The role of EspO1 in *Escherichia coli* pathogenesis

Ph.D. Thesis
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By
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Abstract

Enterohaemorrhagic *E. coli* (EHEC) has been the causative agent of diarrhoeal outbreaks for decades. EHEC virulence relies on a type 3 secretion system (T3SS), which directly translocates T3SS effectors into the host cell cytoplasm. Once translocated, T3SS effectors alter various host cell functions, including manipulating the cell death response, in order to facilitate bacterial colonisation. EHEC is equipped with at least 40 effector proteins; however, not all identified effectors are fully characterised. This study set out to investigate the role of EspO during EHEC infection. EHEC O157:H7 strain EDL933 carries 2 EspO homologs: EspO1 and EspO2.EspO homologs are also found in several enteric pathogens including *Shigella flexneri* (OspE1 and OspE2), *Salmonella enterica* serovar Typhi and Typhimurium (SopD), *Citrobacter rodentium* (EspO), and some enteropathogenic *E. coli* (EPEC) clinical isolates (EspO). It has previously been established that the EspO effector family interacts with integrin linked kinase (ILK) via a conserved tryptophan residue: W68 for OspE1 and W77 for EspO1. EspO1 and EspO2 co-operatively inhibit cell detachment by blocking focal adhesion disassembly, via their interactions with ILK and EspM2, respectively. In a previous study, HAX-1 was identified as the novel interaction partner of EspO1 by a yeast 2 hybrid (Y2H) screen. HAX-1 is an ubiquitiously expressed anti-apoptotic protein that localises to mitochondria. In this study, the EspO1-HAX-1 interaction was confirmed by direct Y2H. Functionally, EspO1 and OspE1 were shown to protect HeLa cells from staurosporine-induced apoptosis during transfection and EspO1 was able to inhibit cell death during *in vitro* infection. Additionally it was established that the EspO1 and OspE1 anti-apoptotic activity is HAX-1 dependent, but ILK independent. To summarise, this study reported that EspO1 displays anti-apoptotic activity in a HAX-1 dependent manner. Therefore, EspO1 serves 2 main functions: inhibition of apoptosis through HAX-1 and blocking of cell detachment through ILK.
Declaration of Originality

I, Sujinna Lekmeechai, hereby declare that this thesis is my own work, and that information derived from work of others has been duly acknowledged.

The mutagenesis and generation of *E. coli* O157:H7 strain 85-170 Δ*nleF* was carried out by Dr. Mitchell Pallett (CMBI, Imperial College London).

The cloning and generation of pRK5::myc-tccP (sakai) construct was carried out by Dr. Junkal Garmentai (CMBI, Imperial College London).

The HAX-1 knockdown (miHAX-1), and the control (miNEG) cell lines were generously provided by Dr. Ewa A. Grzybowska (Maria Skłodowska-Curie Memorial Cancer Center and Institute of Oncology, Poland).
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I would like to thank and acknowledge Dr. Ewa A. Grzybowska for generously providing cell lines that were very useful for this study.

I also appreciate the constructive feedback on my 9 and 18 month reports offered by Dr. Stuart Haslam and Dr. Angelika Gründling.

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<th>Description</th>
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<tbody>
<tr>
<td>A/E</td>
<td>Attaching and effacing</td>
</tr>
<tr>
<td>AA</td>
<td>Aggregative adherence</td>
</tr>
<tr>
<td>AAF</td>
<td>Aggregative adherence fimbriae</td>
</tr>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>Ade</td>
<td>L adenine hemisulfate</td>
</tr>
<tr>
<td>AIF</td>
<td>Apoptosis-induce factor</td>
</tr>
<tr>
<td>Apaf-1</td>
<td>Apoptosis-protease factor-1</td>
</tr>
<tr>
<td>Arp2/3</td>
<td>Actin-related protein2/3</td>
</tr>
<tr>
<td>ASC</td>
<td>Apoptosis-associated speck-like protein containing a CARD</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
</tr>
<tr>
<td>BI-1</td>
<td>Bax inhibitor-1</td>
</tr>
<tr>
<td>BIR</td>
<td>Baculovirus IAP repeat Domain</td>
</tr>
<tr>
<td>BFP</td>
<td>Bundle Forming Pili</td>
</tr>
<tr>
<td>BH</td>
<td>Bcl-2 homology</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CAD</td>
<td>Caspase-Activated DNase</td>
</tr>
<tr>
<td>CARD</td>
<td>Central caspase recruitment domain</td>
</tr>
<tr>
<td>CF</td>
<td>Colonisation factor</td>
</tr>
<tr>
<td>CFA</td>
<td>Colonization factor antigen</td>
</tr>
<tr>
<td>CFTR</td>
<td>Cystic fibrosis transmembrane conductance regulator</td>
</tr>
<tr>
<td>CK18</td>
<td>Cytokeratin18</td>
</tr>
<tr>
<td>CNF1</td>
<td>Cytotoxic necrotising factor 1</td>
</tr>
<tr>
<td>CR3</td>
<td>Complement receptor 3</td>
</tr>
</tbody>
</table>
cyt c  
Cytochrome c

DA  
Diffuse adherence

DAEC  
Diffusely adherent *E. coli*

DAF  
Decay-accelerating factor

DAMPs  
Damage associated molecular patterns

DD  
Death domain

DDO  
Double Dropout

DED  
Death effector domain

DIABLO  
Direct inhibitor of apoptosis-binding protein with low pI

DISC  
Death-inducing signalling complex

DMEM  
Dulbecco's modified eagle medium

DnaJB1  
DnaJ heat shock protein family (Hsp40) member B1

DnaJB6b  
DnaJ heat shock protein family (Hsp40) member B6 variant 2

DTT  
Dithiothreitol

EAEC  
Enteroaggregative *E. coli*

EAF  
Enteropathogenic *E. coli* adherence factor

EAST1  
Enteroaggregative *E. coli* heat-stable enterotoxin 1

EBP50  
ERM-binding phosphoprotein 50

EIEC  
Enteroinvasive *E. coli*

EPEC  
Enteropathogenic *E. coli*

EhaA  
*E. coli* immunoglobulin-binding protein

EHEC  
Enterohaemorrhagic *E. coli*

ER  
Endoplasmic reticulum

ERM  
Ezrin/radixin/moesin

EspFU  
*E. coli* secreted protein F in prophage U
ETEC  Enterotoxigenic *E. coli*
FA    Focal adhesion
FADD  Fas-Associated protein with Death Domain
FAK   Focal adhesion kinase
FcγRs Fcγ receptors
FCS   Fetal calf serum
GADPH Glyceraldehyde-3-phosphate dehydrogenase
Gb3   Globotriaosylceramide
GC    Guanylate cyclase
GlcNAc N-acetylglucosamine
GrlA   Global regulator of LEE activator
GrlR   Global regulator of LEE repressor
HAX-1  HCLS1-associated protein X-1
HBSS  Hank's balanced salt solution
Hcp   Haemolysin co-regulated protein
His   L histidine HCl monohydrate
HlyA   α-haemolysin
HMGB1 High mobility group box 1
HRK   Harakiri (also known as death protein-5)
HUS   Haemolytic uremic syndrome
IκB   Inhibitor of NF-κB
IAP   Inhibitor of apoptosis
IBCs  Intracellular bacterial communities
IHF   Integration host factor
IKK   IκB Kinase
<table>
<thead>
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<th>ILK</th>
<th>Integrin linked kinase</th>
</tr>
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<tbody>
<tr>
<td>IM</td>
<td>Inner membrane</td>
</tr>
<tr>
<td>IMC</td>
<td>Inner membrane complex</td>
</tr>
<tr>
<td>IAP</td>
<td>Inhibitor of apoptosis</td>
</tr>
<tr>
<td>IP3R</td>
<td>Inositol 1,4,5-triphosphate receptor</td>
</tr>
<tr>
<td>IRTKS</td>
<td>Insulin receptor tyrosine kinase substrate</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>LEE</td>
<td>Locus of enterocyte effacement</td>
</tr>
<tr>
<td>LB</td>
<td>Lysogeny Broth</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LA</td>
<td>Localised adherence</td>
</tr>
<tr>
<td>Ler</td>
<td>LEE encoded regulator</td>
</tr>
<tr>
<td>LiAc</td>
<td>Lithium acetate</td>
</tr>
<tr>
<td>LT</td>
<td>Heat-labile enterotoxin</td>
</tr>
<tr>
<td>MAP</td>
<td>Mitogen-activated protein</td>
</tr>
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<td>Double minute 2</td>
</tr>
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<td>MFP</td>
<td>Membrane fusion protein</td>
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<tr>
<td>MLK1</td>
<td>Mixed-lineage kinase domain-like protein</td>
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<tr>
<td>N-WASP</td>
<td>Neural Wiskott-Aldrich syndrome protein</td>
</tr>
<tr>
<td>NC</td>
<td>Needle complex</td>
</tr>
<tr>
<td>NEMO</td>
<td>NF-κB essential modulator</td>
</tr>
<tr>
<td>NMEC</td>
<td>Neonatal meningitis-associated <em>E. coli</em></td>
</tr>
<tr>
<td>NHE3</td>
<td>Na(^+/)H(^+) exchanger</td>
</tr>
<tr>
<td>NHERF1</td>
<td>Na(^+/)H(^+) exchanger regulatory factor 1</td>
</tr>
<tr>
<td>NIK</td>
<td>NF-κB-inducing kinase</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>NK cell</td>
<td>Natural killer cell</td>
</tr>
<tr>
<td>Nle</td>
<td>Non-LEE effector</td>
</tr>
<tr>
<td>NLR</td>
<td>Nucleotide-binding oligomerisation domain-like receptor</td>
</tr>
<tr>
<td>OM</td>
<td>Outer membrane</td>
</tr>
<tr>
<td>OMP</td>
<td>Outer membrane protein</td>
</tr>
<tr>
<td>PAI</td>
<td>Pathogenicity island</td>
</tr>
<tr>
<td>PAMPs</td>
<td>Pathogen-associated molecular patterns</td>
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<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PBST</td>
<td>Phosphate buffered saline containing 0.02% Tween-20</td>
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<tr>
<td>PCC</td>
<td>Protein conduction channel</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>PDZ</td>
<td>Postsynaptic density95/Disc large/Zonula occludens-1</td>
</tr>
<tr>
<td>Pet</td>
<td>Plasmid encoded toxin</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PIGR</td>
<td>Polymeric immunoglobulin receptor</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PMF</td>
<td>Proton motive force</td>
</tr>
<tr>
<td>Pol β</td>
<td>DNA polymerase β</td>
</tr>
<tr>
<td>PRRs</td>
<td>Pattern recognition receptors</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidenedifluoride</td>
</tr>
<tr>
<td>QIRs</td>
<td>Quiescent intracellular reservoirs</td>
</tr>
<tr>
<td>QDO</td>
<td>Quadruple dropout</td>
</tr>
<tr>
<td>REPEC</td>
<td>Rabbit enteropathogenic <em>E. coli</em></td>
</tr>
<tr>
<td>RING</td>
<td>Really interesting new gene</td>
</tr>
<tr>
<td>RPS3</td>
<td>Ribosomal protein S3</td>
</tr>
</tbody>
</table>
SAM  S-adenosyl methionine
SD agar  Synthetic Defined agar
SDS  Sodium dodecyl sulfate
Sec  Secretory
SERCA2  Sarcoplasmic reticulum Ca\(^{2+}\) transport ATPase2
SGLT1  Na\(^+\)/glucose cotransporter
Smac  Second mitochondria-derived activator of caspases
SPATE  Serine protease autotransporters of Enterobacteriaceae
ST  Heat-stable entero toxin
STEC  EHEC autotransporter encoding gene A
STS  Staurosporine
Stx  Shiga toxin
T1SS  Type 1 secretion system
T2SS  Type 2 secretion system
T3SS  Type 3 secretion system
T4SS  Type 4 secretion system
T5SS  Type 5 secretion system
T6SS  Type 6 secretion system
Tat  Twin-arginine translocation
TdT  Terminal deoxynucleotidyl transferase
TIM17b  Translocase of inner mitochondrial membrane 17b
Tir  Translocated intimin receptor
TLR  Toll-like receptor
TNF  Tumour necrosis factor
TNFR  Tumour necrosis factor receptor
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRADD</td>
<td>Tumour necrosis factor receptor type 1-associated death domain</td>
</tr>
<tr>
<td>TRAF</td>
<td>Tumour necrosis factor receptor-associated factor</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP Nick-End Labeling</td>
</tr>
<tr>
<td>TRAIL1-R</td>
<td>Tumour necrosis factor-related apoptosis-inducing ligand receptor 1</td>
</tr>
<tr>
<td>UPEC</td>
<td>Uropathogenic <em>E. coli</em></td>
</tr>
<tr>
<td>UPR</td>
<td>Unfolded protein response</td>
</tr>
<tr>
<td>XIAP</td>
<td>X-linked inhibitor of apoptosis protein</td>
</tr>
<tr>
<td>Y2H</td>
<td>Yeast two hybrid</td>
</tr>
<tr>
<td>YPD</td>
<td>Yeast extract peptone dextrose</td>
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Chapter 1 Introduction
Chapter 1 Introduction

The interaction between pathogens and their hosts is a sophisticated process. While bacterial pathogens have elaborated several strategies to conquer their infectious niche, hosts have multiple defensive mechanisms to counteract pathogens. Bacterial pathogens are equipped with numerous tools including secretion systems, which translocate bacterial proteins to facilitate pathogen infection and persistence. On the other side, hosts have evolved humoral and cell death responses to protect themselves from hazardous invasion. The main focus of this study is to investigate the function of proteins secreted by pathogenic Escherichia coli (E. coli), as well as the host responses they target.

1.1 Escherichia coli

E. coli was discovered in 1885 by Theodor Escherich and was initially described as a non-pathogenic bacteria. It is now classified in the family Enterobacteriaceae and is one of the most widely studied bacterial species (Zargar et al. 2015). Apart from being a versatile laboratory tool, E. coli is present in human as both commensal and bacterial pathogen (Belanger et al. 2011).

1.1.1 Commensal E. coli

E. coli is predominant facultative microflora of the human gastrointestinal tract (Nataro and Kaper 1998, Tenaillon et al. 2010, Belanger et al. 2011). E. coli is among the first bacteria to colonise the human colon (Sweeney et al. 1996, Kaper et al. 2004, Mirpuri et al. 2010). Even though factors that aid in E. coli colonisation remain incompletely elucidated, it is hypothesised that E. coli is able to use gluconate, abundant in intestinal mucus, as a nutrient (Sweeney et al. 1996).
In healthy circumstances, the host benefits from *E. coli* colonisation. Commensal *E. coli* serves as a probiotic due to its ability to prevent the colonisation of pathogens (Leatham *et al.* 2009). Furthermore, commensal *E. coli* strains have been shown to modulate host immune responses through NF-κB signalling pathway (Mirpuri *et al.* 2010, Zargar *et al.* 2015).

### 1.1.2 Pathogenic *E. coli*

Several *E. coli* strains have evolved and become pathogens through the acquisition of virulence genes. These pathogenic strains cause disease even in healthy individuals and can be divided into non-diarrhoeagenic and diarrhoeagenic pathotypes (Kaper *et al.* 2004).

#### 1.1.2.1 Non-Diarrhoeagenic *E. coli*

Non-diarrhoeagenic *E. coli* causes disease in humans and animals. Although they are capable of asymptotically colonising the gut, these strains are characteristically different from commensal *E. coli* and responsible for various diseases such as urinary tract infection, neonatal sepsis, and poultry airway infection. Non-diarrhoeagenic *E. coli* that causes diseases in human include (i.) uropathogenic *E. coli* (UPEC), and (ii.) neonatal meningitis-associated *E. coli* (NMEC) (Johnson and Russo 2002).

**i. Uropathogenic *E. coli***

UPEC is responsible for approximately 80% of reported urinary tract infection cases (Belanger *et al.* 2011). UPEC infection leads to cystitis in the urinary bladder and pyelonephritis in the kidneys. UPEC employs several virulence factors including toxins and multiple pili which are tipped by adhesins; each of which is responsible for different states of infection. The better characterised pili are Type I pili tipped with FimH, and P pili tipped with PapG adhesin (Croxen and Finlay 2010). The pathogenesis of UPEC infection begins with the exposure and colonisation of the pathogen in the periurethral area, followed by the
ascension of UPEC into the bladder or the kidneys (Flores-Mireles et al. 2015). The pathogenesis of UPEC is summarised in Figure 1. Once migrated to the urinary bladder, UPEC produces FimH-tipped TypeI pili. FimH binds mannosylated uroplakins and integrins leading to bacterial internalisation via Rho GTPase activation and actin rearrangement (Martinez and Hultgren 2002).

Inside the bladder cells, UPEC forms intracellular bacterial communities (IBCs) subsequent to intracellular propagation. IBCs promote UPEC survival, antibiotic resistance, and bacterial invasion into adjacent cells (Anderson et al. 2003, De La Fuente-Nunez et al. 2013). Apart from IBCs, UPEC can also exist in a non-replicating mode, which can be re-activated and can survive for months, called quiescent intracellular reservoirs (QIRs) (Hannan et al. 2012). However, lipopolysaccharides (LPS) produced by UPEC also activate toll-like receptor 4 (TLR4) resulting in bacterial exocytosis into the bladder lumen where UPEC transforms into filamentous morphology, which enhances the resistance to innate immune response and phagocytosis (Justice et al. 2006, Horvath et al. 2011). UPEC also secretes α-haemolysin (HlyA) toxin, which mediates cell lysis and facilitates nutrient acquisition. HlyA also induces urinary bladder epithelial exfoliation, promoting the dissemination of the pathogen into new hosts (Dhakal and Mulvey 2012). Furthermore, UPEC induces host cell apoptosis by secreting cytotoxic necrotising factor 1 (CNF1) (Mills et al. 2000). In the kidneys, UPEC utilises PapG-tipped P pili to facilitate renal colonisation via the binding between PapG adhesin and globoside expressed on the surface of renal cells. PapG also downregulates polymeric immunoglobulin receptor (PIGR) via an interaction with TLR4. This leads to the decrease of IgA transportation across the tissue, consequently alleviating UPEC clearance and opsonisation. Thus, PapG enhances bacterial survival and the establishment of its infectious niche (Flores-Mireles et al. 2015).
**Figure 1. Illustration summarising UPEC pathogenesis.** (A) In the bladder, UPEC invades the bladder cells, and either forms the IBCs, or camouflages in QIRs. (B) In the kidneys, UPEC attaches to the renal cells by using P pilus, and dampens the host inflammatory responses by decreasing IgA transportation (Flores-Mireles et al. 2015). QIRs, quiescent intracellular reservoirs; IBCs, intracellular bacterial communities. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Microbiology (Flores-Mireles, Walker et al. 2015), copyright 2015.

**ii. Neonatal Meningitis-Associated E. coli**

NMEC is the prominent causative agent of neonatal meningitis. NMEC infection leads to poor outcome and developmental impairment of the surviving infants (Mirpuri et al. 2010). The majority of NMEC strains possess K₁ polysaccharide capsule which is essential for
bacterial penetration into the central nervous system (Kim et al. 1992). As reviewed by Kaper and colleagues (2004), the pathogen translocates from the gastrointestinal tract, into the bloodstream, and finally enters the central nervous system. K₁ E. coli uses S fimbriae to attach itself to the luminal surface of microvascular endothelial cells. The invasion through the blood brain barrier (BBB) is mediated by outer membrane protein A (OmpA), which binds to the glycoprotein of microvascular epithelial cells. Once penetrated through the BBB, K₁ capsule enhances bacterial survival by promoting immune resistance (Kaper et al. 2004).

1.1.2.1 Diarrhoeagenic E. coli

Diarrhoeagenic E. coli pathotypes establish an infectious niche in the gut and are categorised into 6 pathotypes; (i.) enterotoxigenic E. coli (ETEC), (ii.) enteroinvasive E. coli (EIEC), (iii.) enteroaggregative E. coli (EAEC), (iv.) diffusely adherent E. coli (DAEC), (v.) enteropathogenic E. coli (EPEC), and (vi.) enterohemorrhagic E. coli (EHEC) (Belanger et al. 2011).

i. Enterotoxigenic E. coli

ETEC is defined as E. coli strains encoding either heat-stable (ST) or heat labile (LT) enterotoxin, or both. ETEC is the prevalent cause of diarrhoea in travellers and children in developing countries. The infection is caused by consuming contaminated food and water (Nataro and Kaper 1998). ETEC is also responsible for diarrhoea in cattle and piglets (Nataro and Kaper 1998, Belanger et al. 2011). The symptom of ETEC infection is watery diarrhoea that could either be self-limited or severe diarrhoea (Nataro and Kaper 1998). ETEC pathogenesis begins with bacterial attachment to the small intestine epithelium. The attachment is firstly mediated by long-range flagella, tipped by EtpA (Roy et al. 2009). Then, once the flagella are degraded, colonisation factor (CF) fimbriae are responsible for further attachment (Roy et al. 2009). ETEC also secretes ST and LT which induce watery diarrhoea
(Kaper et al. 2004). ST binds to the intestinal epithelial cell receptor, guanylate cyclase (GC)-C, leading to the increase of cGMP levels. Consequently, the elevation of cGMP concentration inhibits sodium and chloride ion uptake as well as increasing the secretion of Cl\(^-\). This leads to fluid secretion into the intestinal lumen, followed by secretory diarrhoea. LT induces the increase of cAMP levels, resulting in Cl\(^-\) efflux and diarrhoea (Sears and Kaper 1996, Nataro and Kaper 1998, Croxen and Finlay 2010)

**ii. Enteroinvasive E. coli**

EIEC causes watery diarrhoea which can be difficult to distinguish from secretory diarrhoea induced by ETEC. EIEC infection however causes inflammatory colitis. EIEC is a foodborne and waterborne pathogen; however, EIEC can also be transmitted from person-to-person (Nataro and Kaper 1998). EIEC is closely related to *Shigella spp.* in biochemical, genetical, and pathogenic aspects (Kaper et al. 2004). The prominent virulence factor of EIEC is a Type 3 secretion system (T3SS) encoded by the *mxi* and *spa* loci carried on a pINV virulence plasmid (Croxen and Finlay 2010). EIEC and *Shigella* infections involve several steps; EIEC penetration into intestinal epithelium cells in an endocytic vacuole, pathogen-mediated release from the vacuole, intracellular reproduction, lateral movement through the cytoplasm, and invasion into adjacent cells (Nataro and Kaper 1998). The movement of EIEC is mediated by actin ‘tail’ formation caused by IcsA, which is localised at one pole of the bacterial cell (Goldberg and Theriot 1995, Egile et al. 1999). T3SS effectors IpaA, IpaB, IpaC, and IpaD contribute to EIEC invasiveness. IpaC and IpaA mediate actin polymerisation, and depolymerisation, respectively (Bourdet-Sicard et al. 1999, Tran Van Nhieu et al. 1999). IpaB aids in escaping from phagoctytic vacuoles in macrophages (High et al. 1992). IpgD causes host cell membrane blebbing and facilitates bacterial internalisation (Niebuhr et al. 2002). The multiple IpaH effectors are suspected to modulate inflammation (Ashida et al. 2007). In addition to the T3SS, EIEC secretes enterotoxins. Even though the...
role of toxins in EIEC pathogenesis remains unclear, they are believed to contribute to EIEC-induced watery diarrhoea (Kaper et al. 2004, Croxen and Finlay 2010).

iii. Enteroaggregative E. coli

EAEC manifests an aggregative adherence (AA) pathotype named because of the “stacked-brick” adherence phenotype on cultured Hep-2 cells, shown in Figure 3B and can form biofilms on cells and tissue (Jafari et al. 2013). The symptoms of EAEC infection are watery diarrhoea either with or without mucus, abdominal pain, nausea, vomiting, and fever. Normally, diarrhoea caused by EAEC is self-limited; however, persistent diarrhoea can be observed in HIV/AIDS patients as well as malnourished children. In May 2011, a diarrhoeal epidemic caused by EAEC O104:H4, which laterally acquired phage-encoded Stx2, caused hemolytic uremic syndrome (HUS) in approximately 23% of patients (Rasko et al. 2011). EAEC is transmitted via the fecal-oral route (Cennimo et al. 2007). The pathogenic mechanism of EAEC is not completely understood as EAEC is a heterogeneous pathogen equipped with different virulence genes, some of which are also found in ETEC and Shigella. The main virulence factors of EAEC are aggregative adherence fimbriae (AAF) which mediate bacterial adherence to the gut, dispersin which promotes EAEC spreading, and toxins which induce mucosal toxicity (Czeczulin et al. 1997, Sheikh et al. 2002). EAEC pathogenesis can be summarised by the following 3 stages: 1) attachment, 2) mucus production in the gut, and 3) mucosal toxicity caused by toxins e.g. plasmid encoded toxin (Pet), and heat stable toxin EAST-1 (Boisen et al. 2009, Boisen et al. 2012, Philipson et al. 2013)

iv. Diffusely Adherent E. coli

DAEC is characterised by E. coli strains that express a diffuse adherence (DA) pattern on Hep-2 cells, illustrated in Figure 3C. DA pattern is described as a cellular projection that engulfs bacterial cells (Nataro and Kaper 1998). DAEC is thought to be one of the causative
agents of diarrhoea in children aged between 1 to 5 years old (Croxen and Finlay 2010). Even though the pathogenesis of DAEC is the subject of speculation, the DA phenotype was reported to be mediated by Dr adhesin encoded by f1845 gene. Dr adhesin binds to decay-accelerating factor (DAF) on the host cell membrane and induces signal transductions resulting in DAF upregulation which, in turn, enhances bacterial adhesion as well as tumour necrosis factor α (TNFα) and IL-1β synthesis. Apart from the effect on epithelial cells, DAEC also induces the migration and apoptosis of polymorphonuclear leukocytes. Furthermore, DAEC secretes the serine protease autotransporter of Enterobacteriaceae (SPATE), Sat, to induce the disruption of tight junctions and focal adhesions (FAs) leading to cell detachment and cell death (reviewed in Servin 2005, Clements et al. 2012).

v. Enteropathogenic E. coli

EPEC was the first E. coli pathotype discovered in the 1940s as the cause of acute diarrhoea in infants (Kaper et al. 2004). EPEC is defined as pathogenic E. coli strains that manifest attaching and effacing (A/E) lesions (Nataro and Kaper 1998). A/E lesions (shown in Figure 2) are caused by intimate bacterial attachment, massive cytoskeleton re-arrangement into a pedestal-like structure beneath the site of bacterial attachment, and microvilli destruction. Important EPEC virulence genes are encoded in the locus of enterocyte effacement (LEE) pathogenicity island (PAI). The LEE encoded T3SS apparatus and effector proteins are essential for A/E lesion induction (Garmendia et al. 2005).

Within EPEC, there are 2 sub-pathotypes categorised as typical and atypical EPEC. Typical EPEC strains manifest a localised adherence (LA) pattern by forming microcolonies on the surface of Hep-2 cells (Figure 3A) (Hernandes et al. 2009). Microcolony formation is mediated by Bundle Forming Pili (BFP) encoded on the EPEC adherence factor (EAF) plasmid (Trabulsi et al. 2002). O55:H6, O86:H34, O111:H2, O114:H2, O119:H6, O127:H6, O142:H6, O142:H34 are frequently isolated typical EPEC serotypes (Trabulsi et al. 2002).
The prototype EPEC O127:H6 strain E2348/69 used in laboratories worldwide is also a typical EPEC (Iguchi et al. 2009). In contrast, atypical EPEC carries the LEE PAI, but not the EAF plasmid. Therefore, atypical EPEC is able to induce A/E lesions, but do not manifest the LA adherent pattern (Figure 3D). Atypical EPEC include the serotypes O26:H11, O55:H7, O55:H34, O86:H8, O111:H9, O111:H25, O119:H2, and O129:H2 (Trabulsi et al. 2002). Both typical and atypical EPEC are pathogenic; however, these strains affect different geographical areas. While atypical EPEC is more common in industrialised countries such as the USA and the UK, typical EPEC causes mortality and illnesses in underdeveloped countries e.g. Mexico, South Africa, and Brazil. EPEC is transmitted by the ingestion of contaminated food and water (Trabulsi et al. 2002). The mechanism of EPEC pathogenesis is discussed in detail in section 1.3.

Figure 2. Electron micrograph demonstrating A/E lesion induced by EPEC in a piglet.
Figure 3. The adherence pattern of different *E. coli* pathotypes. Hep-2 cells infected with (A) typical EPEC manifesting LA pattern, (B) EAEC exhibiting AA adherent phenotype, (C) DAEC displaying DA phenotype, and (D) atypical EPEC (bacteria are indicated by arrows) (Benevides-Matos et al. 2015).

vi. Enterohaemorrhagic *E. coli*

EHEC is defined as an *E. coli* strain that is able to secrete shiga toxin (Stx), and induce A/E lesions (Frankel *et al.* 1998, Nataro and Kaper 1998). EHEC has been responsible for several outbreaks over the past few decades and is considered an important enteric pathogen in Europe, North America, Australia, Japan, Chile, and South Africa. EHEC is transmitted via the fecal-oral route by either the consumption of contaminated food or through person-to-person contact. Cattle are the most important animal reservoir of EHEC (Nguyen and Sperandio 2012). Recently, the number of reported illnesses associated with the consumption of contaminated food has been increasing (Berger *et al.* 2010). EHEC infection causes a variety of symptoms ranging from mild diarrhoea to fatal HUS defined by acute renal failure,

Like EPEC, EHEC strains contain the LEE encoded T3SS which is required for A/E lesion production, but unlike EPEC, EHEC also produces the phage encoded Stx responsible for HUS (Le Blanc 2003). Stx targets glycolipid globotriaosylceramide (Gb3) which is expressed on renal glomerular cells. Stx inhibits protein synthesis resulting in the death of renal endothelial cells and consequently leads to the occlusion of glomerular microvasculature, the impairment of kidney function, and, ultimately, renal failure. Interestingly, Stx can also be taken up by Gb-3 negative intestinal cells by micropinocytosis, and instead of inhibiting protein synthesis in these cells, Stx is believed to dampen the chemokine expression, therefore reducing inflammatory responses (Malyukova et al. 2009, Croxen and Finlay 2010). In addition to the LEE and the phage-encoded Stx, EHEC contains a conserved pO157 plasmid. The pO157 plasmid encodes several virulence factors that contribute to EHEC pathogenesis, including HlyA hemolysin; EspP which cleaves human coagulation factor V; ToxB which promotes EHEC adherence; a catalase, and StcE, which disrupts the host complement pathway (Mellies et al. 2007). The pathogenesis of each pathotype is summarised in Figure 4.
Figure 4. The pathogenesis of diarrhoeagenic *E. coli*. Each *E. coli* pathotype induces illnesses using unique pathogenic mechanisms, each of which is schematically presented. These proposed mechanisms are still not completely understood, and may not be a full explanation of *in vivo* pathogenesis. (A) EPEC colonises the small intestine and induces A/E lesions. EPEC pathogenesis could be simplified into the following process steps: 1) initial attachment, 2) T3SS effector translocation, and 3) pedestal formation. (B) EHEC exploits T3SS to induce the change of cellular physiological functions as well as secreting Stx which induces fatal HUS. (C) ETEC uses colonisation factors, e.g. CFA, to mediate the attachment before the secretion of LT and ST. Guanylate cyclase, GM1, GD1b are cell surface receptors which bind to ETEC toxin. (D) EAEC attaches to the intestinal epithelium displaying the AA.
phenotype and form a biofilm on the cell surface. The main adherence factor is fimbria called
AAFs. EAEC also secretes toxins. (E) EIEC colonises the large intestine. Similar to Shigella
spp., EIEC invades colonic epithelial cells. (F) DAEC infects the small intestine. The
hallmark of DAEC infection is the DA phenotype. AAF, aggregative adherence fimbriae;
BFP, bundle-forming pilus; CFA, colonisation factor antigen; DAF, decay-accelerating
factor; EAST1, enteroaggregative E. coli, ST1; LT, heat-labile enterotoxin; ShET1, Shigella enterotoxin 1; ST, heat-stable enterotoxin (Kaper et al. 2004). Reprinted by

1.2 Secretion Systems in Gram-negative bacteria

Bacteria have elaborated secretion machineries to interact with other organisms, including
their hosts and other bacteria (Tseng et al. 2009). Apart from the general secretion pathways
(also known as secretory (Sec) and twin-arginine translocation (Tat)), which transport
proteins from the bacterial cytoplasm to the periplasmic space, Gram-negative bacteria
utilises secretion systems to secrete proteins into the external environment. Secretion systems
can be categorised into 2 main groups based on their structures; 1) those that span the inner
membrane (IM) and the outer membrane (OM) (T1SS, T3SS, T4SS and T6SS), and 2) those
that form a pore in the OM only (T2SS and T5SS) (Costa et al. 2015).

1.2.1 General Secretion Pathways: Sec and Tat

The Sec secretion pathway is the first secretory pathway identified. The Sec secretion
pathway is found in various organisms ranging from proteobacteria to eukaryotes (Natale et
al. 2008). However, only the bacterial Sec secretion pathway is discussed herein. In bacteria,
the Sec secretion pathway mediates the translocation of nascent proteins across the bacterial
IM to the periplasmic space. This secretory pathway serves diverse cellular functions
including metabolism, uptake, secretion, and the formation of bacterial cell envelope
structure (Natale et al. 2008). Sec pathway substrates have an N-terminal signal sequence which is essential for substrate recognition. The signalling sequence also mediates an interaction with Sec secretory apparatus. The Sec secretory apparatus is comprised of an ATPase SecA and a protein conduction channel (PCC). SecA functions as an ATPase-dependent motor when associated with the PCC and as a receptor for secretory proteins. The PCC is the oligomer of at least 6 proteins: SecY, SecE, SecG, SecD, SecE, SecY, and SecJ (De Keyzer et al. 2003). The oligomer provides a clamp allowing substrate secretion. Nascent peptides are preserved in an unfolded state by chaperone SecB (Driessen 2001). The translocation is driven by energy from ATP hydrolysis and proton motive force (PMF). Once translocated into the periplasmic space, the N-terminal signal sequence of Sec substrates is cleaved. Then, Sec substrates either associate with the bacterial cell membrane or are further secreted to an extracellular environment by other secretion systems (Stathopoulos et al. 2000, Natale et al. 2008).

In contrast to the Sec pathway, the Tat pathway facilitates the secretion of folded proteins. Tat substrates contain a conserved distinct signal sequence which is Z-R-R-X-Φ-Φ, where Z and Φ stand for any polar and hydrophobic residues, respectively (Chaddock et al. 1995, Natale et al. 2008). Tat substrates are folded proteins which require specific chaperones to prevent the transportation of unfolded proteins to Tat translocase (De Lisa et al. 2003). The Tat translocase of proteobacteria consists of 3 components; TatA, TatB, and TatC. TatA functions as an anchored protein, while TatB associates with TatC resulting in a TatBC complex which aids in substrate binding (reviewed in Natale, Bruser et al. 2008). The brief mechanism of the Sec and the Tat pathway is demonstrated in Figure 5.
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1.2.2 Type 1 Secretion System (T1SS)

The T1SS translocates a large range of proteins which are 1) a great variety in size, 2) mostly acidic, and 3) most of which are glycine-rich repeats (Delepelaire 2004). T1SS secreted proteins are involved with nutrient acquisition such as iron scavenger protein HasA, and virulence e.g. haemolysins of some UPEC isolates (Delepelaire 2004, Costa et al. 2015). The T1SS apparatus spans both the IM and the OM. The T1SS contains 3 major components which are the inner membrane complex (IMC), a periplasmic adaptor protein or membrane...
fusion protein (MFP) and another membrane channel, TolC. IMC is a member of the ATP-binding cassette (ABC) transporter family (Delepelaire 2004). MFP plays a role in substrate recognition, and TolC provides the opening through the OM (Andersen et al. 2001, Balakrishnan et al. 2001). T1SS substrates are recognised by a secretion signal located in the C-terminal that is not cleaved during secretion (Delepelaire 2004). Even though the mechanism of the T1SS remains incompletely understood, it is believed that substrates bind to the IMC before being transported through the MFP cavity by using energy from ATP hydrolysis at the C-terminal of the IMC. The association of substrates and IMC-MFP complex trigger the opening of TolC starting from the periplasmic side, and, consequently, release substrates into the environment (Costa et al. 2015).

1.2.3 Type 2 Secretion System (T2SS)

The T2SS is involved in the secretion of toxins from several pathogens such as ETEC (LT), Vibrio cholerae (Cholera toxin), and Pseudomonas aeruginosa (exotoxin) (Tseng et al. 2009). The detailed structure and mechanism of T2SS are still not fully understood (Costa et al. 2015). Nevertheless, the T2SS is known to secrete proteins from the bacterial periplasmic space into the environment. T2SS machinery consists of 4 parts; OM complex, periplasmic pseudopilus, IM platform, and cytoplasmic ATPase. The IM platform is believed to play a key role on facilitating a signalling pathway amongst the other elements of the system. The pseudopilus localises in periplasm, and unlike normal pilus, it does not protrude outside the OM. The OM complex is referred as a secretion pore providing the opening channel to the environment. Generally, T2SS substrates are translocated into the periplasmic space by either Sec or Tat secretion pathway. Then, the substrates are pushed through the OM complex by the pseudopilus (Korotkov et al. 2012). The assembly of pseudopilus is believed to be energised by GspE ATPase (Costa et al. 2015)
1.2.4 Type 3 Secretion System (T3SS)

The T3SS plays an important role in host-microbe interactions by delivering secretory proteins, known as effectors, directly from the bacterial cytoplasm to the cytoplasm of target cells. The T3SS is identified in various Gram-negative bacterial pathogens such as *Yersinia spp.*, *Salmonella enterica*, *Shigella spp.*, as well as symbionts rhizobium species (Tseng *et al.* 2009, Diepold and Armitage 2015). Furthermore, the T3SS is an essential virulence factor of EHEC and EPEC as it is responsible for A/E lesions and diarrhoea. Despite a wide range of effectors serving different functions, the T3SS machinery, or needle complex (NC), is highly conserved. The NC contains 2 main structures spanning through the IM and the OM: IM ring, OM ring, and hollow filamentous structure resembling a needle protruding from bacterial surface and penetrating into the host cell cytoplasm. The T3SS also has cytoplasmic accessories which are functionally essential like chaperones and ATPase (Costa *et al.* 2015).

In EHEC and EPEC, the IM ring is comprised of EscV, EscR, EscS, EscT and EscU proteins forming a pore in the bacterial IM, as illustrated in Figure 6. The OM ring is formed by the homo-multimeric protein EscC. Lipoprotein EscJ forms a cylindrical structure across the bacterial periplasmic space and connects the IM and the OM ring. EscF serves as a needle for the T3SS NC, out of which a filament consisting of EspA protrudes. T3SS effectors are secreted through a hollow EspA filament within the EscF needle. EspA also provides an adhesive function. The translocation pore that forms on the host cell membrane consists of an EspD and EspB heteropolymer. T3SS biogenesis begins with the insertion of the IM and the OM ring by the Sec secretion pathway prior to the recruitment of cytoplasmic accessories into the NC. Then, the T3SS secretes its own distal part components from the bacterial membrane to the host cell. T3SS chaperones are also essential components for the
recruitment of effectors to the T3SS apparatus, and it is believed that they are involved with secretion hierarchy. The system is energised by the EscN ATPase (Garmendia et al. 2005).

Figure 6. EPEC and EHEC T3SS apparatus. The EHEC and EPEC T3SS NC are comprised of the IM ring (composed of EscR, EscS, EscT, EscU and EscV), and the OM ring (composed of EscC). Both rings are connected by EscJ located in the periplasm. EscF forms a needle which connects to the EspA filament. EspD and EspB associate to form a translocation pore in the host cell membrane. EscN is an ATPase providing energy for the system. SepD and SepL are cytoplasmic components of the T3SS complex (Garmendia et al. 2005).
1.2.5 Type 4 Secretion System (T4SS)

The T4SS mediates the transportation of proteins as well as DNA into a target cell (Tseng et al. 2009). The ability to translocate nucleic acid is unique to the T4SS, and allows it to contribute to the lateral gene transfer of plasmid-borne antibiotic resistant genes (Costa et al. 2015). The T4SS is found in several Gram-negative bacterial pathogens; for example, in Legionella pneumophila, H. pylori, P. aeruginosa, and in plant pathogen Agrobacterium tumefaciens (Tseng et al. 2009). The T4SS of A. tumefaciens is well characterised, and thus is explained herein (Costa et al. 2015). The T4SS of A. tumefaciens consists of 12 proteins. T4SS translocation apparatus contains 3 main structural components: an OM complex, an IM complex, and a central stalk which connects the 2 membrane complexes. Two components of the T4SS; VirB2 and VirB5 associate with the pilus that protrudes into the extracellular space. The pilus contributes to the contact between mating cells as well as DNA transportation. The OM complex is formed by VirB7, VirB8, and the C-terminal of VirB10, and the IM complex is formed by VirB3, VirB4, VirB6, VirB8 and the N-terminal of VirB10. VirB1 is essential for pilus biogenesis (Christie et al. 2014). The mechanism of T4SS function is yet to be elucidated; however, it is proposed that there is a switch between pilus biogenesis and substrate secretion. The switch is regulated by VirB11 (Costa et al. 2015).

1.2.6 Type 5 Secretion System (T5SS)

The T5SS is exclusive in that T5SS secretory proteins form their own translocation pore, and are able to mediate their own secretion, thus T5SS are known as ‘autotransporters’. The T5SS contributes to the secretion of virulence factor of several pathogens; for example, E. coli (adhesins), Neisseria gonorrhoea (IgA protease), Shigella flexneri (SepA), Rickettsia spp. (S-layer protein), H. pyroli (Hsr), and plant pathogen Dickeyadadantii (HecA/HecB) (Tseng et al. 2009). Additionally, the T5SS participates in interbacterial adhesion, and
biofilm formation (Costa et al. 2015). The T5SS is categorised into 5 subclasses: a, b, c, d, and e. The subclass a is regarded as the classical subclass of T5SS, thereby it is discussed herein. Typical T5aSS substrates contain a conserved C-terminal translocator domains which form β-barrels in the bacterial OM, and a translocated N-terminal domain (Tseng et al. 2009, Costa et al. 2015). The secretion mechanism of T5SS is not completely understood. The current proposed model assumes that T5aSS substrates are transported into the periplasmic space by the Sec pathway. In the periplasmic space, ScrA chaperone maintains substrates in an unfolded state. Then, unfolded substrates are targeted to the Bam complex which mediates the insertion of the C-terminal translocator domain of substrates into the OM. Consequently, the N-terminal passenger domain is translocated through the β-barrel pore. Apart from adhesins which remain associated with the bacterial cell, other substrates are cleaved by their own peptidase domain and are released into the extracellular environment (Costa et al. 2015).

1.2.7 Type 6 Secretion System (T6SS)
The T6SS contributes to the virulence of Vibrio cholerae, Edwardsiella tarda, Burkholderia oryzae, P. aeruginosa as well as biofilm formation of free-living Myxococcus xanthus. The T6SS secretes effector proteins into both eukaryotic and prokaryotic cells (Tseng et al. 2009). Currently, the T6SS is prominently associated with bactericidal activity and growth competition in a biofilm community. The biogenesis of T6SS is tightly regulated by environmental factors; for example, quorum sensing, biofilm formation, ion depletion, and salinity (Tang et al. 2016). The T6SS consists of 2 main components; a membrane complex, and a tail complex. The membrane complex contains IM protein TssM which mediates an interaction between TssL and lipoprotein TssJ. The tail complex contains a tube, a tail sheath, and a base plate which functions as a ground for tail complex assembly. The tail tube is the homopolymer of haemolysin co-regulated protein (Hcp). At the distal part of the tube, there is
a spike formed by VgrG homodimer. The tube is surrounded by the tail sheath made of a
TssB-TssC heterodimer. T6SS effectors are hypothesised to be associated with the spike-tube
complex. The mechanism of T6SS secretion resembles contractile phage tails by which the
base plate complex activates tail sheath contraction leading to the translocation of effectors
into target cells (Costa et al. 2015, Tang et al. 2016). The simplified structures of different
secretion systems are illustrated in Figure 7.

Figure 7. Simplified structure of the 6 Gram negative secretion apparatuses. HM, Host
membrane; OM, outer membrane; IM, inner membrane; OMP, outer membrane protein;
MFP, membrane fusion protein (Tseng et al. 2009).

1.3 EPEC & EHEC as Attaching and Effacing (A/E) Pathogens

1.3.1 The Definition of A/E Pathogens

A/E pathogens are enteric bacteria capable of inducing A/E lesions, defined by intimate
bacterial attachment, microvilli destruction, and pedestal formation underneath bacteria.
EPEC, EHEC, rabbit enteropathogenic E. coli (REPEC), and the mouse pathogen Citrobacter
rodentium are A/E pathogens (Deng et al. 2001). Among these pathogens, EHEC and EPEC
are evolutionarily closely related (Donnenberg and Whittam 2001).
1.3.2 Locus of Enterocyte Effacement (LEE)

A/E pathogens are equipped with the LEE PAI that encodes proteins essential for A/E lesion formation (Deng et al. 2001). It is believed that the LEE was introduced into the bacterial genome by lateral gene transfer; however, the origins of the LEE PAI are unknown. The LEE is highly conserved both in terms of linear gene order and predicted protein sequences (Hacker and Kaper 2000, Deng et al. 2001).

The LEE PAI is organised in 5 major operons; LEE1-LEE5. The LEE1, the LEE2, and the LEE3 encode LEE encoded regulator (Ler), T3SS NC spanning the IM and the OM, EscN ATPase, and an OM protein porin. The LEE4 encodes translocators (EspA, EspD, EspB), EscF needle, and the effector EspF. The LEE5 encodes intimin, translocated intimin receptor (Tir), and CesT which is a chaperone for Tir (Garmendia et al. 2005, Mellies et al. 2007).

Intimin is an adhesive protein associated with the bacterial OM, and contributes to tissue tropism. Once translocated through the T3SS, Tir inserts itself into the host cell membrane and serves as a receptor for intimin. Tir-intimin binding promotes intimate bacterial attachment. Apart from LEE encoded effectors, there are T3SS effectors which are not encoded within the LEE, also known as non-LEE effector (Nle) (Bugarel et al. 2011).

EHEC and EPEC virulence factors are regulated in both pre-transcriptional and post-transcriptional levels. Ler lies at the heart of pre-transcriptional regulation by sensing signalling inputs and disrupting the suppression of virulence gene expression by H-NS leading to the expression of proteins encoded within the LEE. The expression of Ler is further regulated by global regulator of LEE activator (GrlA), global regulator of LEE repressor (GrlR), and Per. While GrlA and Per promote Ler expression, GrlR suppresses Ler expression (Deng et al. 2004, Mellies et al. 2007). The expression of Ler is tightly regulated by several regulators e.g. integration host factor (IHF), SOS response and quorum sensing.
(Mellies et al. 2007). In the post-transcriptional level, SepD and SepL govern T3SS function by controlling the switch from translocator to effector secretion (Deng et al. 2004).

EHEC and EPEC LEE have been extensively studied, and the results indicate that they display similar characteristics with only a few differences. EHEC LEE (43kbp) is larger than EPEC LEE (35.6kbp). This is due to a lysogenic bacteriophage insertion into EHEC LEE after the PAI acquisition. Secondly, there are a variety of intimin coding sequences as well as a difference in the number of effector proteins, considering that EHEC O157:H7 encodes 44 effector proteins, while EPEC E2348/69 encodes only 21 effector proteins (Tobe et al. 2006, Iguchi et al. 2009). Thirdly, EHEC LEE is not capable of inducing A/E lesion formation when introduced into a reference E. coli K-12 strain, while EPEC LEE is (Donnenberg and Whittam 2001). This is because the T3SS effector E. coli secreted protein F in prophage U (EspFU or TccP) is required for EHEC-mediated pedestal formation is not encoded within the LEE (Campellone et al. 2004).

1.3.3 The Alteration of Host Cell Physiological Function by T3SS Effectors

Once delivered into host cells, effector proteins hijack host cell physiological functions by mimicking or disrupting the function of host proteins in order to promote bacterial persistence, and to establish an infectious niche (Frankel et al. 1998, Le Blanc 2003). Host physiological functions subverted by T3SS effectors are discussed in the following sections.

1.3.3.1 Actin Cytoskeleton Remodelling

EPEC and EHEC use T3SS effectors to alter the host cytoskeleton in several ways including pedestal formation, Rho GTPases modulation and invasion. Pedestal formation facilitates bacterial attachment and A/E lesion formation. Upon bacterial attachment, Tir is inserted into the host cell membrane in a hairpin topology serving as a receptor for intimin. Tir-intimin
interaction leads to intimate attachment and pedestal formation beneath the bacterial cell (Battle et al. 2014). EPEC and EHEC induce pedestal formation via different mechanisms. During EHEC infection, pedestal formation is the result of actin polymerisation induced by TccP. TccP interacts with insulin receptor tyrosine kinase substrate (IRTKS), which binds Tir, thus IRTKS indirectly links Tir to TccP. TccP also activates neural Wiskott-Aldrich syndrome protein (N-WASP) leading to the recruitment of actin-related protein2/3 (Arp2/3) complex followed by actin polymerisation (Cheng et al. 2008, Vingadassalom et al. 2009). In contrast, EPEC Tir is phosphorylated at tyrosine 474 (Y474) leading to recruitment of the adaptor Nck and activation of N-WASP (Kenny 1999, Schuller et al. 2007). Consequently, N-WASP interacts with the Arp2/3 complex and promotes actin polymerisation (Kalman et al. 1999). The mechanism of pedestal formation is demonstrated in Figure 8.
Figure 8. Illustration summarising the alteration of cytoskeleton by T3SS effectors.

EHEC and EPEC induce pedestal formation via different mechanisms. Tir is translocated into the host cell membrane and serves as a receptor for intimin. EHEC secretes TccP/EspFU which is recruited to the bacterial adhesion site via the TccP-IRTKS interaction. TccP also binds N-WASP leading to actin polymerisation. In contrast, EPEC Tir is phosphorylated at Y474 residue, and directly recruits Nck which mediates actin polymerisation via N-WASP. T3SS effectors also modulate Rho GTPases activity resulting in the alteration of actin cytoskeleton (Stevens et al. 2006, Wong et al. 2011). Moreover, EHEC and EPEC exploit EspH, Map, EspT, and EspM2 to subvert actin cytoskeleton. EspH inhibits Rho GEFs, allowing bacterial Rho GEF (Map, EspT, EspM2) to govern host Rho GTPase functions (Wong et al. 2012). Map and EspM2 induce filopodia formation and stress fibre formation,

Rho GTPases are ubiquitously expressed in mammals and act as molecular switches by cycling between an active GTP-bound and an inactive GDP-bound state. Active Rho GTPases regulate numerous downstream signalling pathways including actin polymerisation, cell migration and cell adhesion. Rho GTPases activity is controlled by Rho GEFs which accelerate the exchange of GDP to GTP, thus positioning Rho GTPases in an active state. Guanine nucleotide dissociation inhibitors (GDIs) maintain Rho GTPases in an inactive state, and GTPase-activating proteins (GAPs) enhance GTP hydrolysis leading to Rho GTPases inactivation. RhoGTPase family members which have been extensively studied are RhoA, Rac1, and Cdc42. RhoA triggers actin polymerisation leading to stress fibre formation and FA assembly. Rac1 promotes lamellipodia formation leading to membrane protrusion during cell migration. Cdc42 regulates membrane rafts and filopodia formation (Raftopoulou and Hall 2004). Due to significant roles in cellular processes, Rho GTPases are targeted by several T3SS effectors.

leading to filopodia formation (Jepson et al. 2003). Apart from directly triggering Cdc42, Map was reported to interact with the ezrin/radixin/moesin (ERM)-binding phosphoprotein 50 (EBP50), also known as the Na\(^+\)/H\(^+\) exchanger regulatory factor 1 (NHERF1). EBP50 binds to ezrin, which links the actin cytoskeleton to the plasma membrane, and plays important role in filopodia formation. The Map-EBP50 interaction is believed to provide a link between Map and the actin cytoskeleton via an EBP50-ezrin interaction (Simpson et al. 2006). EspT is an important virulence factor as EspT producing EPEC strains were reported to be more virulent compared to non-EspT producing strains (Arbeloa et al. 2009). EspT activates Rac1 and Cdc42 resulting in membrane ruffles and lamellipodia followed by the internalisation of EPEC by non-phagocytic cells (Bulgin et al. 2009). EspH hijacks the host Rho GTPase signalling pathway by inhibiting host Rho GEF activity; this facilitates an interesting scenario where a bacterial pathogen subverts host Rho GTPase signalling pathways by replacing host Rho GEFs with bacterial Rho GEFs. Furthermore, EspH significantly impedes Cdc42 and RhoA signalling pathways, as well as interfering with host actin dynamics resulting in filopidia disruption, FA disassembly, and cell rounding (Tu et al. 2003, Wong et al. 2012). Furthermore, EspF interacts with cytokeratin 18 (CK18) and its adaptor protein 14-3-3. This interaction changes an intermediate filament network, resulting in the alteration of epithelial architecture (Viswanathan et al. 2004).

### 1.3.3.2 Modulation of Immune Responses

In order to survive in the host body, EPEC and EHEC have implemented several strategies to evade host immune responses, including innate immunity and phagocytosis. Phagocytosis is a process by which cells uptake particles ≥0.5µm (Caron and Hall 1998). The phagocytosis of foreign bodies by macrophage, neutrophil, and dendritic cells serves as the first line of host defence mechanisms. Additionally, phagocytosis triggers the adaptive immune response by secreting pro-inflammatory cytokines, and by attracting lymphoid cells (Flannagan et al.
Phagocytosis is a complex process composed of several stages by which receptors recognise and mediate the uptake of particles. In macrophages, the best characterised opsonic receptors involved with phagocytosis during infection are complement receptor 3 (CR3) and Fcγ receptors (FcγRs). CR3 and FcγRs interact with C3bi and IgG, respectively (Caron and Hall 1998). The uptake of particles is accomplished by actin remodelling leading to the alteration of cell membrane and the engulfment of the particle, which can then be taken up within a vesicle called phagosome. The phagosome then merges with lysosomes resulting in the digestion of phagosome components (Stuart and Ezekowitz 2008).


NF-κB controls the expression of genes involved with numerous cellular processes, particularly in both innate and adaptive immune responses (Vallabhapurapu and Karin 2009). Under healthy circumstances, NF-κB subunits are suppressed through binding with inhibitors of the NF-κB (IκB) family: IκBβ and IκBα. The activity of IκB family is regulated by IκB Kinase (IKK) complex. The IKK complex contains 2 catalytic subunits: IKKβ and IKKa, and
a regulatory subunit NF-κB essential modulator (NEMO). Upon stimulation, IKK complex modifies IκB complex, leading to the release of NF-κB subunits into the nucleus. NF-κB is activated via 2 pathways; classical (canonical) and alternative (non-canonical) pathway, each of which is triggered by different stimuli. In the classical pathway, adaptor proteins, e.g. tumour necrosis factor receptor-associated factors (TRAFs), activate IKKβ, which phosphorylates p105 and IκBα resulting in the release of a p50 and RELA (p65) heterodimer into the nucleus. For the alternative pathway, NF-κB inducing kinase (NIK) detects a signal from TNF receptor family, and activates IKKα. Then IKKα phosphorylates p100 leading to the cleavage of p100 into p52 that associates with RELB. Consequently, the p52 and RELB heterodimer redistribute into the nucleus. The activation of NF-κB leads to the production of protein mediators essential for the innate immune response. These proteins are chemokines, cytokines, adhesion molecules, secondary inflammatory mediator production enzymes, and apoptosis inhibitors. Moreover, NF-kB products are important for the recruitment of inflammatory cells and phagocytes to the site of infection. NF-κB plays a pivotal role in host resistance prior to the onset of adaptive immune response. Therefore, several pathogens, including EHEC and EPEC, elaborate strategies to hijack NF-κB activation (Beug et al. 2012).

During the early stage of infection, EHEC and EPEC activate the NF-κB pathway in a T3SS independent manner before exploiting T3SS effectors (NleE, NleB, NleC, NleD) to silence immune responses (Yen et al. 2010). NleE exhibits S-adenosyl methionine (SAM) dependent cysteine methyl transferase activity, and methylates adaptor proteins (TAB2 and TAB3) required for NK-κB signalling pathway. This consequently leads to NF-κB inhibition by blocking the nuclear localisation of p65 and c-Rel, hence decreasing pro-inflammatory IL-8 secretion (Nadler et al. 2010). NleE also stabilises IκB during TNF-α and IL1-β treatment (Kunsch and Rosen 1993, Newton et al. 2010). NleB disrupts TRAF2 mediated NF-κB
activation through an interaction with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Kelly et al. 2006, Gao et al. 2013). NleC degrades the DNA-binding domain of p65, thereby inactivating NF-κB (Yen et al. 2010, Muhlen et al. 2011, Hodgson et al. 2015). In contrast to NleB and NleC, NleD cleaves the activation loop of c-Jun N-terminal kinase (JNK), p38. This results in the inhibition of the AP-1 transcription factor that regulates numerous cellular processes including inflammation and apoptosis (Baruch et al. 2011). Furthermore, the NleH family (NleH1 and NleH2) hinders NF-κB activation, subsequent to Ribosomal protein S3 (RPS3) inhibition. RPS3 regulates the specificity of selected target genes transcribed by NF-κB and facilitates NF-κB DNA binding (Gao et al. 2009). The simplified mechanism of immune response modulation by T3SS effector is demonstrated in Figure 9.

Figure 9. Illustration summarising the suppression of immune responses by T3SS effectors. T3SS effectors modulate inflammatory responses by targeting classical NF-κB signalling pathway and phagocytosis process. NleB, NleE, and NleC disrupt NF-κB signaling pathway. NleB and NleE inhibit NF-κB activation by modifying adaptor protein TRAFs and

1.3.3.3 Modulation of Cell Death

EHEC and EPEC infections induce apoptotic cell death in both T3SS-dependent and T3SS-independent manners (Abul-Milh et al. 2001, Malladi et al. 2004, Shankar et al. 2009, Wong Fok Lung et al. 2014). EHEC infection triggers both apoptotic and necrotic cell death. T3SS-independent killing is mediated by EHEC haemolysin (Barnett Foster et al. 2000, Bielaszewska et al. 2013). EPEC infection provokes both intrinsic and extrinsic apoptotic pathways; however, the exact mechanism of EPEC infection-induced cell death is not yet elucidated (Crane et al. 1999, Flynn and Buret 2008). It was reported that OMPs and BFP from EPEC induce apoptosis in vitro (Abul-Milh et al. 2001, Shankar et al. 2009). Moreover, EPEC OMPs also induce the immune response via the activation of NF-κB signalling pathway (Malladi et al. 2004).

In terms of T3SS-dependent mechanisms, the effector proteins EspH, EspF, Map, and Cif trigger cell death. EspH not only subverts actin cytoskeletal network and phagocytosis, but EspH also indirectly instigates cytotoxic effects due to its effects on Rho GTPase signalling pathways (Wong et al. 2012). EspF and Map traffic to mitochondria where they disrupt
mitochondrial structure and induce the loss of mitochondrial membrane potential. Consequently, this results in cytochrome c (cyt c) release, followed by caspase activation (Nagai et al. 2005, Ma et al. 2006, Nougayrede et al. 2007). Cif causes G1/G2 and G2/M cell cycle arrest due to the accumulation of Cyclin-dependent kinase (Marches et al. 2003, Taieb et al. 2006, Samba-Louaka et al. 2008). Recently, it was reported that Cif also sabotages cellular functions and causes cytotoxicity by impairing the degradation of cellular proteins due to the inhibition of NEDD8 ubiquitin ligase activity (Cui et al. 2010).

To counteract these cytotoxic effects, EPEC and EHEC secrete several effectors to rescue host cells in order to maintain an infectious niche in the gut. These apoptotic effectors block several apoptotic cascades; JNK-mediated apoptosis (NleD), the extrinsic apoptotic pathway (NleB and NleF), and the intrinsic apoptotic pathway (NleH, EspZ, and NleF). By cleaving at the JNK activation loop, NleD blocks both apoptosis and the inflammatory response (Dhanasekaran and Reddy 2008, Baruch et al. 2011). EHEC and EPEC carry 2 copies of NleB: NleB1 and NleB2. Both NleB1 and NleB2 bind with DD-containing proteins; however, NleB2 does not manifest anti-apoptotic activity. NleB1 displays N-acetylglucosamine (GlcNAc) transferase activity and modifies a specific arginine residue in the death domain (DD) of DD-containing proteins such as Fas-associated protein with death domain (FADD), RIK1, tumour necrosis factor receptor type 1-associated death domain (TRADD) (Newton et al. 2010). NleB1 impedes the extrinsic apoptotic cell death in vitro by modifying the DD of FADD and TRADD (Li et al. 2013, Pearson et al. 2013). Moreover, NleB was shown to contribute to the colonisation of C. rodentium and the inhibition of the extrinsic apoptotic cell death in vivo according to the attenuation of C. rodentium ΔnleB colonisation and the increase of caspase-8 level in the infected gut, respectively (Kelly et al. 2006, Pearson et al. 2013).
EHEC and EPEC also exploit NleH and EspZ to block the intrinsic apoptotic pathway. NleH inhibits apoptosis via an interaction with Bax inhibitor-1 (BI-1) at the surface of ER (Hemrajani et al. 2010). BI-1 is believed to protect cells from ER stress-induced apoptosis by reducing the level of reactive oxygen species and unfolded protein response (Lee et al. 2007, Trebinska et al. 2010). EspZ, a 9kDa LEE encoded effector, localises to the plasma membrane and mitochondria (Shames et al. 2010, Shames et al. 2011). In mitochondria, EspZ binds to the translocase of inner mitochondrial membrane 17b (TIM17b) which is responsible for protein delivery into mitochondrial matrix (Shames et al. 2011). TIM17b knockdown leads to mitochondrial dysfunction and apoptosis, and the binding of EspZ to TIM17b prevents the loss of mitochondrial membrane potential (Garabedian et al. 2011). At the plasma membrane, EspZ binds to the transmembrane protein CD98 to enhance cell survival via the promotion of FAK phosphorylation, Akt phosphorylation, and β1 integrin activation (Rintoul et al. 2002, Shames et al. 2010). NleF uniquely hinders both the extrinsic and the intrinsic apoptotic pathways by blocking caspase-8 and caspase-9, respectively. NleF inhibits caspase-8 and caspase-9 through the binding between the C-terminal of NleF and caspase active site (Blasche et al. 2013). Furthermore, due to the ability to inhibit caspase-4, NleF is hypothesised to disrupt pyroptosis and inflammasome formation (Newton et al. 2010, Shi et al. 2014). The modulation of cell death responses by EHEC and EPEC is summarised in Figure 10.

Moreover, EHEC is equipped with EspO1 and EspO2 whose functions are to maintain cell adhesion, and are described further below (Morita-Ishihara et al. 2013).
Figure 10. Illustration summarising the alteration of cell death response by EHEC and EPEC T3SS. During infection, Hly from EHEC, as well as OMP and BFP from EPEC induce cell death via unknown mechanisms (Barnett Foster et al. 2000, Abul-Milh et al. 2001, Shankar et al. 2009, Bielaszewska et al. 2013). T3SS effectors (Cif, EspF, EspH, and Map) also provoke apoptosis. Cif induces apoptosis by arresting cell cycle (Taieb et al. 2006, Samba-Louaka et al. 2008). EspF and Map cause mitochondrial membrane permeabilisation, resulting in cyt c leakage (Nougayrede and Donnenberg 2004, Nagai et al. 2005, Ma et al. 2006). EspH indirectly induces cytotoxicity via the subversion of Rho GTPase signalling pathway (Wong et al. 2012). However, EHEC and EPEC employ several effectors to inhibit apoptosis. NleH and EspZ inhibit intrinsic pathway. EspZ impedes the intrinsic apoptotic pathway by preventing mitochondrial membrane perforation and activating Akt signalling pathway through CD98 (Shames et al. 2010, Shames et al. 2011). NleH reduces ER Ca$^{2+}$

1.3.3.4 Induction of Diarrhoea

Diarrhoea is caused by the movement of water across an intestinal epithelial barrier subsequent to the disproportion of ion absorption and secretion. Either the malfunction of ion channels or the disruption of intestinal integrity can cause diarrhoea. Ion and solvent absorption and secretion are controlled by ion channels such as the Na$^+$/H$^+$ exchanger (NHE), Na$^+$/glucose cotransporter (SGLT1), cystic fibrosis transmembrane conductance regulator (CFTR), and aquaporins. Adjacent epithelial cells are held together by junctional complexes which consist of tight junctions, adherent junctions, and desmosomes. While adherent junctions and desmosomes mechanically bind the adjacent epithelial cells, tight junctions regulate an intestinal barrier function (Viswanathan et al. 2009). In terms of pathogenesis, diarrhoea helps bacteria to disseminate into a new environment and a new host.

Diarrhoea caused by EPEC and EHEC infections is the accumulation of different factors including malabsorption and intestinal integrity disruption (Wong et al. 2011). A/E lesions, caused by T3SS, destroy microvilli, and decrease the absorptive surface of the intestine (Viswanathan et al. 2009). The effacement of microvilli is thought to be a 2-stage process: 1) pedestal formation mediated by Tir and intimin as well as intimate bacterial attachment, and 2) microvilli destruction due to cytoskeletal disruption which is mediated by EspF, Map, and EspB (Dean et al. 2006, Iizumi et al. 2007). EspI (also known as NleA) disrupts the structure of tight junctions by blocking the transportation of tight junction proteins to the cell membrane (Kim et al. 2007, Thanabalasuriar et al. 2010). Furthermore, EHEC and EPEC
utilise EspG, EspF, Map, and EspI to hijack water and ion absorption. EspG decreases the membrane availability of Cl⁻/OH⁻ exchanger and hampers receptor trafficking to the plasma membrane (Clements et al. 2011, Selyunin et al. 2011). EspF hinders NHE3 activity (Hodges et al. 2008). Additionally, SGLT1 activity was reported to be redundantly affected by Map, Tir, EspF, and intimin (Dean et al. 2006). In addition to these effects, inflammatory responses initiated during EHEC and EPEC infection also disrupt epithelial integrity (Dean and Kenny 2004). Thus, EHEC and EPEC induced diarrhoea is the accumulation of these destructive mechanisms. The simplified mechanism of how EHEC and EPEC induce diarrhoea is shown in Figure 11.

Figure 11. Diagram summarising T3SS effectors induced diarrhoea. Diarrhoea caused by EHEC and EPEC infection is the accumulation of intestinal integrity disruption, and
malabsorption. The loss of intestinal integrity and barrier function are the result of pedestal formation, T3SS-induced cytockkeletal disruption, and tight junction disruption, which is mediated by EspG and EspI. Furthermore, Map, EspF, Tir, and intimin impede the function of ion channel, leading to malabsorption (Dean and Kenny 2009).

Taken together, EHEC and EPEC pathogenesis is complex and is not yet fully understood. As discussed, the T3SS plays a crucial role in EHEC and EPEC pathogenesis; however, the mechanism and the function of the full effector repertoire remain unclear. While many effectors have been identified, not all of their roles during infection are fully characterised (Tobe et al. 2006). This project will be focusing on the role of one of these effector proteins, EspO and its role in EHEC infection.

1.3.4 The Secretion Hierarchy of T3SS Effector Repertoire

Effector proteins are translocated in a tightly regulated sequential manner. Some effectors have either synergistic or antagonist functions, whilst others are redundant in function (Mellies et al. 2007). The secretion hierarchy is controlled in different levels ranging from the timing of gene expression, to the abundance of effector protein in bacterial cytoplasm, and the interaction between the effectors and the T3SS chaperones. Moreover, effector proteins function in a spatiotemperal manner which is regulated by proteasomal degradation, phosphorylation, and the exploitation of scaffold proteins in the host cell cytoplasm (Wong et al. 2011, Mills et al. 2013).
1.4 The EspO Family of Effectors

1.4.1 Known EspO Homologs

EspO is found in two copies in EHEC O157:H7 sakai strain: EspO1-1 (EspO1) gene ID ECs1567 and EspO1-2 (EspO2) gene ID ECs1821 on Sp6 and Sp9 locus of lambdoid prophage, respectively (Tobe et al. 2006). EspO is homologous to the Shigella T3SS effector OspE. Two ospE genes (ospE1 and ospE2) are found in Shigella flexneri and Shigella sonnei. However, in S. sonnei, only OspE2 is expressed, while both OspE1 and OspE2 are expressed in S. flexneri (Miura et al. 2006, Kim et al. 2009). EspO homologs have also been found in Salmonella enterica serovar Typhimurium, REPEC, and C. rodentium (Tobe et al. 2006, Iguchi et al. 2009). EspO is also found in clinical EPEC isolates, while the reference EPEC strain E2348/69 carries an espO pseudogene (Iguchi et al. 2009, Constantinou 2013).

1.4.2 Previous Studies on OspE and EspO

Shigella OspE (OspE1/OspE2 from S. flexneri, and OspE2 from S. sonnei) is the best characterised homolog among EspO effector family members. OspE provides versatile functions, both inside and outside the host cells. Shigella travels along the intestinal lumen prior to invading the colon and causing shigellosis. When the pathogen is exposed to bile salts in the intestinal lumen, the T3SS is induced resulting in the secretion of OspE. In the presence of bile salts, OspE associates with the Shigella OM to increase Shigella adherence (Faherty et al. 2012). Once Shigella invades the intestinal epithelium, bile salts are absent, triggering OspE release into the host cell cytoplasm.

Inside host cells, OspE is localised to FAs, and exerts several functions; OspE preserves host cell morphology, and inhibits host cell detachment. Until recently, there are 2 identified OspE1 interaction partners: integrin linked kinase (ILK) and PDLIM7 (Kim et al. 2009, Yi et al. 2014). OspE interacts with ILK at FAs and inhibits FA disassembly. The interaction
between OspE and ILK is mediated by a tryptophan (W) residue that is conserved among members of the EspO effector family. OspE inhibits FA disassembly by promoting ILK membrane retention and dampening β1 integrin turnover. However, OspE1 does not affect ILK kinase activity nor the formation ILK-PINCH-Parvin complex during FA formation (Miura et al. 2006, Kim et al. 2009). A recent study showed that OspE contains a Postsynaptic Density95/Disc Large/Zonula Occludens-1 (PDZ) binding domain that allows OspE to interact with PDLIM7. PDLIM7 activates protein kinase C (PKC), which phosphorylates several substrates, and is involved with several cellular processes including cell death, cell survival, cell spreading and cell migration. The binding between OspE and PDLIM7 activates the kinase activity of PKC to further phosphorylate numerous target proteins. It is suspected that OspE also preserves host cell morphology and inhibits cell detachment through its interaction with PDLIM7 (Yi et al. 2014). Furthermore, OspE contributes to intercellular spreading during in vitro infection as the ΔospE mutant induces a smaller plaque in comparison to wild type Shigella (Miura et al. 2006). During in vivo infection, OspE promotes Shigella colonisation and host inflammatory response in an ILK-dependent manner (Kim et al. 2009).

Despite extensive study of Shigella OspE, there is only one publication regarding EHEC EspO1 and EspO2 (Morita-Ishihara et al. 2013). It was confirmed that both EHEC EspO1 and EspO2 interact with ILK via the conserved W residue (W77 for both EspO1 and EspO2). Infection with ΔespO1ΔespO2 Sakai strain induces cell rounding that is decreased by the re-introduction of either EspO1 or EspO2. During in vitro infection EspO1 is localised to FAs, thus it is expected to inhibit cell detachment through its interaction with ILK. Unlike EspO1, EspO2 disperses in the cytoplasm and interacts with both EspM2 and ILK. As mentioned earlier, EspM2 induces cell rounding and cell detachment by promoting RhoA activity and stress fibre formation. EspO2 suppresses the activity of EspM2, thereby preventing cell
contraction and cell detachment. Accordingly, EspO1 and EspO2 are suspected to co-operate with each other to stabilise FAs during EHEC infection via different mechanisms (Morita-Ishihara et al. 2013). In a previous study, a yeast two hybrid (Y2H) screen identified a number of novel interaction partners for EspO1 including HCLS1-associated protein X-1 (HAX-1), DnaJ heat shock protein family (Hsp40) member B1 (DnaJB1), and DnaJ heat shock protein family (Hsp40) member B6 variant 2 (DnaJB6b) (Constantinou 2013). The known function and mechanism of the EspO effector family are illustrated in Figure 12.

**Figure 12. Illustration summarising known function and mechanism of EspO homologs.**

_Shigella_ OspE enhances bacterial adhesion in the intestinal lumen where bile salts are present; in the intestinal epithelium, OspE prevents cell detachment and cell rounding via

1.5 Apoptosis

1.5.1 The Role of Apoptosis in Physiology
Apoptotic cell death plays an important role in maintaining healthy physiological functions and in developmental processes. It is essential to maintain the same number of cells by balancing cell death and cell division phenomena. Therefore, an imbalance between cell death and cell propagation results in several diseases. The prevention of cell death leads to cancer, and autoimmune lymphoproliferative syndrome. On the other hand, excessive apoptotic cell death is responsible for neurodegenerative diseases as well as ischemia-associated injuries (Elmore 2007).

1.5.2 The Hallmark of Apoptosis
The term apoptosis was introduced by Kerr and colleagues in 1972 (Kerr et al. 1972). When visualised under the electron microscope, apoptotic cells exhibit distinct morphological characteristics including nuclear condensation, cytoplasmic shrinkage, plasma membrane blebbing and cellular disintegration into apoptotic bodies containing organelles. Apoptotic cells are also found to detach from a basement membrane (Saraste and Pulkki 2000). Moreover, in apoptotic cells, mitochondria are observed to become fragmented and swollen (Bottone et al. 2013). In addition to cell morphology, apoptotic cells display several biochemical alterations such as DNA cleavage, decrease of cytosolic pH, change of cell
membrane composition, and increase of cytosolic Ca\textsuperscript{2+} (Saraste and Pulkki 2000, Elmore 2007).

### 1.5.3 Apoptotic Pathways

Apoptotic cell death is a highly sophisticated process that occurs in a regulated chronological manner starting from apoptotic initiation, execution pathway, to the clearance of apoptotic bodies which are phagocytosed by leukocytes and adjacent cells (Elmore 2007, Wong Fok Lung et al. 2014).

#### 1.5.3.1 The Initiation of Apoptosis

There are a wide variety of physiology and pathology conditions that induce apoptosis. Additionally, different cell types respond differently to different stimuli. There are 2 classical apoptotic cascades; intrinsic and extrinsic pathways. Both pathways eventually lead to the activation of executioner caspases: caspase-3, caspase-7, and caspase-6, resulting in cell demolition (Elmore 2007, Wachmann et al. 2010, McIlwain et al. 2013).

**i. Extrinsic Pathway**

The extrinsic pathway is mediated by the binding between extracellular ligands and cell surface receptors, thus it is also called the death receptor pathway (Igney and Krammer 2002). The extrinsic pathway is triggered by the transmembrane receptor called tumour necrosis factor receptor (TNFR) family and their specific ligands (Ashkenazi and Dixit 1998). Until recently, the best-characterised receptors have been Fas/CD95/Apo-1, TNFR1/p55/CD120a, TNF-related apoptosis-inducing ligand receptor1 (TRAIL1-R)/DR4, and TRAIL-R2/DR5/Apo2/KILLER. FasL, TNF, and TRAIL/Apo2L are death ligands (Peter and Krammer 1998, Suliman et al. 2001, Rubio-Moscardo et al. 2005). The binding between death receptors and death ligands leads to the recruitment of FADD. FADD, then, enlists
procaspase-8 and pro-caspase-10 via the homophilic interaction of death effector domain present in FADD and initiator caspases (Dobreva et al. 2008). This leads to the formation of the death-inducing signalling complex (DISC) followed by caspase-8 and caspase-10 autolytic activation (Kischkel et al. 1995, Wachmann et al. 2010). Caspase-8, and caspase-10 further activate caspase-3, and caspase-7 which then activate caspase-6 (Elmore 2007).

ii. Intrinsic Pathway: An Interplay Between Mitochondria and ER

The intrinsic apoptotic pathway is initiated by several events such as survival factor withdrawal, energy depletion, DNA damage, oxidative stress response, and the increase of mitochondrial Ca\(^{2+}\) influx (Granerus et al. 2001, Danial and Korsmeyer 2004). The intrinsic pathway can be triggered by 2 organelles; mitochondria and ER (Danial and Korsmeyer 2004).

Mitochondria regulate the intrinsic pathway via the release of cyt c from the mitochondrial matrix into the cytosol. Although the exact mechanism of cyt c release remains unclear, the release of cyt c is the result of mitochondrial membrane rupture which is mediated by Ca\(^{2+}\) and Bcl-2 protein family. The increase of cytosolic Ca\(^{2+}\) induces mitochondrial permeability transition due to the opening of non-specific pores on the mitochondrial OM, resulting in cyt c release. Pro-apoptotic Bcl-2 proteins: Bax and Bak, oligomerise on the mitochondrial OM and cause OM permeabilisation (Garrido et al. 2006). In cytosol and in the presence of ATP/dATP, cyt c binds with apoptosis-protease factor-1 (Apaf-1), followed by the recruitment of pro-caspase-9 through its caspase recruitment domain (CARD), resulting in apoptosome formation (Li et al. 1997). In the apoptosome, pro-caspase-9 undergoes autoproteolytic cleavage resulting in caspase-9 activation (Acehan et al. 2002). Caspase-9 then further activates executioner caspases (Elmore 2007). Mitochondria also release toxic proteins such as apoptosis-induce factor (AIF), second mitochondria-derived activator of caspase (Smac)/direct inhibitor of apoptosis-binding protein with low pI (DIABLO),
Omi/HtrA2 and endonuclease G. AIF and endonuclease G cleave DNA resulting in nuclear condensation. Proteases Smac/DIABLO and Omi/HtrA2 promote apoptosis by cleaving anti-apoptotic proteins such as X-linked inhibitor of apoptosis protein (XIAP) and HAX-1, respectively (Saelens et al. 2004, Bottone et al. 2013).

ER triggers apoptosis through 2 different mechanisms; the induction of cyt c release from mitochondria and unfolded protein response (UPR) (Breckenridge et al. 2003). ER transmits Ca\(^{2+}\) to mitochondria via the ER-mitochondria associated membrane, causing cyt c release consequent to the increase of mitochondrial Ca\(^{2+}\) influx. The Ca\(^{2+}\) channel inositol 1,4,5-triphosphate receptor (IP3R), Bax, and Bak also mediate ER Ca\(^{2+}\) efflux leading to the increase of cytosolic Ca\(^{2+}\) (Breckenridge et al. 2003, Scorrano et al. 2003). The increase of cytosolic Ca\(^{2+}\) activates calpain protease, which cleaves numerous proteins. Caspase-12 was also reported to be involved with ER mediated apoptosis (Nakagawa et al. 2000, Sano and Reed 2013). Moreover, ER activates apoptosis through Mitogen-activated protein (MAP) kinase pathways during UPR (Sano and Reed 2013).

### iii. Cross-talk Between Intrinsic and Extrinsic Pathways

Intrinsic and extrinsic apoptotic pathways are not completely isolated events as there are a few mechanisms mediating cross-talk between them. Firstly, caspase-8 cleaves Bid, and releases the C-terminal of Bid, aka tBid. tBid localises to mitochondria and forms pores in the mitochondrial membrane resulting in cyt c release (Li et al. 1998, Luo et al. 1998). Additionally, capsase-9 cleaves caspase-3 and caspase-7. In turn, caspase-3 activates caspase-6 leading to caspase-8 and caspase-10 activation. Thus, the intrinsic pathway is amplified by the extrinsic pathway and vice versa (Cullen and Martin 2009). The process of apoptosis initiation is summarised in Figure 13.
1.5.3.2 Executioner Pathway

Regardless of the mode of apoptotic initiation, every pathway ultimately leads to the activation of the executioner caspases. Even though these caspases have common substrates, they have different affinity towards different substrates (Cullen and Martin 2009). Caspase proteolytic activity leads to the alteration of cellular structures and the disruption of metabolic processes resulting in the morphological hallmark of apoptosis (Elmore 2007). As reviewed by Cullen and colleagues (2008), executioner caspases degrade cytoskeletal components, resulting in the alteration of cell shape and cell rounding up. Furthermore, caspases also contribute to membrane blebbing and apoptotic body formation. Caspases also digest nuclear lamin, activate CAD endonuclease, and mediate histone 2B phosphorylation; consequently, this leads to nuclear and DNA fragmentation (Leatham et al. 2009). The fragmentation of DNA helps prevent the release of accumulated DNA, which can activate immune responses upon being released into an extracellular environment. Transcription and translation processes are also disrupted by the proteolytic digestion of transcription factors and ribosomal proteins. Moreover, caspases degrade focal adhesion kinase (FAK), catenin, and other structural proteins followed by the destruction of cell-matrix and cell-cell adhesion sites. This leads to cell detachment from an extracellular matrix. Finally, the dying cell is dissociated into apoptotic bodies containing cytosol and organelles enclosed in the cell membrane (Cullen and Martin 2009).
Apoptotic cell death can be triggered by (1) extrinsic pathway, and (2) intrinsic pathway. The extrinsic pathway (1) is activated by death ligands, which interact and activate death receptors resulting in the recruitment of FADD, as well as caspase-8 and caspase-10 activation. Moreover, caspase-8 cleaves Bid and promotes cyt c release via Bid cleavage. In the intrinsic pathway (2), Bak and Bax create mitochondrial membrane pores and cause cyt c release. Once released into the cytoplasm, cyt c associates with Apaf-1 and pro-caspase-9 resulting in caspase-9 activation. Furthermore, ER also regulates the intrinsic pathway by releasing Ca$^{2+}$ which induces cyt c release from mitochondria, and by triggering JNK, p38 pathway through the UPR. Upon activation, caspase-8, caspase-10 and caspase-9 further activate caspase-3, caspase-6, and caspase-7. Apart from Bcl-2 proteins, XIAP hinders apoptotic cell death by blocking caspases activity. DR, death receptors (Scott et al. 2005, Taylor et al. 2008).
1.5.3 Anti-apoptotic Proteins

1.5.3.1 Bcl-2 Protein Family

Bcl-2 oncogene was discovered in B-cell lymphoma in 1987 (Graninger et al. 1987). Bcl-2 family members are defined by the possession of conserved Bcl-2 homology (BH) domains: BH1, BH2, BH3, and BH4 (Walensky 2006). Bcl-2 members are sub-categorised based on their structure and function into 3 groups: 1) executioner pro-apoptotic proteins containing BH1-BH3 domains (Bax and Bak), 2) anti-apoptotic proteins containing BH1-BH4 domains that hinder pro-apoptotic effect of Bax and Bak (Bcl-2, Bcl-Xl, Bcl-2, Mcl-1, A1), and 3) pro-apoptotic BH3-only proteins which sense cellular stresses and activate executioner Bcl-2 members (Bid, Bad, Bim, Bik, NOXA, PUMA). Pro-apoptotic and anti-apoptotic Bcl-2 members form homo- and hetero- polymers to regulate mitochondrial membrane permeabilisation (Garrido et al. 2006, Shamas-Din et al. 2013). During apoptosis onset, the antagonistic effect of anti-apoptotic Bcl-2 proteins is cancelled either by the binding of BH3-only pro-apoptotic Bcl-2 family members or by proteolytic cleavage. This leads to the accumulation and polymerisation of Bak and Bax on the mitochondrial membrane resulting in cyt c release subsequent to mitochondrial membrane rupture (Garrido et al. 2006). The anti-apoptotic mechanism of Bcl-2 protein family is summarised in Figure 14.
Figure 14. Diagram presenting the simplified mechanism of Bcl-2 protein family. Under normal physiological conditions, the pro-apoptotic activity of Bax, which resides on the mitochondrial membrane, is inhibited by Mcl-1 and Bcl-xL. Bcl-xL and Bcl-2 restrain Bax in the cytoplasm, and prevent Bax-induced mitochondrial membrane perforation. In the presence of apoptotic stimuli, BH3 only proteins bind to Bcl-xL, Bcl-1, and Mcl-1, resulting in the cancellation of Bax and Bak inhibition. Consequently, Bax and Bak oligomerise on mitochondrial membrane, resulting in mitochondrial membrane permeabilisation. Even though the precise mechanism of how Bcl-1 protein family control apoptotic process is not yet clear, it is widely accepted that the cell fate is determined by the relative concentration of pro- and anti-apoptotic family members (Garrido et al. 2006, Shamas-Din et al. 2013).

1.5.3.2 Inhibitor of Apoptosis (IAP) Family

Mammals encode 8 IAP family members; however, not all of them display anti-apoptotic activity (Beug et al. 2012). The IAP proteins are comprised of baculovirus IAP repeat (BIR) domain, really interesting new gene (RING), and central caspase recruitment domain
(CARD) (Lopez et al. 2013). The N-terminal BIR domain is conserved and it contains consensus binding motif required for the interaction partner recognition of IAP proteins (Eckelman et al. 2008). RING domain exhibits E3 ubiquitin ligase activity by adding ubiquitin to an ubiquitination chain. CARD regulates E3 ubiquitin ligase activity (Berthelet and Dubrez 2013). Molecular domains of IAPs are illustrated in Figure 15.

![Molecular domains of IAP proteins](image)

**Figure 15. The molecular domain of anti-apoptotic the IAP proteins in mammals.** The IAP proteins contain BIR, RING, and CARD domain. The part of BIR domain which is responsible for caspase inhibition is presented in red (Riedl and Shi 2004). Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Molecular Cell Biology (Riedl and Shi 2004), copyright 2004

XIAP is the most potent anti-apoptotic protein among the IAP family members (Eckelman et al. 2006). XIAP inhibits caspase-3 and caspase-7 activity via the binding between BIR2 domain and caspase active site pocket (Deveraux et al. 1997). Furthermore, XIAP inhibits caspase-9 activation by sequestering pro-caspase-9 autolytic activity (Shiozaki et al. 2003, Zou et al. 2003). XIAP also directly ubiquitinates and promotes active caspase-3 degradation (Suzuki et al. 2001). Apart from apoptosis, XIAP also inhibits necroptosis by blocking RIPK-dependent ripoptosome formation (Silke and Meier 2013). During apoptosis, XIAP is degraded by Smac/DIABLO, and HtrA2/Omi (Berthelet and Dubrez 2013).
1.5.3.3 HCLS1-Associated Protein X-1

HAX-1 was firstly identified as the interaction partner of HS1-associated protein which plays an essential role in receptor-mediated apoptosis and proliferative responses (Suzuki et al. 1997). Ubiquitously expressed HAX-1 is one of the NF-kB target genes (Sharp et al. 2002, Yedavalli et al. 2005). In humans, HAX-1 has 8 splicing variants resulting from alternative splicing (Fadeel and Grzybowska 2009). The expression level of each variant is different across different tissues. However, HAX-1 variant 1 (referred as HAX-1) is the most widely expressed and best characterised (Trebinska et al. 2010). HAX-1 is comprised of N-terminal BH1 and BH2 domains, a PEST sequence, and a C-terminal domain (Figure 16) (Yap et al. 2010, Yap et al. 2011).

![Figure 16. Molecular domains of HAX-1 variant 1. BH1 and BH2 domains are believed to contribute to HAX-1 anti-apoptotic activity, while the PEST sequence mediates the rapid proteasomal degradation of HAX-1 during apoptotic onset (Fadeel and Grzybowska 2009).]

The anti-apoptotic mechanism of HAX-1 is not yet completely understood; however, it is believed that HAX-1 inhibits apoptosis in various subcellular compartments via interactions with numerous proteins; XIAP, phospholamban, sarcoplasmic reticulum Ca\textsuperscript{2+} transport ATPase2 (SERCA2), caspase-3, and caspase-9 (Han et al. 2006, Vafiadaki et al. 2007, Lee et al. 2008, Vafiadaki et al. 2009, Kang et al. 2010).

In the ER, HAX-1 interaction with phospholamban leads to the downregulation of SERCA2. SERCA2 pumps Ca\textsuperscript{2+} into the ER, thus SERCA2 downregulation leads to the reduction of ER Ca\textsuperscript{2+} storage (Vafiadaki et al. 2007, Vafiadaki et al. 2009). In mitochondria, HAX-1
maintains mitochondrial membrane potential by blocking Bax-induced mitochondrial membrane permeabilisation (Sharp et al. 2002, Fadeel and Grzybowska 2009). Apart from the major sites of apoptotic induction like ER and mitochondria, HAX-1 directly inhibits caspase-9 activation and blocks caspase-3 activity in the cytoplasm (Han et al. 2006, Lee et al. 2008). The exact mechanism of caspase-9 inhibition remains unclear; however, it was hypothesised that HAX-1 inhibits caspase-9 autoactivation by either disrupting apoptosome formation or preventing pro-caspase-9 dimerisation (Shaw and Kirshenbaum 2006). Additionally, HAX-1 prolongs the anti-apoptotic activity of XIAP by preventing its degradation (Deveraux et al. 1997, Kang et al. 2010).

During the onset of apoptosis, the level of HAX-1 is tightly regulated by at least 2 proteases; caspase-3 and the mitochondria protease Omi/HtrA2 (Cilenti et al. 2004, Chao et al. 2008, Lee et al. 2008). The PEST sequence, contained within the HAX-1 molecule, also induces rapid HAX-1 proteasomal degradation (Li et al. 2012). Recently, it was reported that HAX-1 variant 4 forms heterodimers with HAX-1 variant 1, and hinders its anti-apoptotic effect. However, the mechanism of apoptotic regulation by different HAX-1 variants remains unclear (Koontz and Kontrogianni-Konstantopoulos 2014). The proposed anti-apoptotic mechanism of HAX-1 is summarised in Figure 17.
Figure 17. The proposed anti-apoptotic mechanism of HAX-1. HAX-1 impedes the apoptotic process via several mechanisms. HAX-1 reduces ER Ca\(^{2+}\) storage via interactions with phospholamban and SERCA2. HAX-1 also preserves mitochondrial membrane potential. Furthermore, HAX-1 also directly inhibits caspase-9 activation, and prolongs XIAP anti-apoptotic activity by blocking XIAP degradation. However, HAX-1 is also rapidly degraded by ubiquitination, Omi/HtrA2, and caspase-3 during apoptosis onset (Cilenti et al. 2004, Shaw and Kirshenbaum 2006, Vafiadaki et al. 2007, Vafiadaki et al. 2009, Kang et al. 2010, Li et al. 2012).

1.6 Cell Death Response during Infection

During infection, phagocytes sense pathogen-associated molecular patterns (PAMPs) from pathogens, leading to the phagocytosis of the pathogens followed by host defense response
activation (Ashida et al. 2011). Moreover, non-phagocytic cells including intestinal epithelial cells also participate in host defense mechanisms. During bacterial infection, epithelial cells sense PAMPs, death ligands, and bacterial components. The sensing of danger stimuli triggers several stress responses as well as cell death and exfoliation in the infected cells and their neighbours. There are 2 modes of cell death triggered in the gut epithelial cells; apoptosis and necroptosis, each of which is mediated through an individual process. While apoptosis silences the immune response, necrotic cell death triggers inflammatory responses which help eliminate pathogens (Lamkanfi and Dixit 2010).

In addition to apoptotic cell death, which is discussed in the previous section, death ligands, such as TNF and Fas, also trigger necroptosis. In contrast to apoptosis, necroptosis is the caspase-independent mode of cell death. The activation of death receptors leads to complex I (comprised of RIPK1, TRADD, cIAP1, cIAP2, TNFR associated factor2 (TRAF2), and TRAF5) formation, resulting in NF-κB activation. cIAPs facilitate RIPK1 ubiquitination, which retains RIPK1 in complex I. Cylindromatosis enzyme deubiquitinates RIPK1 resulting in complex I dissociation. The deubiquitinated RIPK1 is recruited to the ripoptosome, which activates caspase-8. Alternatively, when caspase-8 is inhibited, RIPK1 binds to RIPK3 leading to necrosome formation. The necrosome recruits and phosphorylates mixed-lineage kinase domain-like protein (MLK1). Phosphorylated MLK1 creates pores on cell membrane and induces cell membrane rupture, followed by the release of pro-inflammatory molecules including damage associated molecular patterns (DAMPs) (Fuchs and Steller 2015). The cross-talk between different modes of cell death is summarised in Figure 18.
Figure 18. Diagram presenting simplified cell death response mechanisms during bacterial pathogen infection. Bacterial infection triggers 2 distinct modes of cell death in intestinal epitheliums: apoptosis and necroptosis. The cross-talk between apoptosis and necrosis is mediated by the activation of caspase-8 by ripoptosome. The ripoptosome is inhibited by cIAPs and potent apoptotic inhibitor XIAP. Additionally, the disruption of apoptosis results in necroptosome activation followed by lytic cell death (Fuchs and Steller 2015, de Vasconcelos et al. 2016).
There are tight regulations and cross-talk mechanisms which control cell death and inflammatory responses in order to maintain a balance between adequate inflammatory responses, and tissue damage (Fuchs and Steller 2015). On the other hand, pathogens delicately manipulate host cell death responses to promote their survival and colonisation in the host body. In the early stage of infection, cell death inhibition allows pathogens to propagate and colonise the gut, while the death of an infected cell enhances the spreading of pathogens into neighbouring cells and into a new environment during later stages of infection (Ashida et al. 2011). Furthermore, enteric pathogens such as Shigella can promote the death of harmful phagocytes, while impeding the death of the infectious niche, the intestinal epithelial cells (Lamkanfi and Dixit 2010). Not only Shigella, EPEC and EHEC also hijack cell death pathway to promote the establishment of their infectious niche. Strategies employed by EHEC and EPEC are previously discussed.

1.7 Aims

The aim of this project was to investigate the role of EspO1 during in vitro infection. As mentioned previously, an interaction between EspO1 and HAX-1 was identified by a yeast two hybrid screen and confirmed by pull down (Constantinou 2013). This study set out to further study the interaction between HAX-1 and EspO1, and to investigate the role of EspO1 during in vitro infection.
Chapter 2 Materials and Methods
Chapter 2 Materials and Methods

2.1 Strains, Growth Conditions, and Reagents

Yeast and bacterial strains used in this study are listed in Table 1. *E. coli* was cultured in Lysogeny Broth (LB) at 37°C, 200 rpm with appropriate antibiotics (ampicillin 100µg/ml, kanamycin 50 µg/ml, and chloramphenicol 25 µg/ml) when necessary. *Saccharomyces cerevisiae* was grown at 30°C either in yeast extract peptone dextrose (YPD) media, or on synthetic defined (SD) agar without individual amino acids as required; Quadruple Dropout (QDO):-Leu,-Trp,-Ade,-His, and Double Dropout (DDO):-Leu,-Trp. DDO was prepared by supplementing SD agar with 2% glucose, 20 mg/L arginine HCl, 30 mg/L isoleucine, 30 mg/L lysine HCl, 20 mg/L methionine, 50mg/L phenylalanine, 200 mg/L threonine, 30 mg/L tyrosine, 20/L mg uracil, 150mg/ml valine, 20 mg/L histidine HCl monohydrate (His), and 20mg/L adenine hemisulfate (Ade). QDO was prepared by adding similar supplements except that His and Ade were omitted. Reagents were purchased from Sigma-Aldrich, unless stated otherwise.

2.2 Plasmids and Cloning Technique

Plasmids and Primers used in this study are listed in Table 2. DNA templates can be found in Table 3. EspO1 and EspO2 were amplified from the cDNA of *E. coli* O157:H7 strain EDL933. OspE1 was amplified from *Shigella flexneri* cDNA. Human DnaJB1 and DnaJB6 were amplified from commercial pCMV-sport6::DnaJB1, and pDONR201::DnaJB6 plasmids, respectively. Human HAX-1 variant I truncation was amplified from pGADT7::HAX-1 (Constantinou 2013). Gene amplification was carried by polymerase chain reaction (PCR) using KOD hot start polymerase enzyme (Novagen), unless stated otherwise. Each of 50µl PCR reactions were comprised of DNA template 1µl, forward primer (10pmol/µl) 1.5µl (Thermo Scientific), reverse primer (10pmol/µl) 1.5µl (Thermo Scientific),
dNTPs (2mM each) 5µl, 10x reaction buffer 5µl, 25 mM MgSO₄ 3µl, H₂O 32µl, and 2.5 U/µl KOD DNA polymerase 1µl. The following cycling conditions were used: 95 °C 20 seconds, 54 °C 10 seconds, 70 °C 30 seconds per kilobase, for 30 cycles, followed by 70 °C 2 minutes. PCR products were purified using PCR purification kit (Qiagen). Empty vectors were propagated overnight in *E. coli* and extracted using plasmid miniprep kit (Qiagen). Purified PCR products and vectors were then double digested using appropriate restriction enzymes (NEB); digestion reactions were set following manufacturer instruction. At the end of the incubation period, antarctic phosphatase (0.1 unit, NEB) and antarctic phosphatase buffer (NEB) were added to digestion mixtures. The mixtures were incubated for further 15 minutes at 37 °C prior to adding a loading dye (NEB). Double digestion products were then separated on a 1% agarose gel; appropriate bands were excised and purified following manufacture protocol (Qiagen, gel purification kit).

All ligations were performed by mixing 5µl of instant sticky-end ligase master mix (NEB), 50 ng of vector, the appropriate amount of insert to gain a 5:1 insert : vector ratio, and H₂O to achieve 10µl final volume. The ligations were allowed to occur for 15 minutes at room temperature. Constructs were then transformed into competent cells using appropriate transformation methods. All constructs were verified by colony PCR and DNA sequencing (GATC).

### 2.3 Site-directed Mutagenesis

Point mutations or single amino acid changes were performed using the Quick ChangeII Site-directed mutagenesis kit (Stratagene). According to manufacturer’s instruction, 50µl reactions were comprised of 10x reaction buffer 5µl, forward primer (10pmol/µl) 1.5µl, reverse primer (10pmol/µl) 1.5µl, dNTPs 1µl, DNA template 30ng, and H₂O was added to achieve 50µl reaction volume. Then, PfuUltra HF DNA polymerase 1µl was added to the
mixture. PCR cycling conditions were as follows: 95 °C 30 seconds, 54 °C 1 minute, 68 °C 8 minutes, for 18 cycles. Once PCR cycles were completed, 1 µl of Dpn I restriction enzyme was added to the PCR products prior to 1 h incubation at 37 °C. After the incubation period, PCR products were transformed into competent cells.

2.4 Bacterial Transformation

2.4.1 Heat Shock Transformation

Chemically competent cells were mixed with 10 ng of construct and incubated on ice for 15 minutes, before 1 minute heat shock at 42 °C. Then, 250 µl of super optimal catabolite repression medium (Invitrogen) was added to the transformation reaction. Transformation mixtures were recovered for 1 h at 37 °C before being spread onto LB agar plates containing appropriate antibiotics.

2.4.2 Electroporation Transformation

Electroporation transformation was performed by adding 150 ng of DNA to 50 µl of prepared electroporation competent cells. The transformation mixtures were incubated for 30 minutes on ice. Mixtures were then transferred to a 0.2 ml sterile gene pulser cuvette (Bio-rad) and subjected to a 2.5kV, 200 Ω, 25 µF pulse (Gene PulserII, Bio-rad). After the addition of 800 µl of LB, transformation mixtures were then incubated for 1 h at 37 °C. After incubation, bacterial cells were pelleted at 12,000 rpm, 1 minute, and resuspended in 200 µl of LB before being spread onto LB agar plates containing appropriate antibiotics.

2.5 Direct Yeast Two Hybrid (Y2H)

A single colony of AH109 on a YPDA plate was used to inoculate YPDA broth and cultured overnight at 37°C, 200 rpm. On the following day, for 10-15 transformations, the overnight
culture was diluted 1:10 in 50 ml YPD broth, and grown for 4 h. The culture was centrifuged for 5 minutes at 4000 rpm, washed with 10 ml sterile water, and centrifuged for a further 5 minutes at 4000 rpm. The supernatant was removed, and the pellet was re-suspended in 1ml of 100mM Lithium acetate (LiAc), and transferred to a 1.5 ml eppendorf tube. The cells were then centrifuged at 14,000 rpm for 1 minute, and re-suspended in 500µl 100 mM LiAc. Competent yeast were then aliquoted into eppendorf tubes in 50µl aliquots, before centrifugation and removal of the supernatant. Then, 240 µl of polyethylene glycol, 36 µl of 1 M LiAc, 25 µl of herring sperm DNA, and 200 ng of DNA plasmid were added in that order and mixed gently. All transformation mixtures were then incubated for 30 minutes at 30°C, followed by being transferred to 42°C for 25 minutes. At the end of incubation period, transformation mixtures were centrifuged at 14,000 rpm for 1 minute, supernatant was removed and pellets were resuspended in 200 µl sterile water, prior to being spread onto DDO plates. The plates were incubated at 30 °C for 5 days. Yeast colonies were re-streaked on DDO and QDO. Protein interactions were indicated by growth on QDO plate.

2.6 Cell Lines and Growth Conditions

Cell lines used in this study are listed in Table 1.

2.6.1 HeLa ATCC cell line

HeLa ATCC cells were cultured in Dulbecco Modified Eagle Medium (DMEM) supplemented with 1000 mg/L glucose, 10% fetal calf serum (FCS) (Gibco), 1mM Glutamax (Invitrogen), and 1% non-essential amino acids (Sigma-Aldrich) at 37 °C with 5% CO₂ (v/v).
2.6.2 HeLa miHAX-1 and miNEG Cell Lines

HeLa miHAX-1 and miNEG cell lines were maintained in DMEM supplemented with 1000mg/ml glucose, 10% FCS (Gibco), 2mM Glutamax (Invitrogen), 0.1 mM non-essential amino acids, and 7.5 µg/ml blasticidin (InvivoGen) at 37 °C with 5% CO₂ (v/v).

2.7 Transfections of Mammalian Cells

24 h prior to transfection, cells were seeded in a 24 well plate (BD-Falcon) on glass coverslips (VWR) at a density of 5.5 x 10⁴ cells per well. On the day of transfection, 500 µl of culture medium was removed from each well. Transfection mix was prepared as follows for each well: 0.75 µl GeneJuice transfection reagent (Novagen) and 20 µl Opti-MEM (Gibco) were mixed and incubated for 5 minutes at room temperature. 0.25 µg of mammalian expression vectors were then added to the GeneJuice/Opti-MEM and gently mixed. The transfection mix was incubated for a further 5-15 minutes before 20 µl was added to each well, dropwise. Cells were incubated at 37 °C with 5% CO₂ (v/v) before being processed as described. Mammalian expression vectors used in this study are listed in Table 2.

2.8 Staurosporine-induced Apoptosis

At 21 h post-transfection, apoptosis was induced by the addition of 1 µM staurosporine (STS)/DMEM (1000 mg/ml glucose with supplements). After a 3h incubation period, cells were washed, fixed by paraformaldehyde (PFA) (Agar Scientific) as described below and stored at 4°C until processing. Apoptotic cell death was quantified by use of a Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) assay and cleaved caspase-3 staining.
2.9 Immunofluorescence Staining

2.9.1 PFA Fixation

Cells were washed 3 times with 1 ml phosphate buffered saline (PBS) and fixed with 250 µl of 3.7% PFA at room temperature for 20 minutes. PFA was removed and cells were washed 3 times with 1 ml PBS before being stored at 4°C until being processed as described.

2.9.2 Staining

Fixed cells on glass coverslips were neutralised with 500 µl of 50 mM NH₄Cl/PBS for 10 minutes, permeabilised with 200 µl of 0.1% Triton X-100/PBS for 8 minutes at room temperature and washed 3 times with 1 ml PBS. Non-specific antibody binding was prevented by incubating with 200 µl of 2% bovine serum albumin (BSA)/PBS for 30 minutes. 2% BSA was removed prior to the application of primary antibodies, which were diluted in 1% BSA/PBS for 45 minutes at room temperature. Antibodies used in this study are listed in Table 4. Coverslips were washed 3 times with 1 ml PBS and further incubated with appropriate secondary antibodies diluted in 1% BSA/PBS for 45 minutes at room temperature. Secondary antibodies were washed off 3 times with 1 ml of PBS. Finally, coverslips were briefly immersed in water and mounted on a drop of ProLong® Gold antifade reagent (Invitrogen) on glass slides. Mounted coverslips were dried overnight in the dark at room temperature, then stored at 4°C until being visualised under AxioObserver.Z1 microscope (Zeiss).

2.9.3 TUNEL Assay

The TUNEL assay was performed according to manufacturer’s specification (DeadEnd™ Fluorometric TUNEL System kit, Promega). Fixed cells were treated with 50 mM NH₄Cl/PBS 500 µl for 10 minutes, prior to being permeabilised by 0.1% Triton X-100/PBS
for 5 minutes. Then, coverslips were washed 3 times with PBS and incubated with 80µl of equilibration buffer for 10 minutes at room temperature. For the staining of apoptotic nuclei, 30µl of incubation buffer, assembled according to manufacturer’s instruction, was added to each well. The reaction was allowed to proceed at 37°C in the dark for 1h, before it was stopped by incubating the cells in the SSC solution, provided by the manufacturer, for 15 minutes at room temperature. The coverslips were incubated with 5mg/ml 0.1% Triton X-100/BSA for 5 minutes, followed by 2 PBS washes. Finally, coverslips were blocked with 2% BSA/PBS and immunofluorescence staining was proceeded as described in Section 2.9.2.

2.10 In vitro Infections of HeLa Cells

2.10.1 EPEC Infections

48 h prior to infection, HeLa cells were seeded in 24 well plate (BD Falcon), containing glass coverslips (VWR) when necessary, at a density of 7.5 x 10^4 cells per well. 17 h prior to infection, EPEC strains were grown at 37°C in LB with antibiotics when necessary. On the day of infection, cultures were primed by diluting overnight cultures 1:100 in pre-warmed DMEM (1000 mg/ml glucose) without supplements, and incubating for 3 h at 37°C 5% CO₂ (v/v) static. Before infection, HeLa cells were washed 3 times using PBS before addition of 500µl primed cultures to each well. Cells were incubated at 37°C 5% CO₂ (v/v). At 1 h post-infection, cells were washed 3 times with PBS before adding 500 µl of 300µg/ml gentamicin/DMEM into each well; cells were maintained at 37°C 5% CO₂ for the remainder of the infection.

2.10.2 EHEC Infections

48 h prior to infection, HeLa cells were seeded in 24 well plate (BD Falcon), containing glass coverslips (VWR) when necessary, at a density of 7.5 x 10^4 cells per well. On the day before
infection, EHEC strains were grown in LB with appropriate antibiotics for 8 h at 37°C 200 rpm before being diluted 1:500 in pre-warmed DMEM without supplements and incubated overnight for 14 h at 37°C 5% CO₂ (v/v). Before infection, overnight EHEC cultures were diluted 1:15 in fresh pre-warmed DMEM without supplements. HeLa monolayers were washed 3 times with PBS before adding 500 µl of diluted EHEC culture into each well. The plates were centrifuged at 500 x g for 5 minutes, and incubated at 37°C 5% CO₂. At 2.5 h post-infection, cells were washed 3 times with PBS, then further incubated with 500 µl of 100 µg/ml gentamicin/DMEM without supplements. At the desired time point, cells were washed 3 times with PBS and fixed with PFA. Alternatively, cells were washed and further processed for cell detachment assays.

2.11 Cell Detachment Assay

Post-infection, cells were washed 5 times with PBS. Then, cells were incubated with 200 µl trypsin for 10 minutesat 37°C 5% CO₂, followed by additionof 200 µl DMEM With supplements. The cells were gently dispersed by pipetting before counting using a haemocytometer. Percentage cell detachment was calculated relative to uninfected controls (referred to as 0%).

2.12 HAX-1 Detection by Western Blot Analysis

Cells were seeded in 6 well plates (BD falcon) at a density of 1.5x10⁵ cells per well. When cells reached approximately 95% confluency, cells were washed 3 times with PBS before being lysed in 100µl of Laemmli buffer (2% Sodium Dodecyl Sulfate (SDS), 10% Glycerol, 0.01% Bromophenol Blue, 0.0625 M Tris pH 6.8, and 0.1 M Dithiothreitol (DTT)) and samples were boiled for 5 minutes. The samples were run on 12.5% SDS-PAGE gels and transferred to polyvinylidenedifluoride (PVDF) membranes (Amersham). The membranes
were blocked in 5% skim milk/PBS containing 0.02% Tween-20 (PBST) for 45 minutes. Then, membranes were incubated with primary antibody diluted in 1% skim milk/PBST at 4 °C overnight. On the following day, membranes were washed with PBS for 5 minutes, 3 times. Then, membranes were stained for 1 h with appropriate secondary antibody diluted in 1% skim milk/PBST. After 1 h of incubation, membranes were washed 3 times with PBS, prior to development with Ez-ECL solution (Geneflow) for 2 minutes, and visualisation with an LAS-3000 imager (Fuji).

2.13 Cytoplasmic Ca\(^{2+}\) Detection

Intracellular Ca\(^{2+}\) detection during \textit{in vitro} EPEC infection was performed using the Fluo-4 Direct™ Calcium Assay Kit (Invitrogen), following manufacturer’s instructions. Briefly, 48 h prior to infection, HeLa cells were seeded in black 96 well plates (BD falcon) at a density of 1.5 x 10^4 cells per ml. 17 h prior to infection, EPEC strains were inoculated in 5 ml LB broth, and incubated at 37°C. 3 h prior to infection, EPEC prime cultures were prepared in DMEM without phenol red and supplements. At 1 h before infection, cells were gently washed with Hank's balanced salt solution (HBSS) before adding 50 µl of fresh DMEM (without phenol red and supplements), and calcium probe, then the cells were maintained at 37 °C 5% CO\(_2\). When primed cultures were ready, 25 µl of prime culture was added into each well. The infection was allowed to continue for 1 h at 37 °C 5% CO\(_2\) static. After the incubation period, cytosolic Ca\(^{2+}\) was measured at 494 nm excitation, 516 nm emission, using a fluorescence plate reader (FLUOstar Omega, BMG labtech).

2.14 Image Processing and Statistical Analysis

Data analysis was performed using GrapPad Prism software, using two-way ANOVA or one-way ANOVA where specified, and Bonferroni post-test. Results were judged to be
statistically significant when the P value was <0.05. Immunofluorescence images were visualised and processed using ImageJ (Fiji) software (Schindelin et al. 2012).

Table 1. Bacterial and yeast strains, and cell lines used in this study.

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>description</th>
<th>Source</th>
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<tr>
<td>E2348/69</td>
<td>E. coli O127:H6 strain E2348/69</td>
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<tr>
<td>∆nleH</td>
<td>E. coli O127:H6 strain E2348/69 ∆nleH1∆nleH2, Cm', Kan'</td>
<td>Hemrajani et al. 2010</td>
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<tr>
<td>EDL933</td>
<td>Wild type E. coli O157:H7 stx'</td>
<td>ATCC</td>
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<tr>
<td>EDL933 ∆espO</td>
<td>E. coli O157:H7 strain EDL933 ∆espO1∆espO2</td>
<td>Constantinou 2013</td>
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<td>85-170</td>
<td>Spontaneous Nal' derivative of E. coli O157:H7 85-170</td>
<td>Stevens et al. 2004</td>
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<tr>
<td>85-170∆nleF</td>
<td>E. coli O157:H7 85-170 ∆nleF</td>
<td>Mitchell Pallett, unpublished</td>
</tr>
<tr>
<td>85-170∆nleH</td>
<td>E. coli O157:H7 85-170 ∆nleH1∆nleH2</td>
<td>Hemrajani et al. 2008</td>
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<td>HeLa</td>
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<td>ATCC</td>
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<tr>
<td>miHAX-1</td>
<td>miRNA HAX-1 silencing cell line derived from HeLa</td>
<td>Grzybowska et al., 2013</td>
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<td>miNEG</td>
<td>miRNA negative control silencing cell line derived from HeLa</td>
<td>Grzybowska et al., 2013</td>
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</table>

Cm', Chloramphenicol resistance 10µg/ml; Kan', Kanamycin resistance 50µg/ml; Nal', Nalidixic acid resistance

Table 2. Plasmids and primers used in this study.

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<td>pcDNA</td>
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<td>RS</td>
</tr>
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<td>EcoRI XhoI</td>
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<td>pcDNA::flag-ospE1</td>
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<tr>
<td>pSA10::ha-nleF</td>
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<td>pGADT7</td>
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<td>Clonetch</td>
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<td>DnaJB1 truncation isolated from Y2H screen</td>
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<td>DnaJB6 80-241</td>
<td>DnaJB6 truncation isolated from Y2H screen</td>
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<td>primer 5' to 3'</td>
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<td>pCMV6::myc</td>
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<td>pRK5</td>
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<p>| pGBKT7             | Plasmid for expression of proteins in yeast with a GAL4 activation domain fusion; N-terminal Myc tag; Kan | | | clonetec |</p>
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<tr>
<td>pRK5::myc-gfp</td>
<td>GFP</td>
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<td>N/A</td>
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**Table 3. DNA templates used in this study.**

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<td>pDONR201:DnaJB6</td>
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<td>pCMV::HAX-1</td>
<td>Vafiadaki et al., 2009</td>
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<tr>
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<td>Shigella flexneri strain M90T</td>
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<td>EHEC EDL933</td>
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**Table 4. Antibodies used in this study.**

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<td>Millipore, 05-724</td>
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<td>anti-HA tag, clone16B12</td>
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<td>Cambridge Bioscience, MMs-101P-1000</td>
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<td>anti-Y397 phosphorylated FAK</td>
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<td>anti-TOMM22</td>
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<td>Abcam, ab57523</td>
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<tr>
<td>anti-cleaved caspase-3</td>
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<td>Cell signaling, 9664</td>
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<td>anti-GFP</td>
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<td>Abcam, ab-290</td>
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<td>Antibodies</td>
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<td>anti-HAX-1 (E-20)</td>
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<tr>
<td>anti-FLAG® M2-FITC-conjugated</td>
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### Secondary antibodies used for immunofluorescence staining

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### Chemicals used for immunofluorescence staining

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### Primary antibodies used for western blot analysis

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### Secondary antibodies used for western blot analysis

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Chapter 3 The Characterisation of EspO1
Chapter 3 The Characterisation of EspO1

3.1 Bioinformatic Analysis

3.1.1 The Distribution of EspO1 and Its Homologs in Enteric Pathogens

In order to further investigate the distribution of EspO effector family in enteric pathogens, the nucleotide sequence of EspO1 from *E. coli* O157:H7 strain EDL933 was searched against the ncbi database by using blastx tool (https://blast.ncbi.nlm.nih.gov/, last accessed on 1/7/2016). In addition to *E. coli* EHEC O157:H7 strain EDL933, EspO homologs were found in other EHEC O157:H7 strains such as Sakai, and TW14359 as well as other EHEC serotypes: O145:H28, and O111:H11. The blast result confirmed that EspO homologs are encoded in several enteric pathogen species; *Shigella spp.* (OspE1 and OspE2), *Salmonella enterica* serovar Typhi (SopD), *Salmonella enteric* serovar Typhimurium (SopD), *Salmonella enterica* serovar Tenessee (SopD), and the mouse pathogen *C. rodentium* (EspO). Additionally, an EspO1 homolog was found in *Escherichai albertii* (EspO), which is a newly emerged enteric pathogen. The sequence of EspO1 homologs were aligned using Clustal Omega, and a phylogeny tree was created using Jalview (Waterhouse *et al.* 2009, Goujon *et al.* 2010, Sievers *et al.* 2011, McWilliam *et al.* 2013). Interestingly, EspO1 and EspO2 of EHEC EDL933 are not as closely related as OspE1 and OspE2 of *S. flexneri* (Figure 19).
3.1.2 Similarity of EspO1 and Its Homologs

In order to further investigate the region of similarity among the homologs, the sequence of EspO1 (EHEC O157:H7 strain EDL933), EspO2 (EHEC O157:H7 strain EDL933), EspO (C. rodentium), and the well characterised OspE1 (S. flexneri) were aligned using Clustal Omega (Goujon et al. 2010, Sievers et al. 2011, McWilliam et al. 2013). The amino acid sequence alignment of EspO1 and EspO2 reveals 80.4% similarity (Figure 20). EspO1 and EspO C. rodentium share 69.6% similarity, while EspO1 and OspE1 (S. flexneri) share only 38.9% similarity. EspO C. rodentium and OspE1 S. flexneri are 40.6% similar (Figure 20).
Consistent with the results from the previous study by Kim et al. (2009), the W residue required for ILK interaction is conserved among EspO homologs (Figure 20) (Kim et al. 2009).

Figure 20. Amino acid sequence alignment by Clustal Omega. EspO1 and EspO2 from EHEC EDL933, EspO from C. rodentium as well as OspE1 of S. flexneri were aligned. The conserved W required for ILK binding is highlighted by a red box.

3.1.2 The Predicted Structure of EspO1 and OspE1

To aid in determining potential domains or residues that may relate to function and binding interaction partners, EspO1 and OspE1 structures were predicted using Phyre2 server (Kelley et al. 2015). Phyre2 accepts a target amino acid sequence and detects sequence homology in PSI-blast database, then creates a predicted protein structure based on the folding of known protein structures. The output provides predicted secondary structure indicating the location of α-helix, β-strain, coil, and disordered regions. Disordered regions present an amino acid range that potentially plays a role in protein-protein interaction (van der Lee et al. 2014). Additionally, the output also informs template information indicating the alignment between the target sequence and a protein template used for structure prediction (Kelley et al. 2015). Predicted 3D structures were visualised by Jmol software (http://www.jmol.org/, last accessed on 23/09/2016) (Herraez 2006).
The predicted 3D and secondary structures of EspO1 are shown in Figure 21. The model was created using Immunoglobulin-like β-sandwich protein as a template. Phyre2 predicted that EspO1 mainly contains short β-strands (3-9, 13-16, 33-57, 66-75, and 81-85 amino acids), and short α-helices (17-20, and 76-80 amino acids). The model also suggested that EspO1 contains a disordered region at the N-terminal (7-16 amino acids) and in the middle of the protein (38-58 amino acids). The T3SS effectors typically contain secretion signal sequence situated within the first 20 amino acids (Samudrala et al. 2009). Hence, the disordered region at the N-terminal of EspO1 potentially indicates the secretion signal sequence. Notably, the conserved W77 residue, essential for an interaction with ILK, was predicted to be situated in the α-helix located in the C-terminal region.
**Figure 21. Predicted structure of EspO1 from EHEC EDL933.** (A) The predicted 3D structure of EspO1 visualised by Jmol software. Structural motifs are presented in different colours; blue indicates β-turn, and yellow indicates β-strand. (B) The predicted secondary structure of EspO1.

The structure of OspE1 3D was also predicted by Phyre2. The model was created using a structural protein mkiaa1027 as a template. The predicted 3D and secondary structures of OspE1 are shown in Figure 22A and Figure 22B, respectively. Similar to EspO1, OspE1 mainly contains short β-strands (5-8, 16-21, 24-34, 36-49, 57-62 amino acids), and α-helices
at the C-terminal (63-75, 78-85 amino acids). The C-terminal of both EspO1 and OspE1 contains the conserved W residue that is essential for the interaction with ILK.

Figure 22. Predicted structure of OspE1 from *S. flexneri*. (A) The 3D structure of OspE1 predicted by Phyre2. The protein structure is visualised by Jmol. Each colour shows different structures; blue, yellow, and magenta present β-turn, β-strand, and α-helix, respectively. (B) The predicted secondary structure of OpsE1.

In a previous study, the structure of EspO1 and OspE1 were predicted using Phyre2 and confirmed by I-TASSER (Constantinou 2013). In this study, with a view to confirm models predicted by Phyre2, the secondary structure of EspO1 and OspE1 were also predicted by predictprotein automatic online service (Figure 23) (Yachdav *et al.* 2014). Predictprotein
predicts the secondary structure of proteins by aligning and comparing the target sequence with protein database (SWISSPROT), then the aligned sequences are used for structure prediction. In this study, secondary structures predicted by predictprotein confirmed that both EspO1 and OspE1 mainly comprise of β strands, and α-helices. Consistent with models predicted by Phyre2 and results reported by Constantinou (2013), the W residue was located in an α-helix in the C-terminal of EspO1 and OspE1. Nevertheless, β-strands were found to fall in slightly different amino acid regions from which they were predicted in the models created by Phyre2.

Figure 23. The secondary structure of EspO1 and OspE1 predicted by predictprotein server. The secondary structure of (A) EspO1, and (B) OspE1 predicted by predictprotein service. Prediction results are presented in 3 rows. The top row presents amino acid number of the protein from N-terminal to C-terminal. The middle role shows predicted protein binding region suggesting potential protein-protein interaction sites. The bottom row indicates a predicted secondary structure with blue and red blocks indicating strand, and helix structures, respectively.
3.2 Subcellular Localisation of EspO1 and OspE1 in HeLa Cell

3.2.1 Localisation of EspO1

3.2.1.1 EspO1 Localises to FAs and Mitochondria upon Ectopic Expression

In order to investigate the localisation of EspO1, the effector was ectopically expressed in mammalian cells. In a previous work done by Constantinou (2013), EspO1 was expressed from pRK5 vector, and appeared to aggregate into a vacuole that co-localised with ER marker. In this study, EspO1 was expressed from pcDNA-NTAP vector, and exhibited different phenotypes. In the majority of transfected cells, EspO1 localised at the edge of the cell in FA-like structures (Figure 24A). Moreover, EspO1 was also observed to accumulate in large aggregates resembling mitochondria in the cytoplasm (Figure 24B). As EspO1 contains the conserved W77 residue that mediates the interaction with ILK, we investigated the localisation of ectopically expressed EspO1 W77A. EspO1 W77A did not localise to FA-like structures. Instead EspO1 W77A was observed to be dispersed throughout the cytoplasm (Figure 24C) or accumulate in punctate structure (Figure 24D).
Figure 24. EspO1 and EspO1 W77A localisation during ectopic expression. HeLa cells were transfected with pcDNA constructs expressing the indicated effector (A&B) EspO1 or (C&D) EspO1 W77A with an N-terminal FLAG fusion. Cells were fixed after 24 h of expression and stained with anti-FLAG antibody (red) and DAPI (blue) to visualise the effectors, and DNA, respectively. The result is the representative of 3 biological repetitions. Bar = 20µm.
In order to confirm if EspO1 localised to FAs and mitochondria, ectopically expressed FLAG-EspO1 was co-stained with Y397 phosphorylated focal adhesion kinase (FAK) and inner mitochondrial membrane protein TOMM22 to determine EspO1 subcellular localisation. Untransfected cells, used as controls, showed the expected localisation for each marker. In EspO1 expressing cells, the co-localisation between EspO1 and both organelle markers was clearly observed (Figure 25). Therefore, the results revealed that EspO1 localised to both FAs and mitochondria during transfection.

Figure 25. EspO1 co-localises with FA and mitochondrial markers. HeLa cells were transfected with pcDNA construct expressing EspO1 with N-terminal FLAG fusion for 24 h before being fixed and immunostained. Cells were co-stained with anti-FLAG antibody (red) to visualise EspO1, and either (A) anti-phosphorylated Y397 FAK antibody (green) to visualise FA, or (B) anti-TOMM22 antibody (green) to visualise mitochondria. DNA was visualised by DAPI staining (blue). Bar = 20µm.
3.1.1.2 EspO1 Localises to FAs during In vitro Infection

The result from ectopic expression indicates that EspO1 localises at FAs and mitochondria. However, ectopic expression does not always reflect effector localisation during infection, therefore in vitro infections were performed. In previous works done by Morita-Ishihara (2013), and Constantinou (2013), HA-EspO1 is localised to FA during in vitro EHEC infection. EspO1 polyclonal antibodies were raised in rabbits to help in the detection of endogenous EspO. Unfortunately, the characterisation of anti-EspO1 antibody by immunoblot and immunofluorescence indicated that the antibody was not able to detect the endogenous level of EspO1 (data not shown). Therefore, EspO1 was expressed with a C-terminal 4xHA tag in order to track the protein. In this study, two infection models were tested; EHEC O157:H7 strain EDL933 (EDL933), and EPEC O127:H6 strain E2348/69 (E2348/69). EHEC EDL933 encodes two EspO effectors; EspO1 and EspO2, while EPEC E2348/69 contains a pseudo espO gene, and can therefore be considered as a “null” mutant. HeLa cells were infected with EDL933 ∆espO1∆espO2 (EDL933 ∆espO) and E2348/69 strains overexpressing C-terminal HA tagged EspO1 and EspO1 W77A. EDL933 ∆espO and E2348/69 were used as negative controls. The localisation of EspO1 and EspO1 W77A was studied at different infection time points in order to monitor effector localisation during different stages of infection which are 2.5, 5, 7.5 h post-infection for EDL933 and 1, 3, 6 h post-infection for E2348/69. There was no difference in the localisation of the effectors whether they were delivered by EHEC or EPEC. EspO1 localised to the cell periphery at every time point. EspO1 W77A was diffuse throughout the cytoplasm at every time point. Since there was no difference in EspO1 and EspO1 W77A localisation among time points investigated, the localisation of EspO1 and EspO1 W77A during EHEC (Figure 26) and EPEC (Figure 27) infection is shown by one representative time point.
Figure 26. The localisation of EspO1 and EspO1 W77A at 2.5 h post-EHEC infection. HeLa cells were infected with EDL933 ∆espO, EDL933 ∆espO/pSA10::espO1x4HA (EDL933 ∆espO/pespO), or EDL933 ∆espO/pSA10::espO1 W77AxAHA (EDL933 ∆espO/pespO1 W77A). At 2.5 h post infection, cells were washed and fixed prior to immunofluorescence staining. Phalloidin (red) was used to visualise the actin pedestals, which confirms other T3SS effectors are being translocated. HA-tagged effectors, bacteria, and DNA were stained by anti-HA antibody (green), anti-O157:H7 antibody (cyan), and DAPI (blue), respectively. EspO1 was localised to structures resemble to FAs, and EspO1 W77A was diffused throughout cytoplasm. Bar = 20µm.
Figure 27. The localisation of EspO1 and EspO1 W77A at 1 h post-EPEC infection. At 1 h post-infection with E2348/69, E2348/69/pSA10::espO1x4HA (E2348/69/pespO1), or E2346/69/pSA10::espO1 W77Ax4HA (E2348/69/pespO1 W77A), cells were fixed and stained with anti-HA antibody (green), anti-O127:H6 antibody (cyan), phalloidin (red), and DAPI (blue) to visualise indicated effectors, EPEC, actin, and DNA, respectively. EspO and EspO1 W77A were observed to localise to FA resembling structures and disperse throughout cytoplasm, respectively. Bar = 20µm.
As EspO1 was localised to structures that resembled FAs, EspO1 was co-stained with Y397 phosphorylated FAK to confirm FA localisation. Uninfected cells showed the expected localisation of the FA marker (Figure 28A). The co-localisation between EspO1 and the mitochondrial marker was not investigated because EspO1 did not display the punctate phenotype observed during ectopic expression. Considering there was no difference in EspO1 and EspO1 W77A localisation dependent on whether the effectors were delivered by EHEC or EPEC, the co-staining was only performed only during EHEC infection. The co-localisation between EspO1 and the FA marker was clearly observed in EDL933 ΔespO/pespO1 infected cells (Figure 28B), while there was no co-localisation between EspO1 W77A and the FA marker (Figure 28C). The result suggested that EspO1 localised to FAs during \textit{in vitro} infection, and the interaction with ILK is essential for EspO1 FA localisation.

![Figure 28](image)

**Figure 28.** EspO1 is localised to FAs during \textit{in vitro} infection. (A) The expected localisation of the FA marker in uninfected cells. Cell were infected with EHEC strains over expressing C-terminal 4xHA fusion tagged (B) EspO1 or (C) EspO1 W77A for 2.5 h prior to
fixing. The cells were co-stained with anti-FAK (green), and anti-HA (red) antibodies for FAs and EspO1 visualisation, respectively. DNA was stained by DAPI (blue). Bar = 20µm.

3.1.2 OspE1 from Shigella flexneri Localises to FAs when Ectopically Expressed in HeLa Cells

Ectopically expressed OspE1 was previously observed to localise to FAs, dependent on the OspE1-ILK interaction, which is mediated by the W68 residue (Kim et al. 2009, Yi et al. 2014). In this study, the localisation of OspE1 and OspE1 W68A was investigated to confirm the role of ILK interaction on OspE1 localisation. Similar to EspO1, OspE1 localised to FA-resembling structures (Figure 29A) as well as punctate structures (Figure 29B), while OspE1 W68A appeared diffuse throughout the cytoplasm (Figure 29C).
Figure 29. OspE1 localisation during ectopic expression in HeLa cells. HeLa cells were transfected with pcDNA constructs expressing the indicated effector (A&B) OspE1 or (C) OspE1 W68A with N-terminal FLAG fusion. After 24 h of transfection, the cells were fixed and immunostained with anti-FLAG antibody (red) and DAPI (blue) for effectors and DNA visualisation, respectively. OspE1 localised to FA-resembling and punctate structures, while OspE1 W68A was diffused throughout cytoplasm. Bar = 20µm.

OspE1 and OspE1 W68A were co-stained with the FA marker in order to confirm the localisation of OspE1 at FAs. While OspE1 co-localised with Y397 phosphorylated FAK, OspE1 W68A did not (Figure30). This suggested that, consistent with the role of ILK interaction on the localisation of EspO1, OspE1-ILK interaction is essential for the FA localisation of OspE1. The FA localisation of OspE1 is in line with previous studies;
however, the punctuate phenotype of OspE1 has never been described (Miura et al. 2006, Kim et al. 2009, Yi et al. 2014).

![Image of microscopy results]

Figure 30. OspE1 co-localises with the FA marker, while OspE1 W68A does not. (A) untransfected cells, and cells transfected with pcDNA constructs expressing (B) OspE1 or (C) OspE1 W68A with N-terminal FLAG fusion were fixed, prior to staining with anti-FLAG antibody (red) to visualise the effectors, anti- Y397 phosphorylated FAK antibody (green) to visualise FAs, and DAPI (blue) to visualise DNA. Bar = 20µm.

3.2 HAX-1: The Novel Interaction Partner of EspO Effector Family

3.2.1 HAX-1 is a Putative Interaction Partner of EspO1 and Its Homologs

In a previous study, HAX-1 variant I, DnaJB1 truncation containing 80-241 amino acids, and DnaJB6b truncation containing 73-340 amino acids were identified as novel interaction partners of EspO1 by a Y2H screen (Constantinou 2013). In order to confirm interactions between EspO effector family and newly identified targets, direct Y2H was performed by using full length proteins as well as the truncated versions identified from the screen as preys.
(Figure 31). The growth on selective QDO media (-Ade/-His/-Leu/-Trp) indicated that EspO1 interacted with full length HAX-1 as well as truncated version of DnaJ1 and DnaJB6, but not full length DnaJB1 or DnaJB6 (Figure 31B). EspO1 homologs; EspO2 (EHEC EDL933), OspE1 (S. flexneri), and EspO (C. rodentium) also interacted with HAX-1, but not DnaJB1 nor DnaJB6.

**Figure 31.** EspO1 and its homologs interact with HAX-1 variant I. Potential EspO1 interaction partners identified by Y2H screening were confirmed by a direct Y2H assay. (A) The growth of yeast AH109 on double dropout (DDO) medium (Leu/-Trp) indicated that both plasmids were successfully co-transformed into yeast. (B) Growth on QDO indicated that the two fusion protein reconstitute the GAL4 promoter, allowing growth on selective media and indicating a protein-protein interaction. p53 and SV40 were used as a positive control. aa, amino acids; CR, C. rodentium.
3.2.2 Interaction Region Mapping

3.2.2.1 HAX-1 Interaction Region Mapping

HAX-1 is comprised of 4 known domains; BH1, BH2, PEST, and C-terminal domain (Figure 32A) (Fadeel and Grzybowska 2009). With a view to determine the amino acid region that is responsible for EspO1-HAX-1 interaction, a series of HAX-1 truncations were constructed, and their interactions with EspO1 were tested by direct Y2H (Figure 32). The assay revealed that the interaction region between EspO1 and HAX-1 falls in the C-terminal domain (118-279 amino acids) which has no known functional domain (Fadeel and Grzybowska 2009). Therefore, a specific amino acid region responsible for the interaction with EspO1 was not yet identified.
Figure 32. HAX-1 interaction region mapping by direct Y2H. (A) Diagram presented the molecular domain of full length HAX-1, and HAX-1 truncations tested by direct Y2H. (B) Growth on DDO confirms the presence of both plasmids in yeast. (C) Growth on QDO plate
indicated protein interaction. p53 and SV40 were used as the positive control of protein-protein interaction. aa, amino acid.

### 3.2.2.2 EspO1 Interaction Region Mapping

In order to map an interaction region required for the interaction with HAX-1, interactions between full length HAX-1, and EspO1 mutants as well as EspO1 truncations were tested by direct Y2H (Figure 33A). EspO1 is 91 amino acids in length, and the W77 is the highly conserved amino acid that is required for EspO1-ILK interaction. To begin with, EspO1 was divided into N- and C-terminal domains (1-45 and 46-91 amino acids, respectively); both truncations were tested for interaction with HAX-1. Only the C-terminal fusion interacted with HAX-1.

With a view to further determine potential domains or residues that may be interaction sites, predicted EspO1 structure from predictprotein server was considered (Figure 23A). The HAX-1-interacting C-terminal half of EspO1 contains β-strands (at amino acids 44-56 and 66-77), and an α-helix (at amino acids 74-83). In order to test if inclusion of the predicted β-strand at amino acids 44-56 to the non-interacting N-terminal region of HAX-1 was sufficient to restore the EspO1-HAX-1 interaction, an EspO11-56 amino acids truncation was tested for an ability to interact with HAX-1 by direct Y2H. The truncation was able to interact with HAX-1, indicating that the 46-56 amino acid region might be responsible for EspO1-HAX-1 interaction. In order to confirm if the suspected region mediates the interaction between EspO1 and HAX-1, EspO1 1-50 amino acids truncation was tested. Unexpectedly, partial growth was observed as the colony was pink in comparison to the colony color of positive control. According to the 3D model structure predicted by Phyre2 (Figure 21A), there is a short loop consisting of 2 lysine amino acids: K49 and K50 within this β-strand. The importance of these lysines for the interaction between EspO1 and HAX-1 was therefore
tested using an EspO1 K49A K50A mutant. Growth was still observed by direct Y2H indicating the K49 and K50 amino acid residues did not mediate the EspO1/HAX-1 interaction (Figure 33C). Therefore, while a particular amino acid residue essential for EspO1 and HAX-1 could not be identified, amino acids 46-56, containing part of a predicted β-strand, appears to be essential for this interaction. In addition, the ILK interacting amino acid was altered (W77A) and tested for interaction with HAX-1 (Figure 33C). The EspO1 W77A mutant was able to interact with HAX-1 indicating that W77 was not mediating the interaction with HAX-1.

Figure 33. EspO1 interaction region mapping by direct Y2H. (A) The first row presents diagram showing predicted EspO1 secondary structure where purple and red indicate β-strands and α-helices, respectively. EspO1 truncations used for interaction region mapping
were also listed. (B) Growth of the yeast AH109 on DDO confirmed successful co-transformation. (C) The growth of yeast on QDO indicated protein interaction. p53 and SV40 were used as the positive control of protein-protein interaction. EspO1 requires the 46-55 amino acids for HAX-1 binding. aa, amino acids.

3.2.3 EspO1 and HAX-1 Co-localise in HeLa cells upon Ectopic Expression, but not during *In Vitro* Infection.

3.2.3.1 EspO1 and HAX-1 Co-localised when Co-Expressed in HeLa Cells. Constantinou (2013) reported that endogenous HAX-1 co-localised with EspO1 vacuole; however, the subcellular compartment in which 2 proteins co-localised was not determined. In this study, the localisation of HAX-1 was also investigated. In an attempt to detect endogenous HAX-1, two commercial HAX-1 antibodies were tested for the detection of endogenous HAX-1 by immunofluorescence staining. Endogenous HAX-1 could not be detected (data not shown), and therefore myc-HAX-1 was ectopically expressed in HeLa cells for the purpose of HAX-1 visualisation. Upon ectopic expression, HAX-1 localised to punctate structures resembling mitochondria. To specify the subcellular compartment where HAX-1 localised, ectopically expressed HAX-1 was co-stained with the inner mitochondrial membrane protein TOMM22. The co-localisation between HAX-1 and the mitochondrial marker confirmed that HAX-1 was localised to mitochondria (Figure 34). The result is in line with the previous study which reported that HAX-1 is localised to mitochondria (Yap *et al.* 2010)
Figure 34. HAX-1 localises to mitochondria in HeLa cells. HeLa cells were transfected with pCMV6::myc-hax-1. At 24 h post-transfection, the cells were fixed. (A) untransfected cells, and (B) transfected cells were co-stained with anti-HAX-1 (green), and anti-TOMM22 (red) antibodies for HAX-1, and mitochondria visualisation, respectively. DNA was stained by DAPI (blue). Bar = 20µm.

The localisation of EspO1 and HAX-1 upon co-expression was investigated in order to further investigate EspO1-HAX-1 interaction in mammalian cells. Furthermore, in order to confirm that the W77 residue is not essential for EspO1-HAX-1 interaction, EspO1 W77A and HAX-1 were ectopically co-expressed in HeLa cells. GFP was used as a control to ensure that the co-localisation is reserved for interaction partners. While EspO1, EspO1 W77A and HAX-1 did not co-localise with GFP (Figure 35A, B, and C), both EspO1 and EspO1 W77A co-localised with HAX-1 in punctate structures resembling mitochondria, indicating that EspO1 interacted with HAX-1 in mammalian cells in an ILK-independent manner (Figure 35 D and E).
Figure 35. EspO1 co-localises with HAX-1 upon ectopic co-expression. (A) HeLa cells were co-transfected with pcDNA::flag-gfp & pCMV6::myc-hax-1, and were co-stained with anti-FLAG antibody (green), and anti-myc antibody (red) to visualise FLAG-GFP and Myc-HAX-1, respectively. Cells co-transfected with (B) pcDNA::flag-espO1 & pRK5::myc-gfp, or (C) pcDNA::flag-espO1 W77A & pRK5::myc-gfp were co-stained with anti-FLAG antibody (red) to visualise FLAG-EspO1 and FLAG-EspO1 W77A, as well as anti-myc antibody (green) to visualise myc-HAX-1. The cells co-transfected with (D) pcDNA::flag-espO1 & pCMV6::myc-hax-1, or (E) pcDNA::flag-espO1 W77A & pCMV6::myc-hax-1 were co-stained with anti-FLAG antibody (red), and anti-myc antibody (green) to visualise
FLAG-EspO1 & FLAG-EspO1 W77A, and Myc-HAX-1, respectively. In every condition, DNA was stained by DAPI (blue). Bar = 20µm.

With a view to further investigate the subcellular compartment where interaction partners co-localised, HeLa cells were co-transfected prior to staining for FLAG-EspO1, myc-HAX-1, and the mitochondrial marker TOMM22. It was observed that EspO1 and HAX-1 co-localised at mitochondria (Figure 36).

![Figure 36. EspO1 co-localises with HAX-1 at mitochondria. HeLa cells were co-transfected with pcDNA::flag-espO1 & pCMV6::myc-hax-1. At 24 h post-transfection, cells](image-url)
were fixed and stained with anti-HAX-1 antibody (red), anti-FLAG-FITC antibody (green), anti-TOMM22 antibody (magenta), and DAPI (blue) to visualise HAX-1, EspO1, mitochondria, and DNA, respectively. Bar = 20µm.

Moreover, it was discovered that HAX-1 altered EspO1 subcellular distribution. When co-expressed with GFP, EspO1 mainly localised to FA-resembling structures. Interestingly, EspO1 staining became significantly more prominent in punctate structures similar to mitochondria when HAX-1 was over-expressed (P<0.0001) (Figure 37). This revealed that HAX-1 influenced EspO1 localisation.

![Graph showing EspO1 localization upon co-expressed with GFP and HAX-1.](image)

**Figure 37.** HAX-1 enhances EspO1 mitochondrial localisation upon co-expressed in HeLa cells. HeLa cells were co-transfected with either pcDNA::flag-espO & pRK5::myc-gfp or pcDNA::flag-espO1 & pCMV6::myc-hax-1. 24 h post-transfection, cells were fixed and immunostained to monitor protein localisation. EspO1 phenotype was observed, and categorised based on the subcellular localisation of EspO1. 100 cells expressed both proteins were counted. Significance (P<0.0001) was tested using 2 way ANOVA (Bonferroni test). The result was the average of 3 independent biological repetitions.
3.2.3.2 EspO1 Does Not Co-localise With HAX-1 during *In vitro* Infection.

With a view to further investigating the EspO1-HAX-1 interaction during *in vitro* infection, HeLa cells were transfected with pCMV6 construct expressing HAX-1 with N-terminal myc fusion prior to EDL933 ΔespO1, EDL933 ΔespO1/pespO1, or ΔespO1/pespO1 W77A infection. EspO1 localisation was monitored at 2.5, 5, and 7.5 h post-infection. At every time-point monitored, EspO1 localised solely in FA-resembling structures without co-localisation with HAX-1 (Figure 38).
Figure 38. EspO1 does not co-localise with HAX-1 during infection. HeLa cells were transfected 24 h prior to infection with indicated EHEC strains. The cells were fixed at (A) 2.5, (B) 5, and (C) 7.5 h post-infection, before being stained with anti-HAX-1 antibody (green), anti-HA antibody (red), and DAPI (blue) to visualise HAX-1, EspO1, and DNA, respectively. Bar = 20µm.

3.3 Discussion

Bioinformatic analysis identified EspO homologs in several diarrhoeagenic pathogens including *Salmonella enterica* (serovar Typhi, Paratyp PHI, and Typhimurium), *Shigella sonnei*, *Shigella flexneri*, as well as A/E pathogens including EHEC, EPEC, the mouse pathogen *C. rodentium*, and *Escherichia albertii*. The most recently isolated A/E pathogen *E. albertii* was originally identified in children from Bangladesh (Abbott *et al.* 2003, Huys *et al.* 2003). The EspO effector family is widely distributed among enteric pathogens, suggesting that it is an important virulence factor. In this study, only EspO1 (EHEC O157:H7 strain EDL 933) and OspE1 (*S. flexneri*) were further characterised.

In this chapter, the localisation of EspO1 and OspE1 was examined. In contrast to the previous study reporting that EspO1 aggregated in vacuoles, EspO1 was prominently localised to FAs when ectopically expressed in HeLa cells (Constantinou 2013). The difference in EspO1 localisation when being expressed from pRK5 and pcDNA expression vectors is possibly caused by the difference of expression level. Surprisingly, EspO1 was also found in mitochondria. Although non-ILK binding EspO1 W77A mutant was diffused throughout the cytoplasm, it also accumulated in punctate structure resembling mitochondria. In agreement with the localisation observed by Morita-Ishihara and colleagues (2013), and Constantinou (2013), EspO1 was localised to FAs during *in vitro* infection. EspO1 W77A was observed diffusely throughout the cytoplasm. In line with the findings of several
previous studies, ectopically expressed OspE1 localises to FAs, and OspE1 W68A was observed diffusely throughout the cytoplasm (Miura et al. 2006, Kim et al. 2009, Yi et al. 2014). However, the localisation of OspE1 to mitochondria resembling structures has never been previously described. This suggested that the ILK binding is essential for EspO1 and OspE1 FA localisation, but not for the localisation to mitochondrial-like structure. It can therefore be assumed that EspO1 and OspE1 either interact with another host protein that mediates its mitochondrial localisation, or contain a mitochondrial targeting domain in the molecule.

Both S. flexneri and EHEC EDL933 encode 2 homologs of EspO. According to the phylogenetic tree, OspE1 and OspE2 from S. flexneri are very closely related paralogs with only 1 amino acid difference; both of them inhibit cell detachment and promote cell survival via the interaction with ILK (Kim et al. 2009). On the other hand, EspO1 and EspO2 from EHEC are orthologs, and they hamper cell detachment via different mechanisms (Morita-Ishihara et al. 2013). EspO1 is believed to block cell detachment by inhibiting FA disassembly via the interaction with ILK, while EspO2 indirectly impedes cell detachment by lessening EspM2 activity (Morita-Ishihara et al. 2013). EspM2 induces stress fibre formation followed by cell rounding up and cell detachment (Simovitch et al. 2010). However, the precise mechanism of how EspO homologs inhibit cell detachment and enhance cell survival remains unclear. In a previous study, HAX-1, DnaJB1, and DnaJB6 were identified as novel interaction partners of EspO1 by a Y2H screen (Constantinou 2013). HAX-1 is a ubiquitously expressed anti-apoptotic protein (Yap et al. 2011). DnaJB1 and DnaJB6 are chaperones that bind to Hsp40 and promote its function (Vos et al. 2008). In this study, HAX-1 was verified as a novel interaction partner of EspO homologs: EspO1 (EHEC), EspO2 (EHEC), EspO (C. rodentium), and OspE (S. flexneri).
The interaction region required for EspO1 and HAX-1 binding was also further determined. The results suggested that the C-terminal region of HAX-1 (118-279 amino acids) is crucial for EspO1 binding. Furthermore, a point of significance is that the C-terminal region of HAX-1 is essential for binding with several interaction partners: phospholamban, SERCA2, HtrA2, caspase-9, and HS-1 (Fadeel and Grzybowska 2009). The precise amino acids of HAX-1 required for the interaction with EspO1 was not further dissected because no functional motifs have currently been identified in the C-terminal of HAX-1 (Fadeel and Grzybowska 2009). In terms of EspO1, although no specific amino acid responsible for EspO1-HAX-1 binding interface was identified, the most obvious findings were; 1) the conserved W77 residue, required for ILK binding, is not involved with EspO1-HAX-1 interaction, and 2) the interaction region falls in between the 46-55 amino acid residues. In further investigations, the interaction region between EspO and HAX-1 could be analysed by X-ray crystallography or NMR spectroscopy techniques.

The binding between EspO1 and HAX-1 was further confirmed by co-expressing both interaction partners in HeLa cells. EspO1 and HAX-1 co-localised at mitochondria where HAX-1 resides. Moreover, the overexpression of HAX-1 induces EspO1 re-distribution from FAs to mitochondria. To this extent, it can be implicated that HAX-1 influences EspO1 mitochondrial localisation. Surprisingly, the co-localisation between EspO1 and HAX-1 during in vitro infection was not observed, perhaps because ILK is the preferential target of EspO1. The amount of EspO1 present in cells during infection is less than the amount during ectopic expression and perhaps the amount of mitochondrial-localised EspO1 is insufficient to be detected by immunofluorescence.

HAX-1 has numerous partners including mammalian and pathogenic proteins, as well as EspO1 and OspE1 (Yap et al. 2010). In addition to EspO homologs, HAX-1 binds several pathogenic proteins such as K15 protein of kaposi’s sarcoma-associated herpes virus, human
immunodeficiency virus type 1 viral protein R (Vpr), and influenza A virus PA protein (Sharp et al. 2002, Yedavalli et al. 2005, Hsu et al. 2013). HAX-1 attenuates the cytotoxic effect of Vpr and co-localises with the K15 protein in the ER (Sharp et al. 2002, Yedavalli et al. 2005). The influenza A virus PA protein is an RNA dependent RNA polymerase required for viral propagation. In this scenario, HAX-1 serves a defensive function by blocking the nuclear localisation of the PA protein, and attenuating the propagation of the virus (Hsu et al. 2013). Hence the binding between HAX-1 and pathogenic protein is clearly involved with pathogenesis, the effect of EspO1-HAX-1 interaction on cellular physiological function was further investigated, and thereby it is discussed in the next chapter.
Chapter 4 EspO1 and OspE1 Exhibit Anti-apoptotic Activity
Chapter 4 EspO1 and OspE1 Exhibit Anti-apoptotic Activity

4.1 Anti-apoptotic Activity of EspO1 and OspE1 during Transfection

4.1.1 EspO1 Protects HeLa Cell against STS-induced Apoptosis

HAX-1 is shown to manifest anti-apoptotic activity (Fadeel and Grzybowska 2009). The interaction between HAX-1 and EspO1 shown by Y2H and co-localisation when ectopically expressed; the ability of EspO1 and OspE1 to protect transfected cells against STS-, tunicamycin-, and thapsigargin- induced apoptosis was claimed (Constantinou 2013). Therefore, the function of EspO1, and non-ILK interacting mutant EspO1 W77A in regulating apoptosis was further investigated in this study.

Firstly, the anti-apoptotic activity of EspO1 was preliminary tested in comparison to GFP, TccP (from EHEC O157:H7 strain sakai) that has never been reported to play any role in apoptotic cell death, and the anti-apoptotic effector NleF (Campellone et al. 2004, Garmendia et al. 2004, Blasche et al. 2013). All genes were cloned in the pRK5 vector for these experiments as in the previous study these genes were cloned in this vector. STS was used to induce apoptotic cell death, which was determined by cleaved capase-3 staining. EspO1 and NleF significantly reduced the percentage of apoptotic cell death in comparison to TccP and GFP (P<0.0001) (Figure 39).
Figure 39. EspO1 displays anti-apoptotic activity. HeLa cells were transfected with pRK5 constructs expressing EspO1, TccP, NleF, or GFP with N-terminal myc fusion. At 21 h post-transfection, cells were treated with STS for 3 h before being fixed and stained with anti-myc, and anti-cleaved caspase-3 antibodies. DNA was stained by DAPI. 100 transfected cells were counted. Anti-cleaved caspase-3 staining positive cells were marked as apoptotic cells. Significance (P<0.0001) was measured by 2 way ANOVA (Bonferroni post-test). The result was obtained from 3 biological repetitions.

In these preliminary experiments, genes were cloned into the pRK5 vector. However when EspO1 was expressed in mammalian cells from the pRK5 vector, EspO1 did not localise to FAs (Constantinou 2013). Hence, the experiment was repeated with EspO1 and EspO1 W77A cloned into the pcDNA vector from which EspO1 exhibited FAs localisation when ectopically expressed (Figure 24). The effector NleH1 was used as an anti-apoptotic effector control (Hemrajani et al. 2010). Pan-caspase inhibitor z-VAD fmk (zVAD) was used as a negative control. Apoptotic cells were assessed by TUNEL assay (Figure 40A) and cleaved caspase-3 staining (Figure 40B).
When apoptosis was induced by STS and quantified by TUNEL assay (Figure 40C), the percentage of apoptosis in MOCK-transfected cells was significantly higher than the percentage of apoptosis in EspO1, EspO1 W77A, and NleH1 expressing cells (P<0.0001). zVAD treatment decreased the percentage of apoptotic cell death indicating that STS induced caspase-dependent cell death (P<0.0001). There was no notably apoptotic cell death in transfected cells which were not challenged with STS.

The result observed when the percentage of apoptosis was assessed by cleaved caspase-3 staining is consistent with the result obtained when apoptosis was assessed by TUNEL assay. When cells were challenged with STS, the percentage of apoptosis was significantly reduced in the presence of EspO1, EspO1 W77A, and NleH1 in comparison to the percentage of apoptosis of the MOCK-transfected control (P<0.0001). zVAD treatment significantly decreased the percentage of apoptosis as expected (P<0.0001). There was no remarkable increase in the percentage of apoptosis in the untreated group (Figure 40D). Moreover, there was no statistically significant difference between the percentage of apoptosis in cells expressing either EspO1, or NleH1. Taken together, results suggested that EspO1 displayed anti-apoptotic activity which was as potent as known anti-apoptotic effectors: NleH1, and NleF. The interaction with ILK was not involved in the anti-apoptotic activity of EspO1.
Figure 40. EspO1 protects HeLa cells against STS-induced apoptosis. HeLa cells were transfected with pcDNA constructs expressing either EspO1 or EspO1 W77A with N-terminal FLAG fusion, or pHM6 construct expressing NleH1 with N-terminal HA fusion. At 21 h post-transfection, apoptosis was induced by challenging cells with STS for 3 h, prior to fixing. Cells were stained with anti-FLAG antibody (red) to visualise indicated effectors. Apoptotic cell death was assessed by (A) TUNEL assay (TUNEL positive nucleus was visualised in green), and (B) cleaved-caspase-3 staining (green). DNA was stained by DAPI (blue). Cells marked as apoptotic cells were indicated by white arrows. 100 transfected cells were counted and the percentage of apoptosis in each condition is shown in bar graphs. (C) Bar graph presented the percentage of apoptosis assessed by TUNEL assay. (D) Bar graph showed apoptotic cell death assessed by cleaved caspase-3. Significance (P<0.0001) was calculated by 2 way ANOVA (Bonferroni post-test). Results were obtained from 3 biological repetitions. Bar = 20µm.

4.1.2 OspE1 Protects HeLa Cell against STS-induced Apoptosis

It is well established that OspE1 interacts with ILK. ILK phosphorylates and activates numerous proteins resulting in the activation of downstream signalling pathway. One of ILK substrates is Akt that promotes cell survival (Kim et al. 2009). Constantinou (2013) also reported that OspE1 displays anti-apoptotic activity when ectopically expressed from pRK5 vector. In this study, it was observed that OspE1 also interacted with HAX-1. Thus, the ability of OspE1 to protect HeLa cells from STS-induced apoptosis was investigated. The anti-apoptotic activity of OspE1 and OspE1 W68A was compared with EspO1, and EspO1 W77A. All of effectors tested were cloned in pcDNA vector. The experiment was conducted as described previously. The percentage of apoptosis of OspE1, OspE1 W68A, EspO1, and EspO1 W77A expressing cells was significantly decreased in comparison to the percentage of
apoptosis of MOCK-transfected cells (P<0.0001) (Figure 41). There was no statistically significant difference between the percentage of apoptosis in cells expressing these effectors. This revealed that OspE1 also harbours anti-apoptotic activity and this was not reliant on the interaction with ILK.

Figure 41. OspE1 exhibited anti-apoptotic activity. HeLa cells were transfected with pcDNA constructs expressing OspE1, OspE1 W68A, EspO1, or EspO1 W77A with N-terminal FLAG fusion. Apoptosis was induced as described previously. Apoptotic cells were determined by TUNEL assay. The percentage of apoptosis was calculated from 100 transfected cells. Significance (P<0.001) was determined by 2 way ANOVA (Bonferroni post-test). The result was obtained from 3 independent biological repetitions.
4.2 The Role of EspO1 during In Vitro Infection

4.2.1 EspO1 and EspO2 Double Deletions Do Not Induce the Detachment of EHEC Infected Cells.

EHEC encodes several known anti-apoptotic effectors; EspO1, NleH1, NleH2, and NleF (Tobe et al. 2006). In a previous study performed by Hemrajani and colleagues (2010), a cell detachment assay was introduced to assess cell death during in vitro infection. In this study, the technique was adapted to investigate whether the deletion of either of these apoptotic effectors induce cell death in EHEC infected cells. HeLa cells were infected with EHEC EDL933, EDL933 ∆espO, 85-170, 85-170 ∆nleH1∆nleH2 (85-170 ∆nleH), and 85-170 ∆nleF. Cell detachment was evaluated at 5 h post-infection. Surprisingly, EDL933 ∆espO, 85-170 ∆nleH, and 85-170 ∆nleF infections did not significantly increase the percentage of cell detachment in comparison to wild type EDL933, and wild type 85-170 infections (Figure 42). This suggests that these effectors are redundant in protecting against cell detachment.

Figure 42. EspO1, NleH, and NleF do not affect cell attachment during EHEC infection.

HeLa cells were infected with EHEC strains mentioned earlier. At 5 h post-infection, cells were washed with PBS. The remaining cells were trypsinised and counted. The percentage of cell detachment was calculated relative to uninfected cells (referred to as 0% detachment).
The difference was examined by one way ANOVA (Bonferroni post-test). The result was acquired from 3 different biological repetitions.

4.2.2 EspO1 Inhibits Cell Detachment Induced by EPEC O127:H6 Strain

E2348/69 ΔnleH1ΔnleH2

Hemrajani and colleagues (2010) created EPEC E2348/69 ΔnleH1ΔnleH2 (ΔnleH), which encodes neither nleH1 nor nleH2. They reported that ΔnleH infection rapidly induced apoptosis, which could be complemented by either NleH1 or NleH2 (Hemrajani et al. 2010). In addition to NleH1 and NleH2, NleF is another anti-apoptotic effector known to impede apoptosis by directly blocking caspase-8, and caspase-9 activity (Blasche et al. 2013). Moreover, in the previous study, EspO1 was capable to compliment nleH1 anti-apoptotic activity during ΔnleH infection (Constantinou, 2013). Because EPEC encodes only an espO pseudogene, it can be used as an EspO ‘null’ mutant. Thus, in this study, ΔnleH was used to study the activity of EspO1 during infection rather than creating an EHEC EDL933 ΔnleH1ΔnleH2ΔnleFΔespO1ΔespO2 strain. In this experiment, HeLa ATCC cells were infected with E2348/69 transformed with the empty vector pSA10 (E2348/69/pSA10), ΔnleH transformed with the empty vector pSA10 (ΔnleH/pSA10); ΔnleH complemented with EspO1 (ΔnleH/pespO1); EspO1 W77A (ΔnleH/pespO1 W77A); NleH1 (ΔnleH/pnleH1), and NleF (ΔnleH/pnleF). The control condition in which ΔnleH infected cells were treated with pan-caspase inhibitor zVAD (ΔnleH+zVAD) was included, in order to confirm if the cell detachment observed was the result of cell death. Cell death was assessed by evaluating the percentage of cell detachment as described in section 4.2.1.

ΔnleH/pSA10 infection significantly increased the percentage of cell detachment in comparison to E2348/69/pSA10 infection (P<0.0001). The increase of cell detachment was significantly reduced by zVAD treatment suggesting that the cell detachment observed was
the result of cell death (P<0.0001). The re-introduction of the effectors (EspO1, EspO1 W77A, NleH1, and NleF) significantly reduced the percentage of cell detachment to the same level as the percentage of cell detachment induced by E2348/69/pSA10, suggesting that EspO1 also inhibits cell death during *in vitro* infection in an ILK independent manner (P<0.0001) (Figure 43). Furthermore, the anti-apoptotic effect of NleF during infection has never been reported. Hence, this study also confirmed that NleF inhibited cell death during *in vitro* infection.

![Figure 43](image)

**Figure 43.** EspO1, NleH1, and NleF can individually prevent cell detachment induced by ∆nleH infection. HeLa cells were infected with E2348/69/pSA10, ∆nleH/pSA10, ∆nleH/pespO1, ∆nleH/pespO1 W77A, ∆nleH/pnleH1, or ∆nleH/pnleF. ZVAD treatment was used as the negative control. At 90 minutes post-infection, cells were washed, trypsinised, and counted. The percentage of cell detachment was calculated relative to uninfected cell (referred to as 0%). Significance (P<0.0001) was assessed by one way ANOVA (Bonferroni post-test). The result was acquired from 3 different biological repetitions.
4.3 Discussion

HAX-1 plays a part in several cellular processes. Apart from apoptosis, the ubiquitously expressed HAX-1 also regulates protein expression, actin cytoskeletal polymerisation, and cell migration. HAX-1 is a nucleotide shuttling protein that is involved with mRNA processing under certain cellular stresses (Grzybowska et al. 2013). To date, HAX-1 was reported to associate with mRNA encoding vimentin and DNA polymerase β (Pol β), and post-transcriptionally regulate their expression (Al-Maghrebi, Brule et al. 2002, Sarnowska et al. 2007). Moreover, it was reported that HAX-1 promotes actin polymerisation via RhoA and Rac1 during cancer cell metastasis (Radhika et al. 2004, Gomathinayagam et al. 2014, Zhang et al. 2016). HAX-1 also promotes actin polymerisation via Arp2/3 complex upon interaction with active potassium channel Kv3.3 in nerve cells (Zhang et al. 2016). Furthermore, HAX-1 is involved with PKD2 mediated cell-matrix contact and cell migration by providing the connection between PKD2, cortactin, and cytoskeleton (Gallagher et al. 2000).

Despite the role in protein expression and actin polymerisation, the prominent and most extensively studied role of HAX-1 is in regulating apoptosis. As previously discussed, HAX-1 inhibits the apoptotic cascade at several stages in various cellular compartments (Yap et al. 2011). HAX-1 was reported to exert a protective effect against several apoptotic stimuli such as H$_2$O$_2$, etoposide, STS, hypoxia/reperfusion, and thapsigargin (Han et al. 2006, Vafiadaki et al. 2007, Vafiadaki et al. 2009, Kang et al. 2010, Yap et al. 2010). Moreover, the expression level of HAX-1 is altered in several diseases including cancer, psoriasis, and severe congenital neutropenia (Fadeel and Grzybowska 2009, Trebinska et al. 2010, Yap et al. 2011). It was also discovered that the depletion of HAX-1 increases spontaneous apoptotic cell death (Li et al. 2009). Not only does HAX-1 play a physiological role in apoptotic modulation under normal physiological circumstances, but HAX-1 also interacts with the
virus Vpr protein and attenuates its pro-apoptotic function (Yedavalli et al. 2005). Notably, this study provides another example of the scenario where HAX-1 modulates the cell death response via an interaction with a pathogenic protein.

EspO1 and EspO2 inhibit cell detachment via different mechanisms. Moreover, both EspO1 and EspO2 play a role in preserving cell architecture as the infection with EHEC O157:H7 strain sakai ΔespO1ΔespO2 induces cell rounding (Morita-Ishihara et al. 2013). Although EspO1 is widely known to block FA disassembly via the interaction with ILK, it is important to bear in mind that ILK also promotes cell survival by activating the downstream Akt signalling pathway. Moreover, cell rounding is one of the morphological changes during apoptosis onset (Sakai et al. 2003, Elmore 2007, Morita-Ishihara et al. 2013). However, the function of EspO1 as an anti-apoptotic effector has never been investigated although EspO1 possibly can inhibit cell detachment by promoting cell survival.

In this study, we clearly demonstrated that EspO1 displays anti-apoptotic effect in an ILK-independent manner when ectopically expressed. EspO1 inhibited STS-induced apoptosis when expressed from the pRK5 vector during which it localises to cytoplasmic aggregates (Constantinou 2013), or from pcDNA-NTAP when it localises to FAs and mitochondria (Figure 24). Moreover, EspO1 W77A that did not localise to FAs also protects cells from STS induced-apoptosis. Accordingly, we can infer that the anti-apoptotic activity of EspO1 is not related to its localisation. Additionally, the anti-apoptotic effect of EspO1 is as potent as known anti-apoptotic effectors: NleF and NleH1. EHEC TccP is an essential effector required for the activation of actin cytoskeleton polymerisation and pedestal formation (Garmendia et al. 2004). Nevertheless, the role of TccP on host cell death has never been determined previously. In this study we also showed that TccP neither triggers nor inhibits cell death during transfection.
During EHEC and EPEC infection, there are numerous factors that induce host cell death including LPS, flagella, bacterial attachment, host cell immune response, as well as cytotoxic T3SS effectors (Abul-Milh et al. 2001, Shankar et al. 2009, Wong Fok Lung et al. 2014). EHEC and EPEC are equipped with several anti-apoptotic effectors, out of which are NleH (NleH1 and NleH2) and NleF that specifically impede the intrinsic apoptotic pathway (Hemrajani et al. 2010, Blasche et al. 2013). Blasche et al. (2013) reported that NleF blocks the proteolytic activity of multiple caspases, but surprisingly, they did not observe the significant increase of cell death in E2348/69 ΔnleF infected cell compared with wild type infection. Surprisingly, we found that infection with EHEC 85-170 ΔnleF, 85-170 ΔnleH, or EDL933 ΔespO did not increase the cell detachment in comparison to the detachment of cells infected with wild type strains. In agreement with the finding of Hemrajani et al. (2010), this study found that the infection with EPEC ΔnleH significantly induced rapid cell death-induced cell detachment. This leads to the assumption that the absence of multiple anti-apoptotic effectors (NleH, and EspO) is required to observe the significant changes in viability and detachment of infected cells in comparison to those of cells infected with wild type. The multiple deletions can be complemented by any of NleH1, NleF, EspO1, or EspO1 W77A, indicating that each of these effectors is able to inhibit apoptosis during in vitro infection. This combination of findings provides supportive elements for the conceptual premise that 1) EspO1 inhibits cell death in an ILK-independent manner during in vitro infection, and 2) anti-apoptotic effectors are redundant. Moreover, this study delivered the proof that NleF attenuates cell death during in vitro infection. In a previous study, the anti-apoptotic effect of NleF was not observed, perhaps because the E2348/69ΔnleF deletion mutant EPEC still contains functional intact NleH1 and NleH2 that compensate NleF function (Blasche et al. 2013). Nevertheless, the presence of endogenous NleF is not sufficient to complement nleH1 and nleH2 deletions, indicating that the anti-apoptotic effect
of NleF is not as potent as NleH1 and NleH2 anti-apoptotic effect. NleF also activates inflammatory responses by enhancing NF-κB activation (Pallett et al. 2014). Therefore, NleF displays 2 different effects: pro-inflammatory and anti-apoptotic, perhaps the apoptotic inhibition is not the prominent role of NleF.

*Shigella* OspE is the most studied member amongst EspO homologs. OspE1 inhibits cell detachment and preserves host cell morphology through the interaction with ILK, mediated by the conserved W residue (Miura et al. 2006, Kim et al. 2009). Furthermore, OspE1 also contributes to *Shigella* colonisation *in vivo* (Kim et al. 2009). Nevertheless, the anti-apoptotic function of OspE1 has never been investigated although OspE1 could conceivably hamper cell detachment by promoting cell survival through ILK downstream signalling pathways (Sakai et al. 2003). In this study, the anti-apoptotic activity of ectopically expressed OspE1 was also examined. OspE1 was observed to protect transfected cells against STS-induced apoptosis in an ILK independent manner. This suggested that OspE1 exerts its anti-apoptotic effect through the interaction with HAX-1, not through ILK.

In short, the anti-apoptotic function of EspO1 and OspE1 has been confirmed. OspE1 manifests anti-apoptotic activity upon being ectopically expressed in HeLa cells. EspO1 modulates cell death during both *in vitro* infection and ectopically expressed in HeLa cells. This anti-apoptotic effect is independent of an interaction with ILK. However, the question that remains unanswered is by which mechanism EspO1 and OspE1 exert their anti-apoptotic effect. Attempts to reveal the anti-apoptotic mechanism of EspO1 are further discussed in the following chapter.
Chapter 5 The Anti-apoptotic Mechanism of EspO1
Chapter 5 The Anti-apoptotic Mechanism of EspO1

5.1 The Anti-apoptotic Activity of EspO1 is HAX-1 Dependent.

EspO1 was shown to manifest anti-apoptotic activity. However, the mechanism of EspO1 action is still unknown. Nevertheless, the role of HAX-1 in the anti-apoptotic activity during ectopic expression of EspO homologs was reported in transiently siRNA HAX-1 knockdown cells (Constantinou 2013). However, in transiently knockdown model, there is variation among repetitions, and HAX-1 expression was not completely diminished. Therefore, in this study, EspO1 anti-apoptotic activity was tested in HAX-1 depleted cell lines which were expected to cause less variation among repetitions.

5.1.1 Introduction to HAX-1 Knockdown Cell Lines

5.1.1.1 Verification of HAX-1 Knockdown Cell Lines

In order to investigate the role of HAX-1 on EspO1 activity, it was necessary to perform experiments in HAX-1 depleted conditions. An miRNA HAX-1 knockdown cell line (miHAX-1) and a negative control (miNEG) cell line were generously provided by Dr. Ewa A. Grzybowska (Grzybowska et al. 2013). Firstly, HAX-1 expression level was investigated by western blot analysis (Figure 44). The amount of HAX-1 in the control miNEG cell line was similar to HeLa ATCC, while HAX-1 level was remarkably reduced in the miHAX-1 cell line. Notably, there was more reduction of HAX-1 expression in miHAX-1 cell line than the reduction of HAX-1 expression in siRNA HAX-1 knockdown cells used in the previous study (Constantinou 2013). Thus, miHAX-1 and miNEG were used to investigate the role of HAX-1 on EspO1 anti-apoptotic activity.
Figure 44. Confirmation of reduced HAX-1 expression in miHAX-1 cell line. HeLa ATCC, miHAX-1, and miNEG cells were seeded in 6 wells plate and harvested after 48 h. Proteins were separated by SDS-PAGE and analysed by western blot using anti-α tubulin and anti-HAX-1 antibodies followed by anti-mouse-HRP antibodies. α- tubulin was monitored as a loading control to ensure that the same amount of protein was loaded in each lane. Protein bands were visualised by S3000 Fuji Imager machine. The result is representative of 2 independent biological repetitions.

5.1.1.2 EspO1 Localisation in The Absence of HAX-1

i. EspO1 Localised to FA- and Mitochondria- Resembling Structures in miHAX-1 and miNEG Cell Lines during Transfection.

In order to investigate if HAX-1 affects EspO1 localisation, the localisation of EspO1 was monitored. Firstly, EspO1 and EspO1 W77A were ectopically expressed in miHAX-1 and miNEG cell lines. Consistent with previous results using HeLa ATCC, EspO1 was localised to FA-like and punctate structures in both miHAX-1 and miNEG cell lines (Figure 45A). EspO1 W77A displayed a diffuse phenotype in both cell lines (Figure 45B). Accordingly, this could be implied that HAX-1 did not affect EspO1 localisation upon ectopic expression.
**Figure 45.** EspO1 and EspO1 W77A localisation during ectopic expression in miHAX-1 and miNEG cell lines. Both cell lines were transfected with pcDNA constructs expressing EspO1 or EspO1 W77A with N-terminal FLAG fusion for 24 h, then processed as earlier described. The effectors were stained by anti-FLAG antibody (red), and DNA was stained by DAPI (blue). (A) The localisation of EspO1 in (A-I&A-II) miHAX-1 and in miNEG (A-III&A-IV) cell lines. (B) The localization of EspO1 W77A in miHAX-1 (B-I), and in miNEG (B-II) cell lines. Bar = 20µm.

**ii. EspO1 Localises to FA-Resembling Structures in miHAX-1 and miNEG Cell Lines during In Vitro Infection.**

EspO1 localisation during *in vitro* infection was also investigated. In accordance with previous results, there was no difference in EspO1 localisation either when the effector was delivered by EHEC or EPEC T3SS. Thus, in this experiment, EspO1 was delivered by EPEC T3SS. Consistent with the results obtained from HeLa ATCC, EspO1 was localised to FA-resembling structures and EspO1 W77A displayed the diffuse phenotype in both miHAX-1 (Figure 46) and miNEG (Figure 47) cell lines. This indicated that HAX-1 had no effect on EspO1 localisation during *in vitro* infection.
Figure 46. EspO1 and EspO1 W77A localisation in miHAX-1 cell line at 1 h post-EPEC infection. miHAX-1 cells were infected with EPEC strain E2348/69, E2348/69 /pespO1, or E2348/69 /pespO1 W77A, then processed as described earlier. Effectors, actin, EPEC and DNA were stained by anti-HA antibody (green), phalloidin (red), anti-O127:H6 antibody (cyan), and DAPI (blue), respectively. Bar = 20µm.
Figure 47. EspO1 and EspO1 W77A localisation in miNEG cell line at 1 h post-EPEC infection. miNEG cells were infected and processed as previously described. Effectors, actin, EPEC, and DNA were visualised by anti-HA antibody (green), phalloidin (red), anti-O127:H6 antibody (cyan), and DNA (blue) staining, respectively. Bar = 20µm.
5.1.2 The Anti-apoptotic Activity of EspO1 and OspE1 is HAX-1 Dependent

5.1.2.1 EspO1 Loses Anti-Apoptotic Activity in miHAX-1 Cell Line

With an intention to investigate if the anti-apoptotic activity of EspO1 is HAX-1 dependent, the experiment was performed as described earlier (section 4.1.1). When apoptosis was quantified by TUNEL assay, in the miHAX-1 cell line, there was no statistically significant difference between the percentage of apoptosis either in cells expressing EspO1, EspO1 W77A, or MOCK-transfected cells. EspO1 and EspO1 W77A lost protection against STS-induced apoptosis, while zVAD treatment, and NleH1 did not. However, in the control miNEG cell line, EspO1, EspO1 W77A, NleH1 expression, and zVAD treatment significantly decreased the percentage of apoptosis in comparison to MOCK-transfection (P<0.0001) (Figure 48A). Similar result was observed when apoptosis was assessed by cleaved caspase-3 staining (Figure 48B). This revealed that the anti-apoptotic activity of EspO1 and EspO1 W77A was HAX-1 dependent, whilst that of NleH1 was not.
Figure 48. EspO1 anti-apoptotic activity is HAX-1 dependent. Both cell lines were transfected with pcDNA constructs expressing either EspO1 or EspO1 W77A with N-terminal FLAG fusion, or pHM6 construct expressing NleH1 with N-terminal HA fusion for 21 h prior to challenging with STS for 3 h. Apoptotic cell death was assessed by (A) TUNEL assay and (B) cleaved caspase-3 staining as described earlier. Significance (P<0.0001) was calculated by 2 way ANOVA (Bonferroni post-test). The results were obtained from 3 biological repetitions.
5.1.2.2 The Anti-apoptotic Activity of OspE1 Partially Depends on HAX-1

With a view to investigating the role of HAX-1 on OspE1 anti-apoptotic activity, OspE1, OspE1 W68A, EspO1, and EspO1 were ectopically expressed in miHAX-1 and miNEG cell lines. The experimental procedure was performed as previously described. STS significantly induced apoptotic cell death in the MOCK-transfected group in both miHAX-1 and miNEG cell lines. Cells expressing EspO1, EspO1 W77A, and OspE1 W68A were unable to protect against STS-induced apoptosis in miHAX-1 cell line, but not in miNEG cell line (P<0.0001). Cells expressing OspE1 also had reduced protection against STS-treatment in the miHAX-1 cell line compared with in the miNEG cell line (P<0.001), although this was an intermediate phenotype (Figure 49).

![Graph](image)

Figure 49. OspE1 anti-apoptotic activity partially depends on HAX-1. Both cell lines were transfected with pcDNA constructs expressing EspO1, EspO1 W77A, OspE1, or OspE1 W68A with N-terminal FLAG fusion. At 21 h post-transfection, cells were challenged by STS for 3 h prior to being fixed and processed as previously described. Apoptotic cell death was quantified by TUNEL assay, and the percentage of apoptosis was calculated from 100
transfected cells. Statistical difference was examined by 2 way ANOVA. The result was obtained from 3 biological repetitions.

5.1.3 EspO1 Anti-apoptotic Activity Is HAX-1 Dependent during In Vitro Infection

The role of HAX-1 in the anti-apoptotic activity of EspO1 during in vitro infection has never been investigated. Therefore, to further determine if HAX-1 is relevant to EspO1 activity during in vitro infection, both cell lines were infected with E2348/69/pSA10, AnleH/pSA10, AnleH/pespO1, AnleH/pespO1 W77A, AnleH/pnleH1, or AnleH/pnleF. Pan-caspase inhibitor zVAD was used to inhibit cell death-induced cell detachment as a negative control. Cell death was assessed by calculating the percentage of cell detachment relative to uninfected cells as described earlier. In miHAX-1 cell line, AnleH/pSA10 infection significantly increased the percentage of cell detachment in comparison to E2348/69/pSA10 infection (P<0.0001). EspO1 and EspO1 W77A lost their ability to protect against AnleH/pSA10-induced cell detachment. Conversely, zVAD treated cells, and AnleH/pnleH1 and AnleH/pnleF infected cells had a similar level of cell detachment to cells infected with E2348/69/pSA10 (Figure 50).

Consistent with the result obtained from the experiment in HeLa ATCC, AnleH/pSA10 infection also significantly increased cell detachment relative to E2348/69/pSA10 infection in the miNEG cell line (P<0.0001). Nevertheless, EspO1, EspO1 W77A, NleH1, and NleF complementation as well as zVAD treatment significantly decreased the percentage of cell detachment to a similar level as E2348/69/pSA10 infected cells (P<0.0001). The percentage of cell detachment induced by AnleH/pespO1 and AnleH/pespO1 W77A infection in miHAX-1 cell line was significantly higher in comparison to the percentage of cell detachment induced by AnleH/pespO1 and AnleH/pespO1 W77A infection in miNEG cell
This revealed that the anti-apoptotic activity of EspO1 during in vitro infection was HAX-1 dependent, but the anti-apoptotic activity of NleH1 and NleF was not.

**Figure 50.** The anti-cell death activity of EspO1 during in vitro infection is HAX-1 dependent. Both cell lines were infected with E2348/69/pSA10, ∆nleH /pSA10, as well as ∆nleH complemented with EspO1, EspO1 W77A, NleH1, and NleF. zVAD was included as a negative control. The cell detachment assay was performed, and the percentage of cell detachment was calculated as mentioned earlier. Statistical difference was examined by 2 way ANOVA. The result was obtained from 3 biological repetitions. ****, P<0.0001

### 5.2 EspO1 Does Not Reduce Cytoplasmic Ca^{2+} during ∆nleH-induced Cell Death

HAX-1 reduces ER Ca^{2+} storage by interacting with phospholamban and SERCA2 via an unknown mechanism. In turn, the reduction of ER Ca^{2+} storage decreases the release of Ca^{2+} from ER during apoptosis, hence attenuating mitochondria perforation and apoptosis (Vafiadaki et al. 2007, Vafiadaki et al. 2009). ∆nleH infection induces the increase of cytoplasmic Ca^{2+}, which could be reduced by NleH1 re-introduction (Hemrajani et al. 2010).
Furthermore, the ability of EspO1 to prevent cell detachment induced by ΔnleH is HAX-1 relevant. Thus, it was hypothesised that EspO1 inhibits apoptosis by decreasing cytosolic Ca\(^{2+}\) in the presence of apoptotic stimuli.

With a view to further elucidate the mechanism of action of EspO1, cytoplasmic Ca\(^{2+}\) was measured at 1 h after HeLa ATCC cells were infected with EPEC. The cytoplasmic Ca\(^{2+}\) was measured by Fluo-4 Direct™ Calcium Assay Kit (Invitrogen). As expected, the cytosolic Ca\(^{2+}\) level of ΔnleH /pSA10 infected cells was significantly higher than the cytosolic Ca\(^{2+}\) level of E2348/69 /pSA10 infected cells (P<0.05). NleH1 complementation remarkably reduced cytoplasmic Ca\(^{2+}\) induced by ΔnleH /pSA10 infection (P<0.05) (Figure 51). However, EspO1 and EspO1 W77A did not significantly reduce cytoplasmic Ca\(^{2+}\) (Figure 51). This suggested that EspO1 did not inhibit cell death by preventing the rising of cytoplasmic Ca\(^{2+}\).

![Figure 51. EspO1 does not decrease cytoplasmic Ca\(^{2+}\) during ΔnleH/pSA10 infection.](image)

HeLa cells were infected with E2348/69/pSA10, ΔnleH/pSA10, ΔnleH/pespO1, ΔnleH/pespO1W77A, and ΔnleH/pnleH1. Cytoplasmic Ca\(^{2+}\) was measured at 1 h post-infection. The amount of cytoplasmic Ca\(^{2+}\) was presented in terms of fluorescence intensity.
relative to uninfected cell. Statistical difference was examined by 2 way ANOVA. Different
was compared with the fluorescence intensity of cells infected with ∆nleH/pSA10. The result
was obtained from 3 biological repetitions.

5.3 Discussion
Previously, we verified that the anti-apoptotic activity of EspO1 and OspE1 is ILK-
independent. In this chapter, we further investigated the anti-apoptotic mechanism of EspO1
and OspE1. Firstly, miNEG and miHAX-1 cell lines were validated, and the localisation of
EspO1 and OspE1 in both cell lines was determined. There was no difference in EspO1 and
OspE1 localisation in both cell lines. Furthermore, EspO1 and OspE1 were still observed in
punctate structures resembling mitochondria when ectopically expressed in the miHAX-1 cell
line. It is therefore likely that HAX-1 has no role in EspO1 and OspE1 mitochondrial
localisation. The mitochondrial localisation of EspO1 and OspE1 is presumably induced by
another factor apart from HAX-1 binding. This could be either binding of an unidentified
interaction partner residing in mitochondria, or the presence of a motif that targets the
effectors to mitochondria. The online protein localisation tool TargetP1.1
(http://www.cbs.dtu.dk/services/TargetP/, last accessed on 08/08/2016), which provides the
localisation prediction based on the N-terminal amino acid sequence, suggested that EspO1
localise to mitochondria (see appendix) (Emanuelsson et al. 2007).

Initially, the anti-apoptotic activity of EspO1 and OspE1 was examined during HAX-1
depletion to confirm if HAX-1 is essential for the anti-apoptotic activity. The results
indicated that EspO1 and EspO1 W77A lose their protection against cell death induced by
STS and EPEC ∆nleH infection in the miHAX-1 cell line. This clearly indicated that the anti-
apoptotic effect of EspO1 relies on EspO1-HAX-1 interaction. Nevertheless, as expected,
neither NleH1 nor NleF lost the protection against death stimuli used in this study. This is
consistent with results from previous works showing that, unlike EspO1, NleH1 and NleF do not target the HAX-1 protein (Hemrajani *et al.* 2010, Blasche *et al.* 2013). NleH1 inhibits apoptosis by decreasing ER Ca\(^{2+}\) release through an interaction with BI-1 protein (Hemrajani *et al.* 2010). NleF directly binds and inhibits caspase-8, and caspase-9 by inserting its C-terminal into caspase active site pocket (Blasche *et al.* 2013). Thereby, the anti-apoptotic effects of NleH and NleF are not altered by the absence of HAX-1. The simplest interpretation is that EHEC and EPEC secrete several anti-apoptotic effectors targeting different apoptotic pathways at different stages in order to ensure apoptotic inhibition.

One of the HAX-1 anti-apoptotic mechanisms is to downregulate SERCA2 resulting in the decrease of ER Ca\(^{2+}\) storage via HAX-1-phospholamban interaction (Vafiadaki *et al.* 2007, Vafiadaki *et al.* 2009). ER Ca\(^{2+}\) release sensitises cells to apoptotic stimuli and induces the leakage of cyt c from mitochondria, thus the decrease of ER Ca\(^{2+}\) storage leads to apoptotic modulation (Pinton *et al.* 2008). Moreover, the increase of cytosolic Ca\(^{2+}\) is, in turn, one of the cellular alterations during apoptosis onset (Pinton *et al.* 2008). The rising of cytosolic Ca\(^{2+}\) is also detected in cells infected with EPEC ΔnleH (Hemrajani *et al.* 2010). In this study, the role of EspO1 in the increase of cytosolic Ca\(^{2+}\) pool during apoptosis was investigated. Disappointingly, EspO1 did not reduce cytosolic Ca\(^{2+}\), suggesting that EspO1 inhibits apoptosis via other mechanisms. EspO1 can possibly protect against apoptotic cell death either by hampering HAX-1 degradation leading to the extension of HAX-1 anti-apoptotic activity, or by enhancing other HAX-1 anti-apoptotic mechanisms which are 1) preventing cyt c release from mitochondria, 2) blocking caspase-9 activation, 3) impeding XIAP degradation and 4) inhibiting caspase-3 activity. Unfortunately, due to time constraints, the anti-apoptotic mechanism of EspO1 was not further investigated and remains subject to speculation.
The anti-apoptotic activity of OspE1 was also tested in miHAX-1 cell lines. As expected, OspE1 W68A completely lost protection against STS-induced apoptosis in the absence of HAX-1. Surprisingly, OspE1 was still able to exert some protection against apoptosis when ectopically expressed in miHAX-1 cells, albeit a reduced effect compared to when OspE1 was expressed in the control miNEG cell line. Considering the fact that ILK promotes cell survival through the Akt signaling pathway, OspE1 possibly can also enhance cell survival through the interaction with ILK (Widmaier et al. 2012).

In essence, in this study, the dominant role of HAX-1 in the anti-apoptotic activity of EspO1 and OspE1 is validated. Even though little is known about the anti-apoptotic mechanism of EspO1 and OspE1, it is clear that EspO1 does not inhibit apoptosis by decreasing the cytosolic Ca\(^{2+}\), and that OspE1 promotes cell survival through interactions with both ILK and HAX-1.
Chapter 6 General Discussion
Chapter 6 General Discussion

6.1 Overview of EHEC and EPEC Infection

EHEC and EPEC are A/E enteric pathogens responsible for diarrhoeal outbreaks worldwide (Kaper et al. 2004, Revenu et al. 2004). The laterally acquired LEE PAI is an important virulence factor of EHEC and EPEC. The highly conserved LEE encodes the T3SS and effectors required for A/E lesion formation, colonisation and pathogenesis (Garmendia et al. 2005). During infection, EHEC and EPEC secrete T3SS effectors into infected host cells. Once secreted, effector proteins intervene with host physiological functions including host cell death responses, to favour pathogen persistence and infection (Frankel et al. 1998). Currently, the function of several identified effectors remains unknown or incompletely characterised. This study set out to investigate the role of EspO1 in EHEC pathogenesis.

6.2 EspO1 and Its Role in EHEC Infection

The EspO effector family is widely distributed in several diarrhoeagenic bacterial pathogens including EHEC, EPEC, Salmonella enterica, Shigella flexneri, and Shigella sonnei. It is known that ILK is an interaction partner of the EspO effector family (Kim et al. 2009, Morita-Ishihara et al. 2013). ILK is one of the best characterised FA proteins. ILK binds integrin, and plays a pivotal role in numerous processes such as FA assembly, actin rearrangement, cell adhesion, cell spreading, cell migration as well as cell survival and proliferation (Widmaier et al. 2012). Results from several studies indicate that EspO1 and OspE inhibit cell detachment and maintain epithelial architecture by impeding FA disassembly via the interaction with ILK (Kim et al. 2009, Morita-Ishihara et al. 2013). Furthermore, OspE preserves intestinal epithelial architecture during infection (Miura et al. 2006). EspO1 also possibly serves the same function as an infection with EHEC ΔespO1 deletion mutant significantly causes cell rounding compared with an infection with EHEC
wild type (Morita-Ishihara et al. 2013). On the other hand, EspO2 indirectly hampers cell rounding and cell detachment by suppressing EspM2 activity (Morita-Ishihara et al. 2013). EspM2 mimics Rho GEF function, and activates RhoA, resulting in stress fibre formation; this consequently lead to cell rounding, cell detachment and the corruption of intestinal epithelial architecture (Arbeloa et al. 2008, Arbeloa et al. 2010, Simovitch et al. 2010). This is in line with the finding that infection with EHEC ΔespO2 deletion mutant induces cell rounding (Morita-Ishihara et al. 2013).

In this study I confirmed that HAX-1 is a putative interaction partner of the EspO family of T3SS effectors. The interaction region falls in the C-terminal (118-279 amino acids) of HAX-1, and 46-56 amino acids of EspO1. Furthermore, it is clear that the conserved W residue required for the ILK interaction is not involved with EspO1-HAX-1 interaction. We also reported that EspO1 displays anti-apoptotic activity both during transfection and in vitro infection. Additionally, OspE1 manifests anti-apoptotic activity when ectopically expressed in HeLa cells.

The findings suggested that HAX-1 is essential for the anti-apoptotic activity of EspO1 and OspE1. Unfortunately, the exact mechanism of how the EspO1-HAX-1 interaction hampers apoptosis was not successfully disclosed. Nevertheless, the result indicated that EspO1 does not decrease cytosolic Ca\(^{2+}\) during apoptosis onset. EspO1 possibly attenuates apoptosis through other known HAX-1 anti-apoptotic mechanisms, as discussed in the previous chapter. Furthermore, it was recently proposed that HAX-1 acts as nucleotide shuttling protein and regulates protein expression under specific cellular stress conditions. This raises another possible mechanism by which HAX-1 promotes cell survival through the regulation of protein expression (Grzybowska et al. 2013). Nevertheless, this newly hypothesised mechanism requires further investigation. In addition, HAX-1 was recently reported to interact with ILK, which enhances cell survival through the Akt signalling pathway (Dobreva...
et al. 2008, Widmaier et al. 2012). Even though a pro-survival mechanism mediated by ILK-HAX-1 interaction has never been elucidated, it is still possible that ILK and HAX-1 collaboratively enhance cell survival (Dobreva et al. 2008). Therefore, EspO1 also conceivably promotes cell survival via ILK and HAX-1 interaction network. However, this hypothesis cannot be investigated unless the cellular function of ILK-HAX-1 interaction is revealed. The function and possible mechanism of EspO1 are summarised in Figure 52.

![Diagram](image)

**Figure 52. Potential functions and mechanisms of EspO1 activity.** EspO1 has previously been shown to inhibit cell detachment via the interaction with ILK, which blocks FA disassembly. The various mechanisms by which EspO can block apoptosis via the interaction with HAX-1 are presented. These include: 1) stabilising HAX-1, 2) blocking XIAP
degradation, 3) inhibiting cyt c release, 4) impeding caspase-9 activation, and 5) hindering caspase-3 activity.

HAX-1 is involved with the activation of RhoA and Rac1, as well as actin polymerisation (Radhika et al. 2004, Cavnar et al. 2011, Gomathinayagam et al. 2014). RhoA controls cell polarity and actin dynamics during cell motility (Heasman and Ridley 2008). Although the role of HAX-1 in the regulation of neutrophil motility is not yet clear, it was reported that HAX-1 localised to the leading edges and activates RhoA during neutrophil chemotaxis (Cavnar et al. 2011). In addition to immune cell motility, HAX-1 plays a part in cancer cell migration (Ramsay et al. 2007, Gomathinayagam et al. 2014, Wei et al. 2014). In ovarian cancer cells, Ga13 stimulates HAX-1, which in turns promotes actin polymerisation by enhancing Rac1 and cortactin association (Radhika et al. 2004, Gomathinayagam et al. 2014). HAX-1 also plays a pivotal role in $\alpha_v\beta_6$ integrin mediated migration (Ramsay et al. 2007). Furthermore, in colorectal cancer, the high level expression of HAX-1 is significantly associated with lymph node metastasis, clinical stages, and resistance to genotoxic stimuli, suggesting that HAX-1 is involved with the regulation of cell death and cell migration in intestinal cells (Wei et al. 2014). Even though little is known about the physiological function of HAX-1 in the regulation of Rac1 and RhoA signalling pathway as well as actin polymerisation, it is clear that Rac1, RhoA and actin cytoskeleton play important roles in cell adhesion and cell morphology (Chauhan et al. 2011, Citalan-Madrid et al. 2013). Rac1 is known to promote actin polymerisation during lamellipodia formation, and membrane ruffling. RhoA is responsible for stress fibre formation (Heasman and Ridley 2008). Furthermore, both Rac1 and RhoA alternatively regulate FA assembly in different stages. In other words, Rac1 promotes the formation of FA in the nascent stage of cell adhesion, while RhoA activity is required during FA maturation when Rac1 is inactivated. Therefore, the
balance between Rac1 and RhoA activity regulates cell adhesion and migration (Guilluy et al. 2011). Moreover, the integrity of actin cytoskeletal network, and the preservation of actin dynamic are essential for the maintenance of cell morphology, including microvilli structure. The fixed structure of microvilli is owing to the precise balance between actin polymerisation and de-polymerisation (Revenu et al. 2004). Additionally, HAX-1 is also part of the ILK interactome. On the basis of current evidence, it seems fair to suggest that HAX-1 could conceivably be involved with cell adhesion and cell spreading through the interaction with ILK, and the role in Rho GTPase activation. Additionally, EspO1 also plays a part in the maintenance of host cell architecture as an EHEC ΔespO1 infection causes cell rounding (Morita-Ishihara et al. 2013). Furthermore, OspE1 is also known to preserve host cell morphology, allowing Shigella to efficiently invade the intestinal epithelium (Miura et al. 2006). Although the role of EspO1 in the maintenance of host cell morphology was not investigated in this study, it is noteworthy that EspO1 might also preserve cell morphology via interactions with HAX-1 and ILK.

The baseline activity of Rho GTPase: RhoA, Rac1, and Cdc42, is implied to be essential for the maintenance of intestinal barrier function. The activation of Rac1 stabilises the barrier function. Conversely, the inhibition of Rac1 activity decreases intestinal barrier function and integrity. However, the balance of RhoA activity needs maintenance in order to preserve barrier function, as both RhoA activation and inactivation result in the loss of barrier function (Schlegel et al. 2011). Given that HAX-1 activates Rac1 and RhoA activity, we could confer that HAX-1 possibly plays a partial role in the maintenance of intestinal epithelial barrier function and integrity. Therefore, via the interaction with HAX-1 and ILK, EspO1 conceivably maintains epithelial architecture and barrier function.

To summarise, this study showed that EspO1 displays anti-apoptotic effect via the interaction with HAX-1. The combination of findings lead to the interpretation that EspO1 is a
multifunctional effector that inhibits both cell death and cell detachment during in vitro infection. However, the mechanism of how EspO1 impedes apoptosis, and if EspO1 displays anti-apoptotic activity in vivo, are questions that remain unanswered. Furthermore, whether or not EspO1-HAX-1 interaction is involved with the maintenance of host cell morphology remains a subject of speculation. These questions warrant further research to unveil the complete picture of the role of EspO1 in EHEC pathogenesis.

6.3 Subversion of Host Cell Death by T3SS Effectors

The induction of cell death is one of the host defence mechanisms. The death and subsequent removal of infected cells reduces the immediate pathogen burden and the preferred site for infection. Furthermore, the death and the shredding of infected epithelium also prevents further pathogen invasion into the deeper layer of tissue or bloodstream (Labbe and Saleh 2008). On the contrary, bacterial pathogens also elaborate strategies to subvert host cell death to facilitate persistence and to establish an infectious niche. Apart from cell death, enteric pathogens also impede cell detachment, ensuring the infected cells remain in contact with a basement membrane. Basically, the inhibition of cell detachment and cell death results in the prolonging of an infectious niche (Raymond et al. 2013).

As mentioned earlier, EHEC and EPEC exploit numerous effectors to secure their infectious niche. While NleH, NleF, EspZ inhibit the intrinsic apoptotic pathway, NleB and NleF impede the extrinsic pathway. NleD blocks the JNK pro-apoptotic pathway. Apart from EHEC and EPEC, other enteric bacterial pathogens, such as Shigella and Salmonella, also elaborate strategies to inhibit apoptotic cell death (Raymond et al. 2013). Shigella was reported to inhibit STS-induced apoptosis in a T3SS dependent manner (Clark and Maurelli 2007). In addition to inhibiting cell detachment by OspE1 and OspE2, Shigella impedes apoptosis by targeting the p53 apoptotic pathway (Schroeder and Hilbi 2008). Shigella IpgD
suppresses p53 activity by stabilising the double minute 2 protein (MDM2) that represses p53 pro-apoptotic activity. *Shigella* also uses VirA to indirectly degrade p53 via calpain activation (Bergounioux *et al.* 2012). *Salmonella* secretes AvrA and SopB which hamper apoptosis by inhibiting JNK pro-apoptotic signalling and promote Akt pro-survival pathway, respectively (Jones *et al.* 2008, Cooper *et al.* 2011).

In this study, we reported that EspO1 hampers intrinsic apoptotic cell death via the interaction with HAX-1. The results also provide further support for the anti-apoptotic role of NleF during *in vitro* infection. EHEC and EPEC anti-apoptotic effectors (NleH1, NleH2, NleF, and EspO1) are redundant as deleting one of NleH, NleF, and EspO1 does not significantly alter infected cell survival during EHEC infection, but deleting multiple of these effectors lead to rapid cell death and cell detachment. The re-introduction of any one of these effectors recues the infected cells. Taking into consideration the fact that EHEC and EPEC use several T3SS effectors targeting different pathways to hijack cell death responses, it seems that the manipulation of cell death is one of EHEC and EPEC virulence strategies.
References


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promoting factors to recruit junctional proteins into pedestals for pedestal maturation and disruption of paracellular permeability. Infect Immun 76(9): 3854-3868.


Appendices
Appendices

Appendix I. The Prediction of EspO1 Localisation

The prediction in a figure below indicates that EspO1 is potentially localised to mitochondria (0.331), and other possible subcellular compartment within the cells (0.630).

![TargetP 1.1 Server prediction results](image)

mTP, mitochondrial target protein; SP, secretion protein; Loc, possible localisation; RC, Reliability class

Appendix II. Permission for the use of Figure 1. Illustration summaries UPEC pathogenesis.

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Appendix III. Permission for the use of Figure 2. Electron micrograph demonstrates A/E lesion induced by EPEC in a piglet

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