors (84 % sequence identity), which have been demonstrated to modulate activation of NF-κB pathway and apoptosis (Grishin et al., 2014). NleH1 has been reported to block IKKβ-dependent phosphorylation of RPS3 at Ser209, and subsequently inhibit nuclear translocation of RPS3, resulting in subverting the activation of NF-κB pathway (Gao et al., 2009; Pham et al., 2012; Wan et al., 2011). Whereas, NleH2 can promote the phosphorylation of RPS3 and mildly activates the NF-κB pathway (Pham et al., 2012). Unlike EHEC and EPEC, *C. rodentium* just encodes one NleH effector that is functionally identical to NleH1 in EHEC and EPEC (Pham et al., 2012).

The mouse inbred line C57BL/6J is widely used in studying bacterial pathogenesis. C57BL/10ScNJ mice are derived from C57BL/6J mice containing a 74.4 kb deletion in *tlr4* gene resulting in the absence of functional TLR4. The inbred mice line C3H/HeJ carries a point mutation in *tlr4* gene making it hyporesponsive to bacterial LPS. The C3H/HeOuJ mouse is closely related to C3H/HeJ mouse, which possesses a functional TLR4-LPS signaling pathway.

Previous studies have demonstrated that transplantation of microbiota is able to alter host resistance to enteric pathogens (Bakken et al., 2011; Willing et al., 2011). In this study, we aimed to evaluate the impact of microbiota on colonization of *C. rodentium* in mouse model through transplantation of gut microbiota between different genetic background of mouse, as well as the impact of T3SS-dependent effector NleH on colonization of *C. rodentium* in mouse model.

Materials and Methods

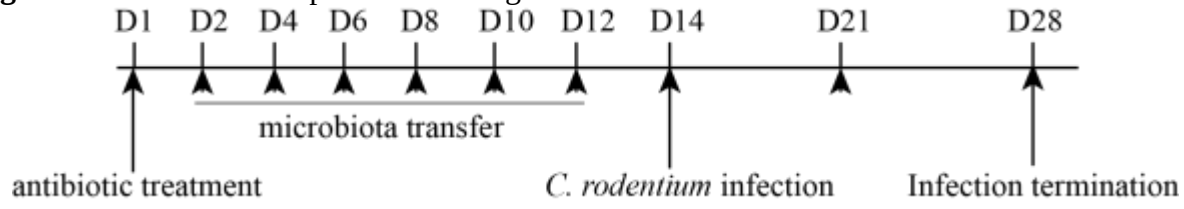
Ethics statement. All animal experiments were performed in strict accordance with the guidelines of Institutional Animal Care and Use Committee at Kansas State University. The protocol was approved by Kansas State University Animal Care Committee (IACUC #3323). This institution complies with all applicable provisions of the Animal Welfare Act and other Federal statutes and regulations relating to animals.

Bacterial strains and reagents. C. rodentium WT (Crepin et al., 2016) and *ΔnleH* mutant (Pham et al., 2012) were used in animal experiments, and were maintained in Luria–Bertani (LB) broth and LB agar plate at 37 °C. antibiotics and MacConkey agar used in this study was obtained from Fisher Scientific. LB broth and LB agar were purchased from MidSci. QIAamp DNA Stool Mini Kit was obtained from Qiagen.

Mice and microbiota transplantation. Three to four-week-old female C57BL/6J, C3H/HeJ, and C3H/HeOuJ mice were obtained from Jackson Laboratory (Bar Harbor, Maine). C57BL/10ScNJ mice were bred at College of Veterinary Medicine, Kansas State University. All mice were housed (5 per group) in sterilized cages, fed autoclaved food and water under specific-pathogen-free and controlled temperature and photoperiod conditions. The microbiota transplantation was carried out as previously described (Willing et al., 2011). Briefly, the native microbiota of mice was depleted by a single oral dose (20 mg/mouse) of streptomycin (D1) 24 hours prior to the first time of microbiota transplantation. Fresh fecal pellets from 3 – 4 donor mice were collected and placed in 1 ml transfer buffer (pre-reduced sterile phosphate buffered saline containing 0.05 % cysteine HCl) on ice. The fecal pellets were homogenized and centrifuged at 800 x *g* for 2 min and the supernatant was collected and diluted (1:3) in transfer buffer. One hundred microliter (100 µl) of diluted fecal supernatant was introduced into recipient mice by oral gavage for 6 times (D2, D4,

D6, D8, D10, and D12). A control group that received fecal transplantation from the same strain of mouse was included in this study.

Figure 13 Overview of experimental design.



C. rodentium infections. A single colony of mouse-specific pathogen *C. rodentium* and isogenic mutant *C. rodentium ΔnleH* was inoculated into 5 ml LB broth and aerobically cultured with shaking (200 rpm/min) overnight at 37 °C. The cultures were diluted (1:100) into 200 ml LB broth and aerobically cultured with shaking (200 rpm/min) overnight at 37 °C. Cells were harvested by centrifugation at 3,000 x g for 15 min at 4 °C and washed 3 times with 20 ml of ice-cold PBS. Cells were resuspended into 2 ml of ice-cold 1x PBS and 100 µl aliquots were used to infect mice by oral gavage 2 days after the last microbiota transfer (D14). The actual infection dose was determined by plating 100 µl aliquots of successive dilutions (10^{-1} – 10^{-8}) on LB agar. Mice were monitored twice daily for clinical signs (dehydration, rectal prolapse and loss of responsiveness to stimulation) and weight loss. Mice were euthanized when the body weight loss was greater than 20 % of the initial weight and other clinical signs of infection (dehydration, rectal prolapse and loss of responsiveness to stimulation).

Bacterial colonization. Colon samples (approximately 4 cm) were collected and stored on ice at necropsy (D28). Feces were removed before weighing tissue. Colon samples were homogenized in 1 x PBS (100 mg colon *vs* 5 ml 1x PBS), serially diluted (10^{-1} – 10^{-8}), and 100 µl aliquots of serial dilutions were plated onto MacConkey agar and incubated for 24 h at 37 °C.

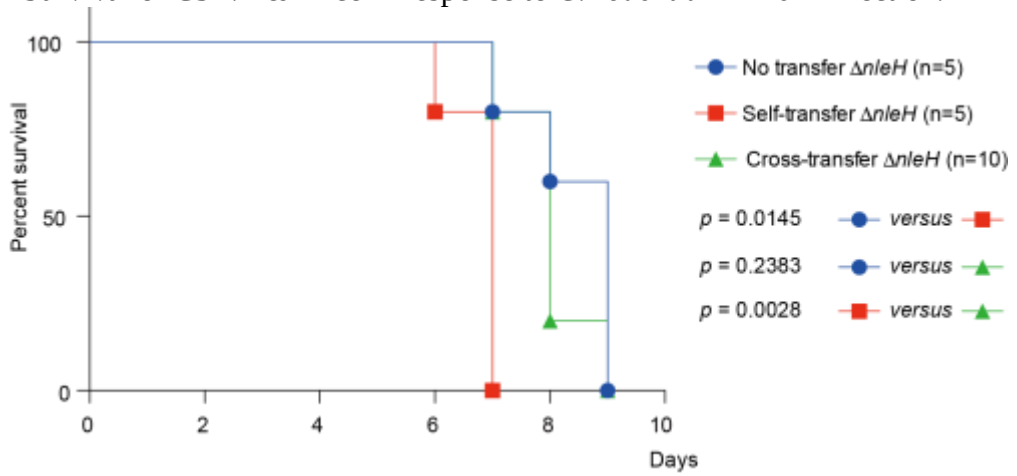
Fecal DNA extraction. Three-four fecal pellets from each mouse were collected on the day of first microbiota transfer (D1), on the day of infection (D14), and on the day of infection end (D28). After collection, the fecal pellets samples were stored at -80 °C. DNA extraction was performed by using QIAamp DNA Stool Mini Kits (Qiagen) following the manufacturer's instruction. The concentration of DNA was determined by using Nanodrop 2000 (Fisher Scientific, USA).

Microbial community analysis. The microbial community profile was assessed by using 16S ribosomal DNA sequencing. Genomic DNA samples from stool samples were used to perform Ion Torrent 16S Ribosomal amplicon library construction. Approximately 40,000 reads per sample were obtained in 400 bp reads.

Results

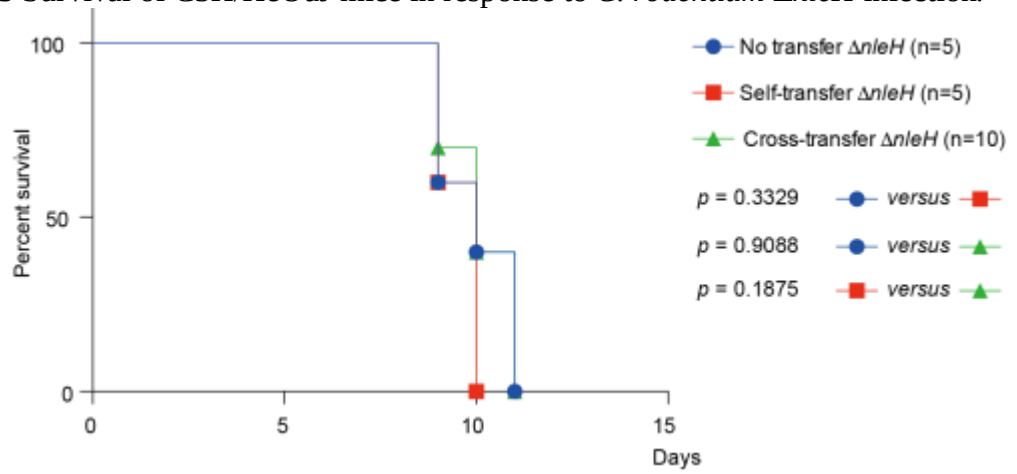
Survival of infected mice at the end of infection. The C57BL/6J mice are more resistant to *C. rodentium* infection, whereas C3H/HeJ mice are more susceptible to *C. rodentium* infection (Vallance et al., 2003). In this study, all C57BL/6J mice challenged by wildtype (WT) *C. rodentium* or *ΔnleH* mutant survived by the end of experiment. By contrast, the C3H/HeJ mice infected with WT *C. rodentium* survived at the end of experiment, whereas the C3H/HeJ mice challenged by *C. rodentium ΔnleH* mutant were euthanized due to loss of great than 20 % of initial weight (**Figure 14**). The similar phenomenon was also observed in C3H/HeOuJ mice (**Figure 15**). The statistical analysis was performed by using Log-Rank (Mantel-Cox) test.

Figure 14 Survival of C3H/HeJ mice in response to *C. rodentium ΔnleH* infection.



Note: No transfer, C3H/HeJ mice (n=5) in this group were not subjected to microbiota transplantation; Self-transfer, C3H/HeJ mice (n=5) in this group were subjected to transplantation of microbiota acquired from C3H/HeJ mice; Cross-transfer, C3H/HeJ mice (n=10) in this group were subjected to transplantation of microbiota acquired from C3H/HeOuJ mice; The *p* values are for log rank test; $p < 0.05$ was considered as significant difference.

Figure 15 Survival of C3H/HeOuJ mice in response to *C. rodentium ΔnleH* infection.

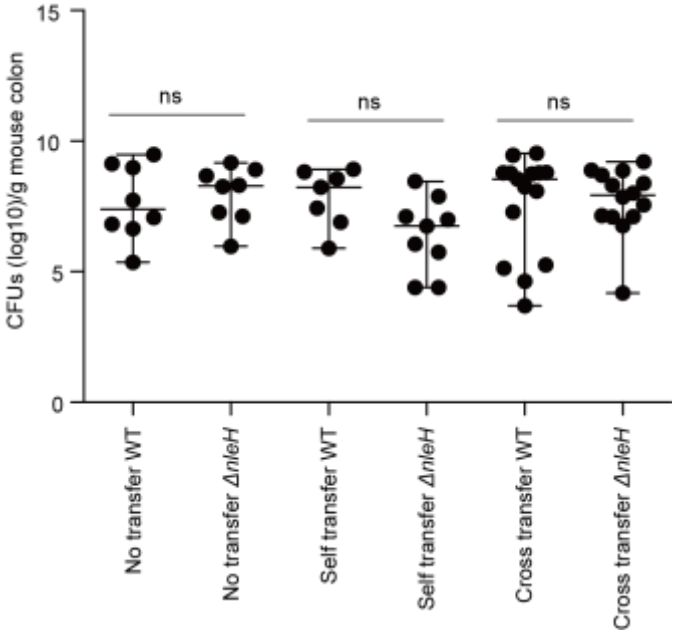


Note: No transfer, C3H/HeOuJ mice (n=5) in this group were not subjected to microbiota transplantation; Self-transfer, C3H/HeOuJ mice (n=5) in this group were subjected to transplantation of microbiota acquired from C3H/HeOuJ mice; Cross-transfer, C3H/HeJ mice (n=10) in this group were subjected to transplantation of microbiota acquired from C3H/HeJ mice; The *p* values are for log rank test; $p < 0.05$ was considered as significant difference.

Colonization of C. rodentium in mice by the end of infection. Next, bacterial colonization was investigated based on genetic background of mice. The pathogen load (cfu/g mouse colon) was subjected to statistical analysis by using unpaired t-test (Mann-Whitney test). In C57BL/6J mice, the mice were more resistant to *C. rodentium* Δ*nleH* infection than WT *C. rodentium* infection (**Figure 16**) in No transfer group. The similar phenomenon was also observed in self-transfer group

(both the donor mice and recipient mice are the same strain) (**Figure 16**). By contrast, transplantation of microbiota from C57BL/6J mice into C57BL/10ScNJ mice reversed the bacterial colonization in C57BL/6J mice in response to WT *C. rodentium* infection and *C. rodentium* Δ*nleH* infection (**Figure 16**). The statistical analysis was performed by using Mann-Whitney test. Our results suggested that T3SS-dependent effector NleH did not significantly contribute to colonization of *C. rodentium* in mouse. In addition, microbiota transfer altered host resistance to colonization of *C. rodentium* in mouse, which was not a significant alteration.

Figure 16 Bacterial colonization in C57BL/6J mice.

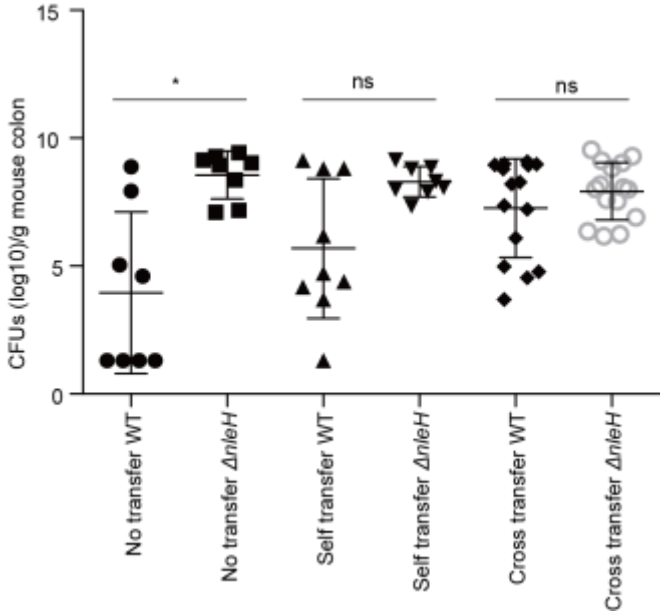


Note: No transfer, C57BL/6J mice (n=8) in this group were not subjected to microbiota transplantation; Self-transfer, C57BL/6J mice (n=9) in this group were subjected to transplantation of microbiota acquired from C57BL/6J mice; Cross-transfer, C57BL/6J (n=15) mice in this group were subjected to transplantation of microbiota acquired from C57BL/10ScNJ mice; WT, wildtype *C. rodentium* infection; Δ*nleH*, *C. rodentium* Δ*nleH* infection; $p < 0.05$ (∗), significant difference; ns, non-significant difference.

In C57BL/10ScNJ mice, the mice were more resistant to WT *C. rodentium* infection than *C. rodentium* Δ*nleH* infection (**Figure 17**) in no-transfer group (negative control). The similar phenomenon was observed in self-transfer group (**Figure 17**). By contrast, transplantation of microbiota from C57BL/6J mice into C57BL/10ScNJ mice altered the resistance to WT *C.*

rodentium infection. The statistical analysis was performed by using Mann-Whitney test. Our results suggested that T3SS-dependent effector NleH significantly resulted in bacterial colonization in C57BL/10ScNJ mice. Microbiota transplantation was able to alter host resistant to colonization of *C. rodentium* WT strain in C57BL/10ScNJ mice.

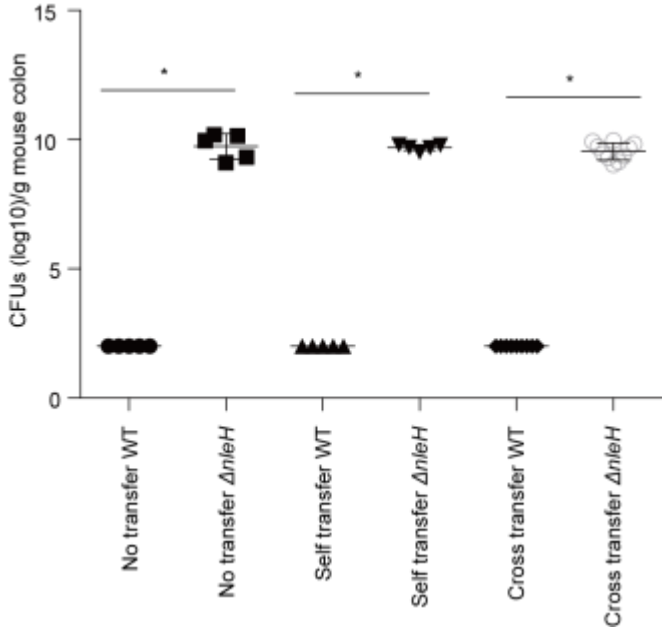
Figure 17 Bacterial colonization in C57BL/10ScNJ mice.



Note: No transfer, C57BL/10ScNJ mice (n=8) in this group were not subjected to microbiota transplantation; Self-transfer, C57BL/10ScNJ mice (n=9) in this group were subjected to transplantation of microbiota acquired from C57BL/10ScNJ mice; Cross-transfer, C57BL/10ScNJ mice (n=15) in this group were subjected to transplantation of microbiota acquired from C57BL/6J mice; WT, wildtype *C. rodentium* infection; Δ*nleH*, *C. rodentium* Δ*nleH* infection; $p < 0.05$ (∗), significant difference; ns, non-signicant difference.

In C3H/HeJ mice, the mice were resistant to colonization of WT *C. rodentium* (**Figure 18**) in no-transfer, self-transfer group, and cross-transfer group (the genetic background of donor mice is different from that of recipient mice). By contrast, C3H/HeJ mice were more susceptible to *C. rodentium ΔnleH* infection (**Figure 18**). The statistical analysis was performed by using Mann-Whitney test. The survival data suggested that modulation of inflammation byT3SS-dependent effector NleH was crucial for disease severity in C3H/HeJ mice.

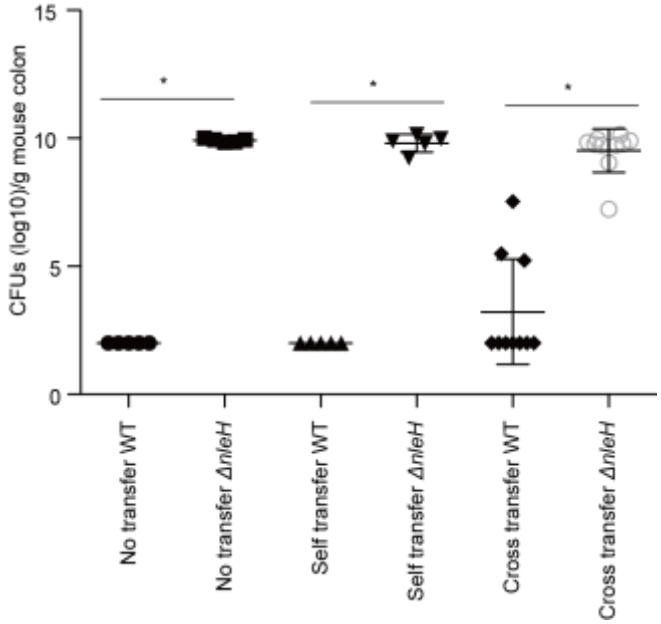
Figure 18 Bacterial colonization in C3H/HeJ mice.



Note: No transfer, C3H/HeJ mice (n=5) in this group were not subjected to microbiota transplantation; Self-transfer, C3H/HeJ mice (n=5) in this group were subjected to transplantation of microbiota acquired from C3H/HeJ mice; Cross-transfer, C3H/HeJ mice (n=10) in this group were subjected to transplantation of microbiota acquired from C3H/HeOuJ mice; WT, wildtype *C. rodentium* infection; Δ*nleH*, *C. rodentium* Δ*nleH* infection; $p < 0.05$ (∗), significant difference; ns, non-signicant difference.

In C3H/HeOuJ mice, the mice were resistant to colonization of WT *C. rodentium* (**Figure 19**) in no-transfer and self-transfer groups, which was inconsistent with previous study. However, transplantation of microbiota from C3H/HeJ mice into C3H/HeOuJ mice increased the bacterial colonization (**Figure 19**). By contrast, C3H/HeOuJ mice were more susceptible to *C. rodentium ΔnleH* infection (**Figure 19**). The statistical analysis was performed by using Mann-Whitney test. These results indicated that inflammation rather than microbiota caused higher mortality in C3H/HeOuJ mice.

Figure 19 Bacterial colonization in C3H/HeOuJ mice.



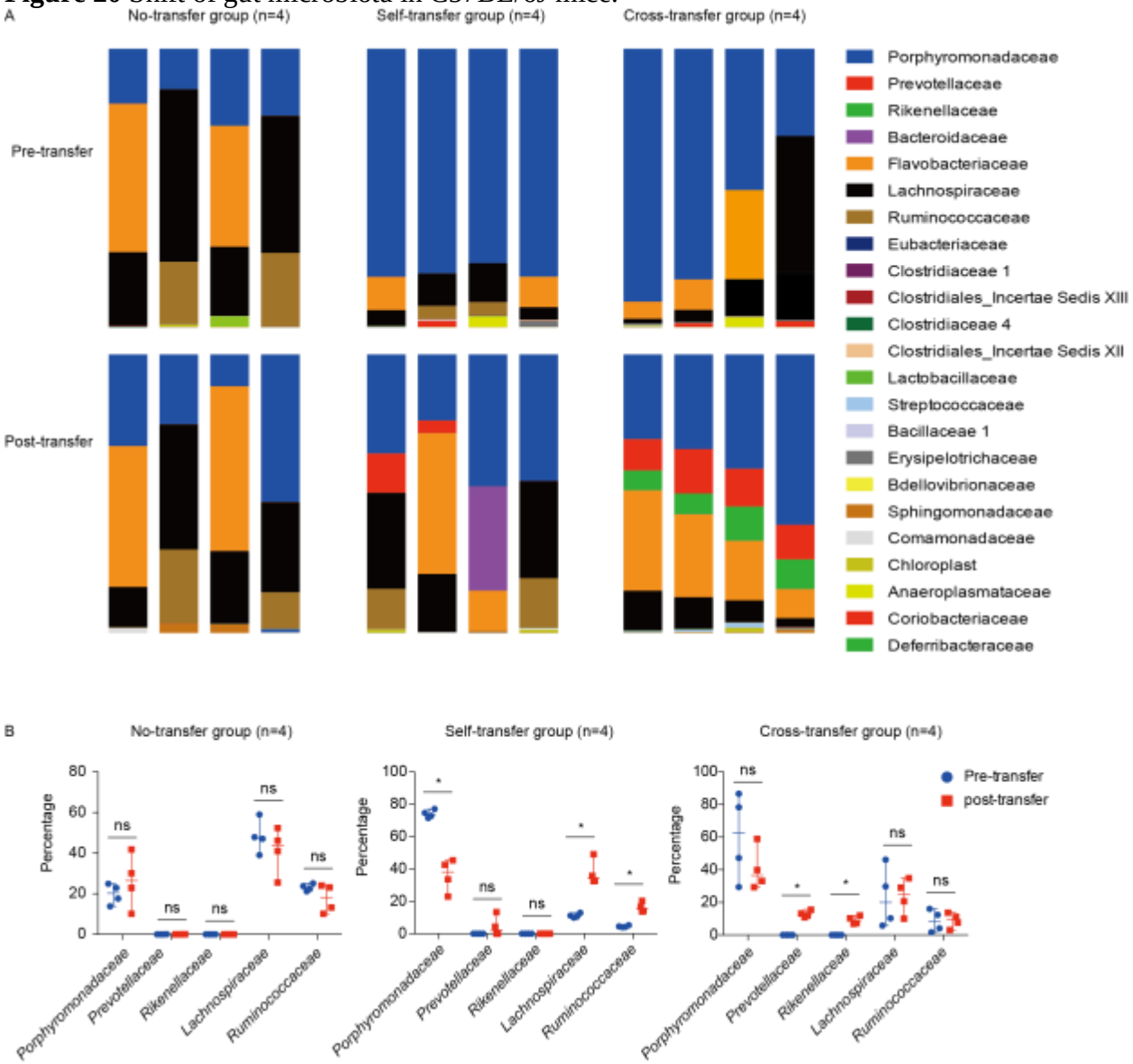
Note: No transfer, C3H/HeOuJ mice (n=5) in this group were not subjected to microbiota transplantation; Self-transfer, C3H/HeOuJ mice (n=5) in this group were subjected to transplantation of microbiota acquired from C3H/HeOuJ mice; Cross-transfer, C3H/HeOuJ mice (n=10) in this group were subjected to transplantation of microbiota acquired from C3H/HeJ mice; WT, wildtype *C. rodentium* infection; Δ*nleH*, *C. rodentium* Δ*nleH* infection; $p < 0.05$ (∗), significant difference; ns, non-signicant difference.

The composition of gut microbiota after microbiota transplantation. By using 16S rDNA sequencing, we evaluated the efficiency of microbiota transplantation. For C57BL/6J mice, transplantation of microbiota derived from C57BL/10ScNJ mice altered the microbiota composition resulting in a high diverse gut microbiota (**Figure 20A**). We next performed statistical analysis of the composition shift in C57BL/6J mice by choosing 5 bacterial families (*Porphyromonadaceae, Prevotellaceae, Rikenellaceae, Lachnospiraceae,* and *Ruminococcaceae)* that were the most abundant bacterial families in experimental mice. In No-transfer group, there was not significant difference (**Figure 20B**). By contrast, microbiota transfer resulted in significant increased abundance of *Lachnospiraceae* and *Ruminococcaceae,* and significant decreased abundance of *Porphyromonadaceae* in Self-transfer group (**Figure 20B**), which might be caused by donor mice or stress such mouse handling. In Cross-transfer group, microbiota transfer

significantly altered the abundance of *Prevotellaceae* and *Rikenellaceae* (**Figure 20B**), which suggested that the microbiota transfer successfully altered the composition of intestinal microbiota in C57BL/6J mice. The statistical analysis was performed by using multiple t tests (Holm-Sidak test) without assuming a consistent SD. Each row was analyzed individually, $p < 0.05$ was considered as significant difference.

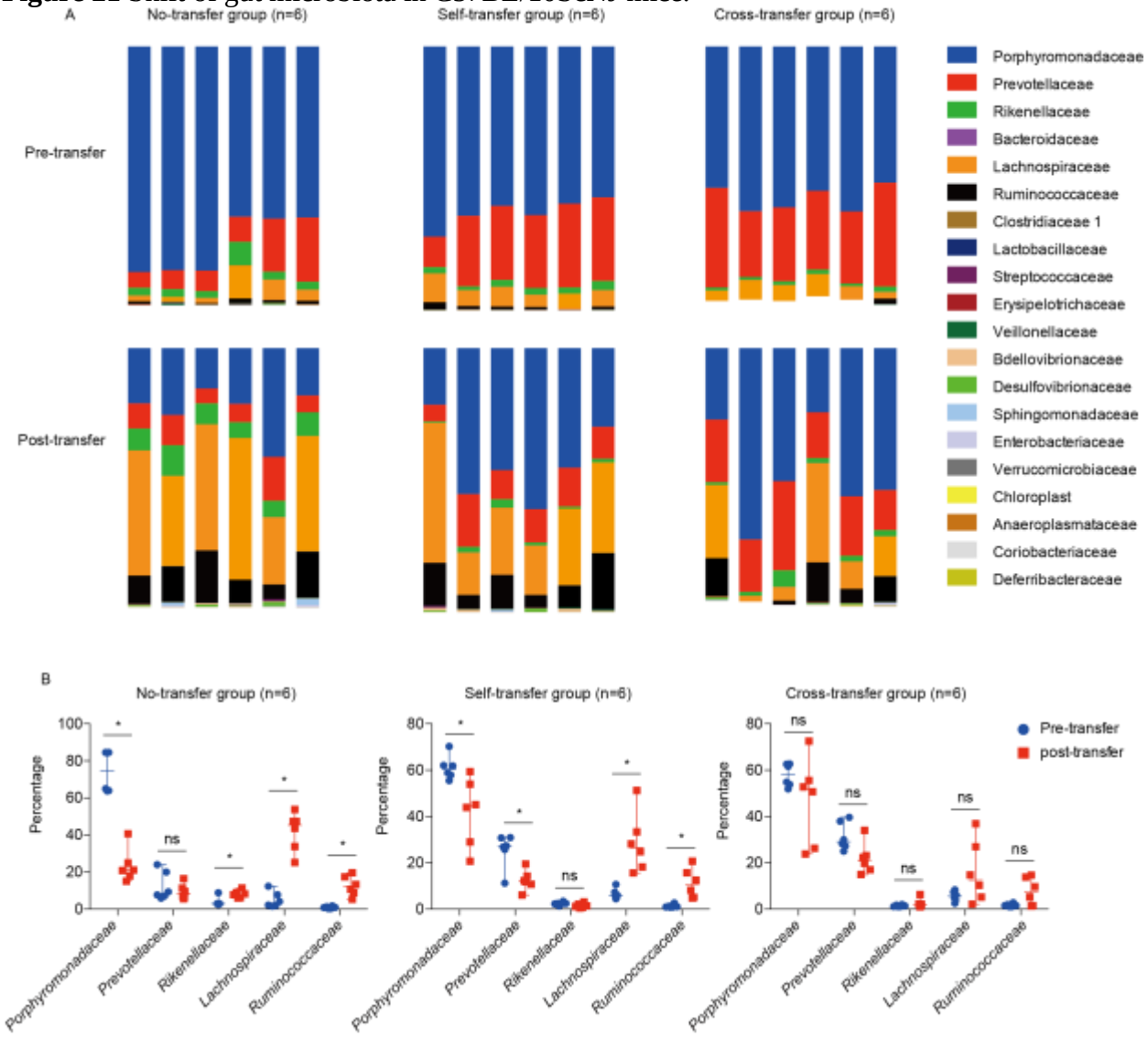
For C57BL/10ScNJ mice, transplantation of microbiota derived from C57BL/6J mice resulted in the shift of microbiota composition (**Figure 21A**). We next performed statistical analysis of the composition shift in C57BL/6J mice by choosing 5 bacterial families (*Porphyromonadaceae, Prevotellaceae, Rikenellaceae, Lachnospiraceae,* and *Ruminococcaceae)* that were the most abundant bacterial families in experimental mice. In Cross-transfer group, there was not significant difference (**Figure 21B**). By contrast, microbiota transfer resulted in significant increased abundance of *Lachnospiraceae* and *Ruminococcaceae,* and significant decreased abundance of *Porphyromonadaceae* and *Prevotellaceae* in Self-transfer group (**Figure 21B**), which might be caused by donor mice. In No-transfer group, the composition of intestinal microbiota was defined by decreased abundance of *Porphyromonadaceae,* and increased abundance of *Rikenellaceae Lachnospiraceae* and *Ruminococcaceae* (**Figure 21B**), which might attribute to age. The shift of microbiota in C57BL/10ScNJ mice suggested that microbiota transfer successfully altered the composition of intestinal microbiota. The statistical analysis was performed by using multiple t tests (Holm-Sidak test) without assuming a consistent SD. Each row was analyzed individually, $p < 0.05$ was considered as significant difference.

Figure 20 Shift of gut microbiota in C57BL/6J mice.



Note: No transfer, C57BL/6J mice (n=4) in this group were not subjected to microbiota transplantation; Self-transfer, C57BL/6J mice (n=4) in this group were subjected to transplantation of microbiota acquired from C57BL/6J mice; Cross-transfer, C57BL/6J (n=4) mice in this group were subjected to transplantation of microbiota acquired from C57BL/10ScNJ mice; $p < 0.05$ (∗), significant difference; ns, non-significant difference.

Figure 21 Shift of gut microbiota in C57BL/10ScNJ mice.



Note: No transfer, C57BL/10ScNJ mice (n=6) in this group were not subjected to microbiota transplantation; Self-transfer, C57BL/10ScNJ J mice (n=6) in this group were subjected to transplantation of microbiota acquired from C57BL/6J mice; Cross-transfer, C57BL/10ScNJ J (n=6) mice in this group were subjected to transplantation of microbiota acquired from C57BL/6J mice; $p < 0.05$ (∗), significant difference; ns, non-significant difference.

Composition shift after C. rodentium infection in C57BL/10ScNJ mice. We next analyzed the composition of gut microbiota prior to and post-infection by choosing 5 bacterial families that were the most abundant bacterial families in experimental mice. In response to WT *C. rodentium* infection, the composition of microbiota was characterized by increased abundance of *Porphyromonadaceae*, and decreased abundance of *Rikenellaceae*, *Lachnospiraceae*, and

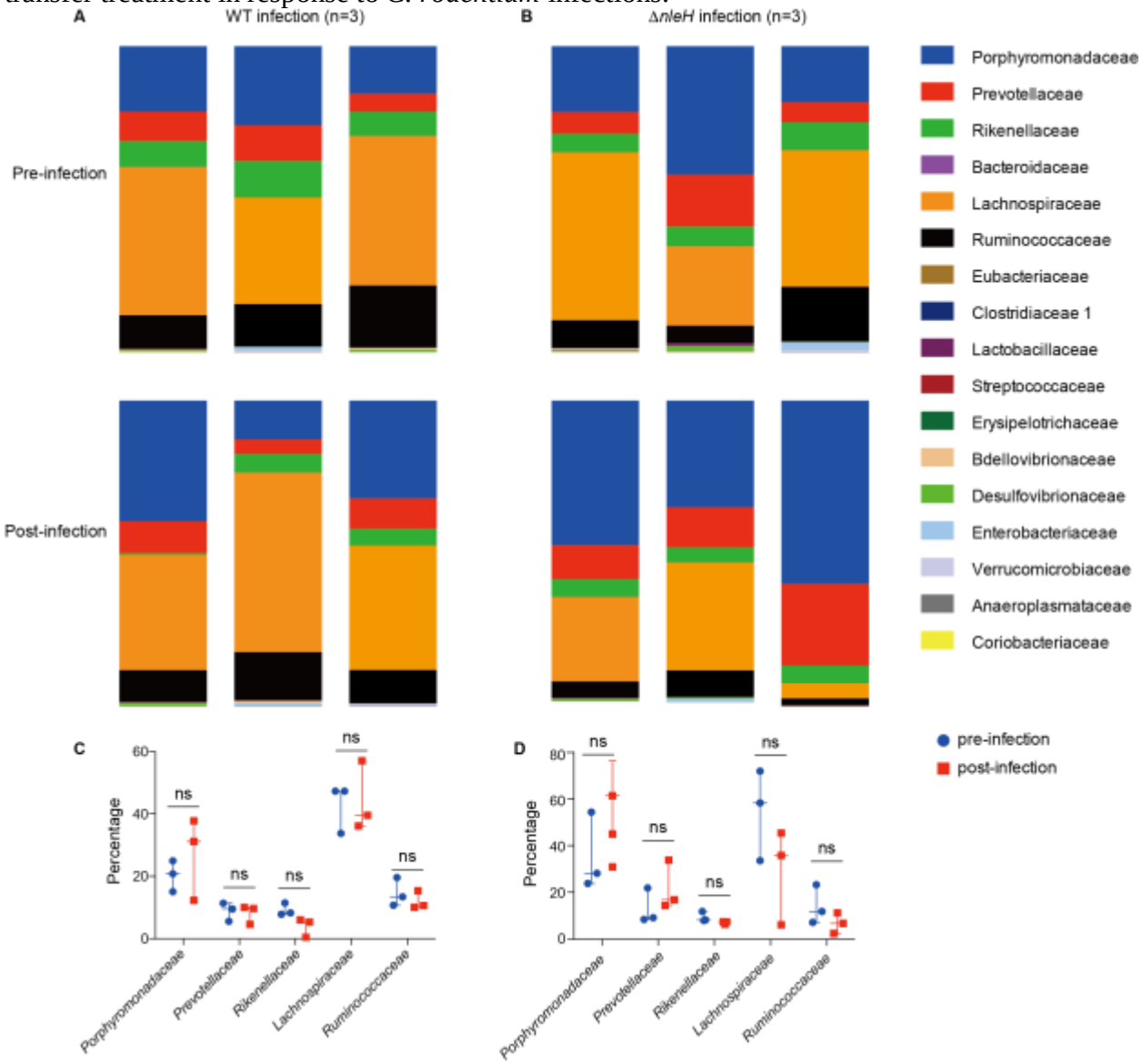
Ruminococcaceae (**Figure 22A** and **22C**). By contrast, the composition of microbiota was defined by increased abundance of *Porphyromonadaceae* and *Prevotellaceae*, and decreased abundance of *Rikenellaceae*, *Lachnospiraceae* and *Ruminococcaceae* in mice challenged by *C. rodentium ΔnleH* strain (**Figure 22B** and **22D**). However, statistical analysis indicated that the alteration of intestinal microbiota was not significant (**Figure 22C** and **22D**). The statistical analysis was performed by using multiple t tests (Holm-Sidak test) without assuming a consistent SD. Each row was analyzed individually, $p < 0.05$ was considered as significant difference.

In Self-transfer group, WT *C. rodentium* infection resulted in the shift of intestinal microbiota characterized by decreased abundance of *Porphyromonadaceae* and *Ruminococcaceae*, and increased abundance of *Prevotellaceae*, *Rikenellaceae*, and *Lachnospiraceae* (**Figure 23A** and **23C**). By contrast, the composition of microbiota was defined by increased abundance of *Porphyromonadaceae* and *Prevotellaceae*, and decreased abundance of *Lachnospiraceae* and *Ruminococcaceae* in mice challenged by *C. rodentium ΔnleH* strain (**Figure 23B** and **23D**). However, statistical analysis indicated that the alteration of intestinal microbiota was not significant (**Figure 23C** and **23D**). The statistical analysis was performed by using multiple t tests (Holm-Sidak test) without assuming a consistent SD. Each row was analyzed individually, $p < 0.05$ was considered as significant difference.

In Cross-transfer group, WT *C. rodentium* infection caused alteration of gut microbiota defined by decreased abundance of *Porphyromonadaceae* and *Prevotellaceae*, and increased abundance of *Rikenellaceae*, *Lachnospiraceae*, and *Ruminococcaceae* (**Figure 24A** and **24C**). By contrast, the composition of microbiota was defined by increased abundance of *Porphyromonadaceae* and *Prevotellaceae*, and decreased abundance of *Rikenellaceae*, *Lachnospiraceae* and *Ruminococcaceae* in mice challenged by *C. rodentium ΔnleH* strain (**Figure 24B** and **24D**).

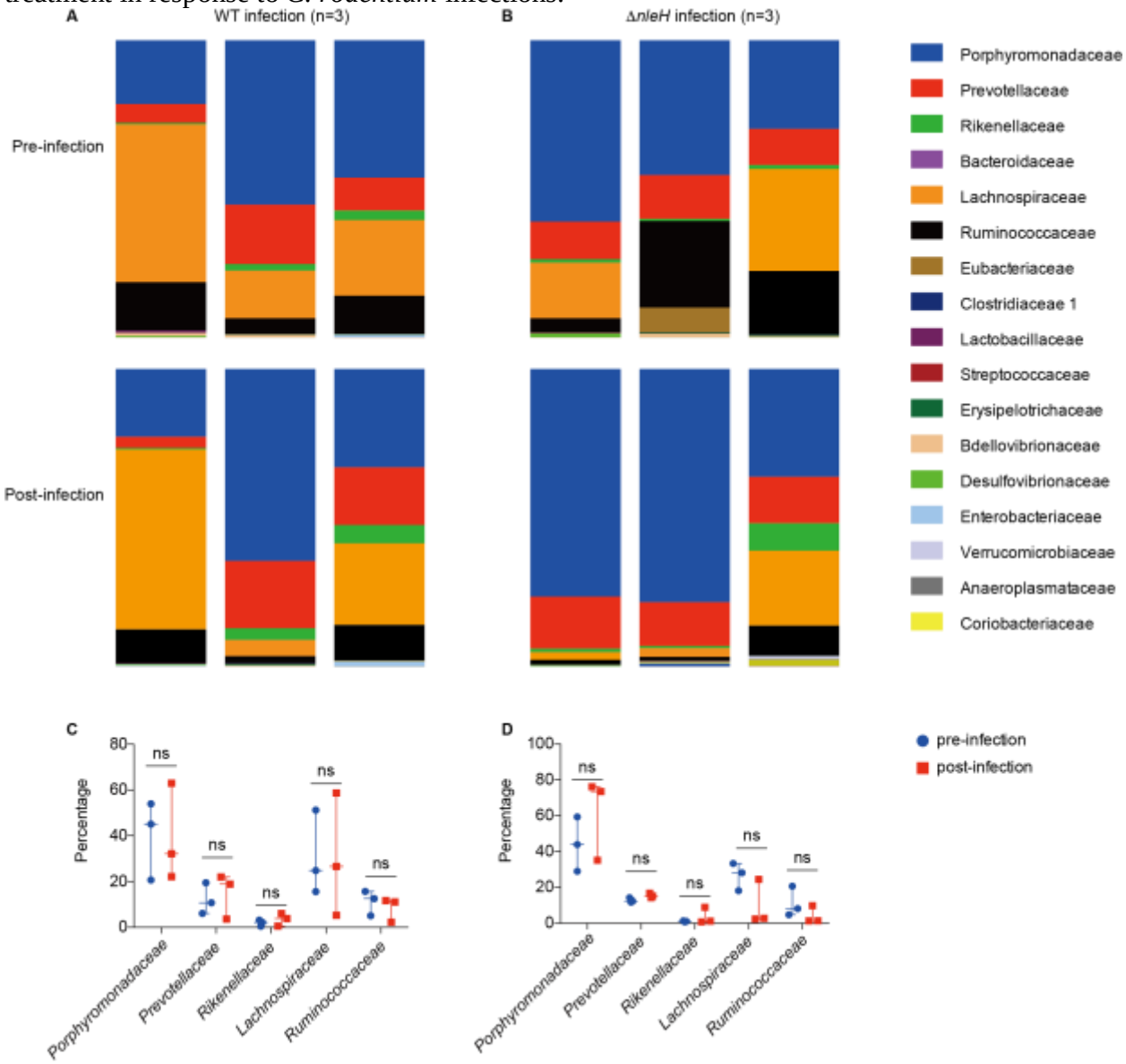
However, statistical analysis indicated that the alteration of intestinal microbiota was not significant (**Figure 24C** and **24D**). In summary, *C. rodentium* infections resulted in the changes of intestinal microbiota, but not significant alterations of the 5 bacterial families in our study (**Figure 22C** and **22D**, **23C** and **23D**, **24C** and **24D**). The statistical analysis was performed by using multiple t tests (Holm-Sidak test) without assuming a consistent SD. Each row was analyzed individually, $p < 0.05$ was considered as significant difference.

Figure 22 Composition of intestinal microbiota in C57BL/10ScNJ mice without microbiota transfer treatment in response to *C. rodentium* infections.



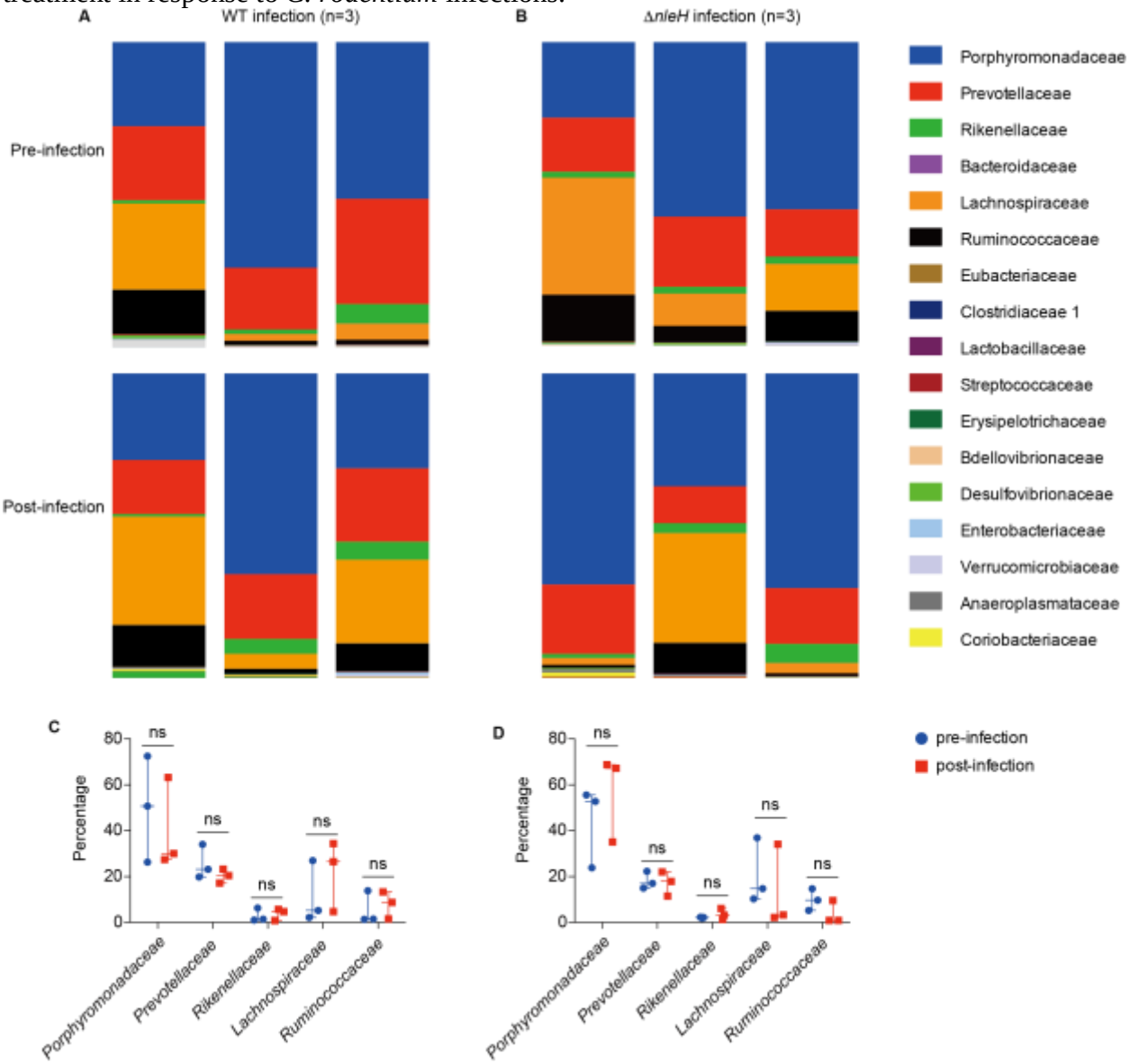
Note: No transfer, the C57BL/10ScNJ mice (n=3) in this group were not subjected to microbiota transfer treatment; ns, non-significant difference.

Figure 23 Composition shift of gut microbiota in C57BL/10ScNJ mice with Self-transfer treatment in response to *C. rodentium* infections.



Note: Self-transfer, the C57BL/10ScNJ mice (n=3) received microbiota transfer from C57BL/10ScNJ (donor mouse); ns, non-significant difference.

Figure 24 Composition shift of gut microbiota in C57BL/10ScNJ mice with Cross-transfer treatment in response to *C. rodentium* infections.

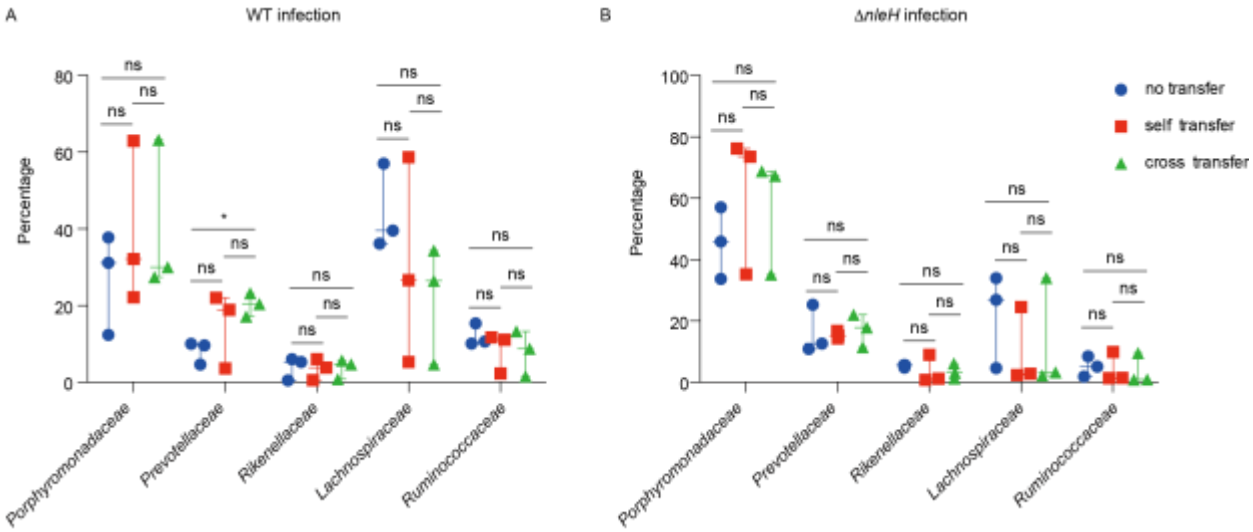


Note: Cross-transfer, the C57BL/10ScNJ mice (n=3) received microbiota transfer from C57BL/6J mice (donor mouse); ns, non-significant difference.

In **Figure 17**, lower bacterial colonization of *C. rodentium* WT in mice that were not subjected to microbiota transfer was observed, whereas higher bacterial colonization of *C. rodentium ΔnleH* was observed. We picked 5 bacterial familes (*Porphyromonadaceae, Prevotellaceae, Rikenellaceae, Lachnospiraceae,* and *Ruminococcaceae*) and compared the abundance of these 5 bacterial families in response to *C. rodentium* infections. In response to *C. rodentium* WT

infection, the abundance of *Prevotellaceae* in Cross-transfer group was significantly higher than that in No-transfer group (**Figure 25A**). However, the role of *Prevotellaceae* should be further studied to determine whether the bacteria in *Prevotellaceae* family are responsible for bacterial colonization in host. By contrast, the abundance of these 5 bacterial families slightly shifted without significant difference in response to *C. rodentium ΔnleH* infection (**Figure 25B**). Our analysis of 5 major bacterial families suggested that *C. rodentium* Δ*nleH* infection did not cause significant alteration of the abundance of the 5 bacterial families, which might indicate that other bacterial families contribute to bacterial colonization in host. The statistical analysis was performed by using multiple t tests (Holm-Sidak test) without assuming a consistent SD. Each row was analyzed individually, $p < 0.05$ was considered as significant difference.

Figure 25 Comparison of the alteration of intestinal microbiota in response to *C. rodentium* infections in C57BL/10ScNJ mice.



Note: No-transfer, the C57BL/10ScNJ mice (n=3) did not receive any microbiota transfer; Self-transfer, the C57BL/10ScNJ mice (n=3) received microbiota transfer from C57BL/10ScNJ mice (donor mouse); Cross-transfer, the C57BL/10ScNJ mice (n=3) received microbiota transfer from C57BL/6J mice (donor mouse); $p < 0.05$ (*), significant difference; ns, non-significant difference.

Discussion

In this study, we used 16S rDNA sequencing technology to address the composition shifts of gut microbiota in response to *C. rodentium* infection. We found that the composition of gut microbiota was dominated by *Bacteroidetes* phylum and *Firmicutes* phylum (**Figure 26A**), which was consistent with previous finding (Eckburg et al., 2005). The composition of gut microbiota varies among individual mice, indicating that diet and environmental stresses (mouse handling) also affect the composition of gut microbiota (**Figure 26A**). Statistical analysis of 10 most abundant bacterial families also demonstrated that the microbiota composition of C57BL/6J mouse was significantly different from that of C57BL/10ScNJ mouse (**Figure 26B**).

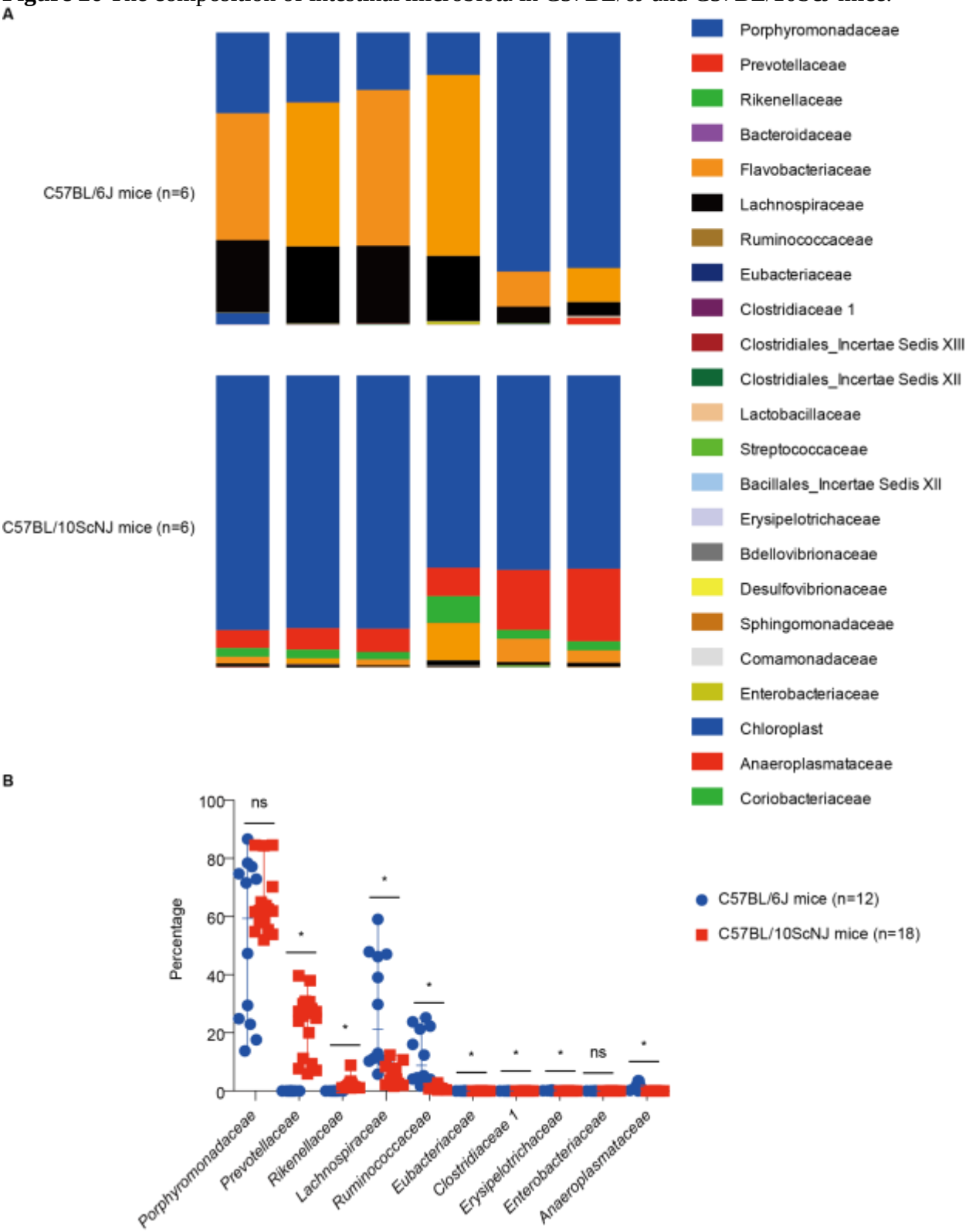
Previous study has demonstrated that transplantation of gut microbiota from strains of mice that are susceptible to *C. rodentium* infection induces similar susceptibility in mice that are previously resistant to *C. rodentium* infection (Willing et al., 2011). In this study, we performed the microbiota transfer between two closely related mouse strains (C57BL/6J mouse *vs* C57BL/10ScNJ mouse, and C3H/HeJ mouse *vs* C3H/HeOuJ mouse). We found that transferring the microbiota of C57BL/6J mouse into C57BL/10ScNJ mouse indeed altered the host susceptibility to WT *C. rodentium* infection (**Figure 17**).

In this study, we observed a higher colonization of *C. rodentium ΔnleH* mutant rather than WT *C. rodentium* in C57BL/10ScNJ mice, which was consistent with a previous study (Feuerbacher and Hardwidge, 2014), suggesting that T3SS-dependent NleH effector contribute to host resistance to WT *C. rodentium*. Transplantation of microbiota derived from C57BL/6J mice into C57BL/10ScNJ mice appeared to contribute to alter host resistance to WT *C. rodentium* infection. We also found that both C3H/HeJ mice and C3H/HeOuJ mice were resistant to WT *C. rodentium* infection, whereas both of the two mouse strains were susceptible to *C. rodentium ΔnleH* mutant,

which was inconsistent with previous finding (Vallance et al., 2003; Willing et al., 2011). Given the role of NleH effector in modulation of NF-κB signaling (Gao et al., 2009), we speculated that the higher mortality in both C3H/HeJ mice and C3H/HeOuJ mice attributed to intestinal inflammation, which was not associated with TLR4-mediated NF-κB signaling.

Here we presented observational data on colonization of *C. rodentium* in mouse model. We found that genetic factor (*tlr*4 deletion) affected the composition of gut microbiota in C57BL/10ScNJ mouse, and the gut microbiota in C57BL/10ScNJ mouse promoted the resistance to WT *C. rodentium* infection compared to *C. rodentium* Δ*nleH* infection. Microbiota transfer indeed altered the resistance of C57BL/10ScNJ mouse to WT *C. rodentium* infection. The microbial community analysis was performed at family level. A deep analysis of the shift of composition of gut microbiota might provide insight into the bacterial family or species that account for the alteration of resistance to *C. rodentium* infection.

Figure 26 The composition of intestinal microbiota in C57BL/6J and C57BL/10ScJ mice.



Note: The *p* value was calculated by using Holm-Sidak test; $p < 0.05$ (∗), significant difference; ns, non-significant difference.

Chapter 5 - Conclusions

To survive and multiply in host, diarrheagenic *E. coli* develops strategies to subvert host immune responses, compete with gut microbiota for nutrition, and sense environmental signals to regulate the expression of virulence factors. In this thesis work, we focused on diarrheagenic *E. coli* signaling and interaction with host NF-kB signaling pathway and intestinal microbiota, which somehow contribute to colonization of diarrheagenic *E. coli* in host intestine.

In the first project of this thesis (Chapter 2), we aimed to identify the ESF and elucidate the mechanism by which ESF blocks TNF-induced NF-κB activation. By employing fast protein liquid chromatography, we divided ETEC-M9 supernatant into different fractions and ESF-containing fractions were separated on SDS-PAGE and in-gel digested. Our work indicated that ETEC flagellin was necessary and sufficient to block IκBα degradation in response to TNF. We subsequently aimed to characterize ETEC flagellin. Our work demonstrated that only full length of flagellin can block IκBα degradation in response to TNF, which was in a TLR5-independent manner. However, the mechanism was not fully understood. ETEC flagellin was reported to contribute to ETEC adherence to intestinal cells, which was independent of serotype (Roy et al., 2009). Our work and previous finding (Wang and Hardwidge, 2012) indicate ETEC flagellin has additional function. Our work might provide potential target for development of anti-inflammatory agent targeting NF-κB signaling. For the future work, the efforts can be made to first determine whether ETEC flagellin enters HCT-8 cells to block IκBα degradation in response to TNF. Second, another direction is to determine whether TNFR is subjected to internalization of in HCT-8 cell in response to ETEC flagellin, which might contribute to address the mechanism.

In the Chapter 3, we aimed to identify cAMP importer in ETEC. By employing Tn5 transposome technology, we successfully constructed mutant library and screened mutants for the hyposensitivity to extracellular cAMP. However, none of the disrupted genes derived from the mutant library were related to cAMP importer. Although the results were negative, our efforts benefit us to identify cAMP importer in future. Given that cAMP regulates transcription of genes related to virulence factor such as enterotoxins and colonization factors in ETEC strains, we reasonably believe that identification of cAMP importer might advance our understanding of interaction of ETEC bacterium with host contributing to its colonization in small intestine, and might provide insight into developing potential pharmaceutical agents for treatment and/or prevention ETEC infections. In this study, the commercial Tn5 transposome kit has been demonstrated to be a suitable tool for preparation of mutant library by using ETEC H10407 Δ*cyaA* strain. For future direction, a reporter plasmid that can interact with intracellular cAMP or activated CRP might be a potential method for screening for the mutants. In our hypothesis, extracellular cAMP can enter ETEC H10407 cells, which might be related to transmembrane proteins. Another direction is to explore the transmembrane proteins that can interact with cAMP in ETEC H10407.

In the Chapter 4, we aimed to evaluate the influence of gut microbiota and T3SS effector on colonization of A/E pathogens in mouse model. We performed microbiota transfer between to genetically related mouse strains. In this study, we observed a higher colonization of *C. rodentium ΔnleH* mutant in C57BL/10ScNJ mice and microbiota transfer affected the colonization of WT *C. rodentium* in C57BL/10ScNJ mice in this study. Unexpectedly, the C3H/HeJ mice in our study were more resistant to WT *C. rodentium* infection, but more susceptible to *C. rodentium ΔnleH* mutant infection. For the future direction, it is necessary to perform additional animal experiments to confirm the susceptibility of C3H/HeJ mice or C3H/HeOuJ mice to *C. rodentium* infection. We

next used 16S rDNA sequencing technology to study the composition of gut microbiota in response to *C. rodentium* infection, and tried to address the difference from the point of gut microbiota. Our preliminary analysis suggested that the relative abundance of *Bacteroidetes* phylum and *Firmicutes* phylum might account for the bacterial colonization. For future direction, a more powerfully advanced software should be employ to analyze the sequencing data of intestinal microbiota, which enable us to analyze the microbiota data, if possible, at genus or species level.

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