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# Role of Type III secretory effectors EspF and SopB in enteric pathogenesis of *Escherichia coli* and *Salmonella enterica* serovar Typhimurium

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## Declaration

I hereby declare that this thesis is of my own composition, and that it contains no material previously submitted for the award of any other degree. The work reported in this thesis has been executed by myself, except where due acknowledgement is made in the text.

## Publications

1. Tahoun et al., 2011 Comparative analysis of EspF variants in the inhibition of *E. coli* phagocytosis by macrophages and the inhibition of *E. coli* translocation through Human- and Bovine-derived M-cells. Infect. Immun. PMID: 21875965

# Abstract

The EspF protein is translocated into host cells by the type III secretion system of enteropathogenic and enterohemorrhagic *Escherichia coli* (EPEC and EHEC). EspF sequences differ between EPEC and EHEC serotypes in terms of the number of SH3-binding polyproline rich repeats and specific residues in these regions as well as residues in the amino domain involved in cellular localization. In this study we have compared the capacity of different *espF* alleles to inhibit: (i) bacterial phagocytosis by macrophages; (ii) translocation through an M-cell co-culture system; (iii) uptake by and translocation through cultured bovine epithelial cells. The $espF_{O157}$ allele was significantly less effective at inhibiting phagocytosis and also had reduced capacity to inhibit *E. coli* translocation through a human-derived *in vitro* M-cell co-culture system in comparison to $espF_{O127}$ and $espF_{O26}$. In contrast, $espF_{O157}$ was the most effective allele at restricting bacterial uptake into and translocation through primary epithelial cells cultured from the bovine terminal rectum, the predominant colonisation site of EHEC O157 in cattle and a site containing M-like cells. As functional differences could not be simply assigned to variation in established interactions of EspF with Sorting Nexin 9 and N-WASP, yeast-2-hybrid screening was used to identify additional host proteins that may interact with EspF. The anaphase promoting complex inhibitor, Mad2L2, was identified from this screen. Mad2L2 was then demonstrated to interact with EspF variants from EHEC O157:H7, O26:H11 and EPEC O127:H6 by Lumier assays. While Mad2L2 has been shown to be targeted by the non homologous Shigella effector protein IpaB to limit epithelial cell turnover, we presume that EspF interactions with this protein may indicate a similar function to promote EPEC and EHEC colonization.

The final section of work addressed whether bacterial interactions can actually induce M-cell differentiation on follicle-associated epithelium. The work focused on bovine rectal primary cell cultures interacting with Salmonella enterica serovar Typhimurium. The type III secreted protein, SopB, was required for Salmonella to:

(i) activate parts of epithelial to mesenchymal transition (EMT) pathway; (ii) transform a subset of epithelial cells to a cell type that phenotypically and functionally resembles specialized antigen sampling M cells; (iii) induce RANKL and downstream RelB dependent NFκB signaling. The work suggests that Salmonella may induce this cellular transformation to promote its invasion and colonization of intestinal mucosa.

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# Abbreviations

| | |
|---|---|
| **aa** | Amino acids |
| **Ab** | Antibody |
| **AAF** | aggregative adherence fimbriae |
| **A/E** | attaching and effacing |
| **Amp** | Ampicillin |
| **β** | Beta |
| **BSA** | bovine serum albumin |
| **°C** | Degrees Celsius |
| **CAM** | chloramphenicol |
| **cDNA** | Complementary DNA |
| **CFU** | colony forming units |
| **DAEC** | diffusely adherent *E. coli* |
| **DMEM** | Dulbecco's modified eagle medium |
| **DNA** | deoxyribonucleic acid |
| ***E. coli*** | *Escherichia coli* |
| **EAEC** | enteroaggregative *Escherichia coli* |
| **EDTA** | ethylenediamine tetraacetic acid |
| **EHEC** | enterohaemorrhagic *Escherichia coli* |
| **EIEC** | enteroinvasive *Escherichia coli* |
| **EPEC** | enteropathogenic *Escherichia coli* |
| **ETEC** | enterotoxigenic *Escherichia coli* |
| **Esp** | *Escherichia coli* secreted protein |
| **Esc** | *Escherichia coli* secretion |
| **FAC** | follicular associated crypts |
| **FAE** | follicle associated epithelium |
| **FBS** | foetal bovine serum |
| **G** | gram |
| **GFP** | green fluorescent protein |
| **G proteins** | guanine nucleotide binding proteins |
| **h** | Hour(s) |
| **H** | flagellar antigen |
| **HC** | haemorrhagic colitis |
| **HK ST** | heat killed *Salmonella* Typhimurium |
| **HUS** | haemolytic uremic syndrome |
| **IPTG** | isopropyl β-D-1-thiogalactopyranoside |
| **K** | capsular antigen |
| **Kan** | kanamycin |
| **kb** | kilobase pairs |
| **kDa** | kilodalton |
| **λ** | lambda |
| **LB** | Luria-Bertani |
| **LEE** | locus of enterocyte effacement |
| **LF** | lymphoid follicle |

| | |
|---|---|
| **Ler** | LEE encoded regulator |
| **LPS** | lipopolysaccharide |
| **LT** | heat-labile |
| **M** | molar |
| **MAD2L2** | mitotic arrest deficient-like 2 |
| **Map** | mitochondrion-associated protein (Map), |
| **MEM** | minimal essential medium |
| **mg** | milligram ($10^{-3}$ gram) |
| **min** | minute(s) |
| **ml** | millilitre ($10^{-3}$ litre) |
| **mM** | millimolar ($10^{-3}$ molar) |
| **NFIC** | nuclear factor 1 C-type |
| **NF κB** | nuclear factor kappa-light-chain-enhancer of activated B cells |
| **ng** | nanogram ($10^{-9}$ gram) |
| **NM** | non-motile |
| **N-WASP** | neuronal Wiskott-Aldrich Syndrome protein |
| **O** | somatic antigen |
| **OC** | ordinary crypts |
| **$OD_{600}$** | optical density at 600 nm |
| **OPG** | osteoprotegerin |
| **ORFs** | open reading frames |
| **PAI** | pathogenicity islands |
| **PBS** | phosphate buffered saline |
| **PCR** | polymerase chain reaction |
| **PFA** | paraformaldehyde |
| **PP** | Peyer's patches |
| **RANKL** | receptor activator of nuclear factor kappa-B ligand |
| **RNA** | ribonucleic acid |
| **rpm** | rounds per minute |
| **RT** | room temperature |
| **RTPCR** | real time PCR |
| **SDS** | sodium dodecyl sulphate |
| **SDS-PAGE** | sodium dodecyl sulphate-polyacrylamide gel electrophoresis |
| **sec** | second |
| **siRNA** | small interfering RNA |
| **SNX9** | Sorting nexin 9 |
| **SPI-1** | *Salmonella* pathogenicity island 1 |
| **SPI-2** | *Salmonella* pathogenicity island 2 |
| **SQRT-PCR** | Semi quantitative real time PCR |
| **ST** | heat-stable |

| | |
|---|---|
| **STEC** | Stx-producing strains of *Escherichia coli* |
| **Stx** | Shiga toxin |
| **T3SS** | type III secretion system |
| **TBE** | tris-borate/EDTA |
| **TCA** | trichloroacetic acid |
| **TFB** | transformation buffer |
| **Tir** | translocated intimin receptor |
| **TRIC** | Rhodamine |
| **Tris** | trishydroxymethylaminomethane |
| **µg** | microgram ($10^{-6}$ gram) |
| **µl** | microlitre ($10^{-6}$ litre) |
| **µM** | micromolar ($10^{-6}$ molar) |
| **U** | unitt |
| **V** | volt |
| **Vtx** | Vero toxin |
| **VTEC** | Vtx-producing strains of *Escherichia coli* |
| **Y2H** | Yeast-two- hybrid |
| **WT (ST)** | wildtype *Salmonella* Typhimurium |

# *Chapter 1*

## *Introduction*

# 1. Introduction

*Enterobacteriaceae* are a large family of bacteria. The members of the *Enterobacteriaceae* are rod-shaped, and their length about 1-5 μm. They are facultative anaerobes. These bacteria are able to ferment sugars to produce lactic acid and different other end products. The majority also reduce nitrate to nitrite and have peritrichious flagella that are used for movement. However a few genera are non-motile. The members of this family are non-spore forming. The catalase reaction varies among the *Enterobacteriaceae*. Most bacteria of this family are a normal part of the gut flora established in humans and other animals' intestines. Certain genera and species in this family are capable of causing a broad spectrum of intestinal and systemic diseases, e.g. *Salmonella typhi*, *Shigella dysenteriae* and *Yersinia pestis* (edwarda & Ewing 1972).

**1.1 *Escherichia coli (E. coli)*** *E. coli* was named after the German paediatrician, Theodore Escherich, who first identified and described it in 1885 from the faecal materials of healthy persons. Many strains/serotypes of *E. coli* are predominantly found as commensal gut flora that colonises the gastrointestinal tract of newborns within a few hours of life. The majority do not cause any disease unless the host is malnourished, immuno-suppressed or there has been a disturbance in the intestinal barrier. Under these circumstances even commensal *E. coli* can cause disease (Nataro & Kaper, 1998). Serologically each *E. coli* isolate can be grouped by its lipopolysaccharide O-antigen, flagellar H-antigen, K-capsule and F fimbriae/pili (Robins Browne & Hartland, 2002). The pathogenic strains include six distinct categories of *E. coli* that cause diarrhoea (Nataro & Kaper, 1998). These can be referred to as pathotypes: (1) Enterotoxigenic *E. coli* (ETEC), (2) Enteroinvasive *E. coli* (EIEC), (3) Enteropathogenic *E. coli* (EPEC), (4) Enteroaggregative *E. coli* (EAEC), (5) Diffuse Adherent *E. coli* (DAEC**),** and (6) Enterohaemorrhagic *E. coli* (EHEC). Key factors that define these pathotypes are genetically-encoded in clusters on virulence-related plasmids, chromosomal pathogenicity islands or on phages. *E. coli*, like any other mucosal pathogen, has evolved the strategy to colonize the

mucosal surface, evade or modulate the host immune responses and secrete virulence factors that help its colonization and persistence.

**1.1.1 Enterotoxigenic *E. coli* (ETEC):** are considered to be the major cause of diarrhoea for travellers to developing countries and to be a very important cause of childhood diarrhoea in developing countries. ETEC are also associated with diarrhoea in animals (Kaper *et al.*, 2004). ETEC cause watery diarrhoea due to colonization of the intestinal mucosa via non-intimate diffuse adherence with fimbriae and production of enterotoxins (heat stable and/or heat labile toxins) that repress gut absorption and increase intestinal secretion. Moreover, ETEC cause diarrhoea in animals and F4 (K88) and F5 (K99) antigens are associated with ETEC infections of pigs and cattle, respectively (Kaper *et al.*, 2004). ETEC serotypes examples include: O6:H16, and O8:H9.

**1.1.2 Enteroinvasive *E. coli* (EIEC):** are a significant reason of illness in children in developed countries and are biochemically, genetically, and pathogenically intimately related to *Shigella spp.* (Kaper *et al.*, 2004). They cause diarrhoea by invasion of epithelial cells followed by multiplication and lysis of the infected cell. This can lead to inflammatory colitis and/or secretory bowel syndrome (Kaper *et al.*, 2004). EIEC serotypes examples include: O28:NM, O29:NM, O112:NM, O124:H30, O124:NM, O136:NM, O136:NM, O143:NM, O144:NM, , and O167:NM.

**1.1.3 Enteroaggreative *E. coli* (EAEC):** are a cause of persistent mucoid diarrhoea in children and adults in both developing and developed countries (Kaper *et al.*, 2004). They adhere to epithelial cells and each other in an aggregative manner by using fimbrial structures known as aggregative adherence fimbriae (AAFS) and produce an enterotoxin that can cause mild damage and inflammation of mucosal surfaces (Kaper *et al.*, 2004). They do not induce attaching and effacing (A/E) lesions (Clarke, 2001). EAEC serotypes examples include: O3:H2, O15:H18, O44:H18, O86:H18, O77:H18, O111:H21, and O127:H2.

**1.1.4. Diffuse adherent *E. coli* (DAEC):** are characterised by a diffuse adherence pattern to HEP-2 cells. They are known to be a cause of diarrhoea in children less than 1 year old. DAEC strains exhibit non-intimate adherence in contrast to EPEC and EHEC adherence, as described in the relevant sections below. DAEC produce fimbriae called F1845 that mediate the diffuse adherence (Nataro & Kaper 2004). Serotypes are not well defined, but include O126:H27.

**1.1.5 Enteropathogenic *E. coli* (EPEC):** are medically-important diarrhoeagenic Gram negative pathogens that colonize the gut mucosa. EPEC can cause serious intestinal disease in many animal species including cattle and are associated with severe diarrhoeal outbreaks among groups of young children in nurseries and generally in developing countries (Nataro & Kaper, 1998; Kenny, 1999; Kaper *et al.*, 2004). The disease process is dependent on intimate attachment with the formation of attaching and effacing (A/E) lesions on intestinal mucosa leading to effacement of microvilli and associated cytoskeletal re-arrangement. Actin-rich pedestals are formed under the bacteria with tight junction damage and eventually cell death. This process occurs through injection of effector proteins directly into host cells (Nataro & Kaper, 1998). Human EPEC infections are considered to arise from human-to-human infection (Spears *et al.*, 2006). It was in 1945, when a severe outbreak of infant diarrhoea in United Kingdom led Bray to identify EPEC pathotype of *E. coli* for the first time **(**Frankel & Phillips 2008). Although recently, it is rare for this strain to cause large outbreaks in industrialised countries, it is still an important cause for potentially fatal infant diarrhoea in advanced countries. For long time, the mechanisms by which EPEC caused diarrhoea have been undefined and this pathotype could only be identified on the basis of serotyping. However, highly advanced techniques since 1979, were used in understanding the pathogenesis of EPEC diarrhoea. For these reasons, EPEC is now among the best understood of all pathogenic *E. coli* (Kaper *et al.*, 2004). Examples of EPEC serotypes are O26, O55, O86, O111, O114, O119, O125, O126, O127, O128, O142, and O158 (Trabulsi *et al.*, 2002).

**1.1.6 Verotoxin (VT) producing *E. coli* (VTEC) or Shiga toxin (Stx) producing *E. coli*:** are defined by the expression of one or more Shiga toxins also known as Verotoxins. In this study they will be referred to as Shiga-toxigenic *E. coli.* These strains may or may not cause disease according to the presence or absence of other virulence factors. So they can be divided into two categories:

1- Typical Enterohaemorrhagic *E. coli* (EHEC), which are STEC strains that are associated with haemorrhagic colitis (HC) and haemolytic-uraemic syndrome (HUS), express Stx, form A/E lesions on epithelial cells and possess a 92Kbp plasmid.

2- Atypical EHEC, which are STEC strains that do not produce A/E lesions and/or possess the 92Kbp plasmid (Nataro & Kaper, 1998).

EHEC are emerging zoonotic pathogens, particularly in industrialised countries (Beutin, 2006), although there is very limited data on epidemiology in most developing countries. EHEC O157:H7 causes sporadic outbreaks of severe disease in humans, the most important being HC and HUS, the latter results in kidney damage and may lead to death (Moon *et al.*, 1983; Griffin & Tauxe, 1991; Boyce *et al.*, 1995). EHEC O157:H7 is the serotype associated with most EHEC infections in United Kingdom, USA and Japan. As well as the production of Stx, EHEC strains produce A/E lesions on intestinal mucosa and in some ways can be considered as EPEC strains that have acquired Shiga toxins (Tesh & O'brien, 1991; Naylor *et al.*, 2005b). Ruminants, particularly cattle, are the main reservoir for certain EHEC serotypes, including *E. coli* O157:H7. EHEC O157:H7 colonises the bovine gastrointestinal tract, especially at the terminal rectum, without causing overt disease (Naylor *et al.*, 2003). In part this reflects the absence of Shiga toxin receptors (CD77) on bovine endothelial cells.

### 1.1.7 Epidemiology of EHEC O157

#### 1.1.7a Animals as reservoirs of EHEC

Cattle are one of the most important host or carrier species of EHEC. High rates of colonisation by Stx-positive *E. coli* have been found in bovine herds in many countries ( Wells *et al.*, 1991; Clarke *et al.*, 1994; Hancock *et al.*, 1994; Burnens *et al.*, 1995). The rates of carrier animals vary from 10 to 60%. STEC strains are usually isolated from healthy animals, but it may cause diarrhoea in young animal

followed by asymptomatic colonization (Nataro & Kaper, 1998). Transmission to human occurs through various routes; the most important is oral route through water, crops, meat, and dairy products intended for human consumption contaminated with faecal matter (Akashi *et al.*, 1994; Yarze & Chase, 2000; Licence *et al.*, 2001).

*E. coli* O157:H7 may persist in a viable state in faeces for more than 20 months (Beutin, 2006). *E .coli* O157:H7 has been isolated in many parts of the world, including Europe, Asia, Africa, and South America (Griffin, 1995). Various epidemiological studies on farms and slaughterhouses have documented a worldwide presence of *E. coli* O157:H7. Faecal pat studies from cattle have shown that *E. coli* O157:H7 occurs more frequently in Canada and United States (Griffin & Tauxe, 1991) with a prevalence of 1.4% in Japan (Miyao *et al.*, 1998), 1.9% in Australia (Cobbold & Desmarchelier, 2000), 0.2 to 60% in USA (Wells *et al.*, 1991; Hancock *et al.*, 1994; Faith *et al.*, 1996), 8.6% in Scotland (Synge, 2000) and 4.7% in England and Wales (Paiba *et al.*, 2002).

Seasonal variation in the prevalence of *E. coli* O157:H7 has been demonstrated, with the highest prevalence occuring during the late summer and early autumn (Chapman *et al.*, 1997; Hancock et al., 1997; Tutenel et al., 2002). In contrast Ogden *et al.* (2004) revealed that the occurrence in cattle was higher throughout cooler periods but that levels of bacterial shedding were highest during warmer periods of the year. In addition to cattle, *E coli* O157:H7 strains have been isolated from a variety of animal species including sheep, goats, wild deers, pigs, and birds (Chapman, 2000).

### 1.1.7b Mode of transmission of STEC O157:H7

The first identified outbreaks of *E. coli* O157:H7 were associated with consumption of ground beef and more outbreaks were associated with consumption of undercooked beef and other bovine products, such as unpasteurised milk (Riley *et al.*, 1983). Bovine faeces contaminate meat during slaughter or meat processing (Armstrong *et al.*, 1996; Barlow *et al.*, 2006). Other animal products, such as raw goat's milk (Bielaszewska *et al.*, 1997), deer jerky (Keene *et al.*, 1997; Renter *et al.*, 2001), porcine meat, avian and sheep products ( Griffin & Tauxe, 1991; Griffin, 1995) have also been implicated in human outbreaks of *E. coli* O157:H7. Vegetables and fruits contaminated with animal faeces also play an important role in

transmission ( Ackers *et al.*, 1998; Fukushima *et al.*, 1999; Duffy, 2003; Karch *et al.*, 2005). Furthermore, some outbreaks occur when *E. coli* O157:H7 contaminates unpasteurised apple juice and fermented salami. This illustrates the potential acid tolerance of *E. coli* O157:H7, which potentially can grow and survive in extremely acidic conditions that destroy other pathogens (Nataro & Kaper, 1998).

### 1.1.7c Water sources

Water sources including the one for recreational uses (Keene *et al.*, 1994), well water and even a municipal water system (Swerdlow *et al.*, 1992) have been associated with outbreaks. Several outbreaks of *E coli* O157:H7 have been associated with consumption of contaminated drinking water especially from private water supplies ( Swerdlow *et al.*, 1992; Jones & Roworth, 1996) or from swimming in lakes or paddling pools (Brewster *et al.*, 1994; CDC, 1996).

### 1.1.7d Contact with animals

Transmission through direct contact with animals has been documented in outbreaks and sporadic infections by *E. coli* O157:H7 due to farm visits (Renwick *et al.*, 1993; Shukla *et al.*, 1995). Also infections have been reported from visiting petting zoos and farms, where direct contact with animals or animal faeces can easily occur (Caprioli *et al.*, 2005). A high profile outbreak in a UK petting farm occurred in 2009 (http://www.griffininvestigation.org.uk/), in which nearly 100 children were infected with 78 people showing symptoms; 22% of them had HUS and 8 patients required dialysis and some of them were left with permanent kidney damage. This farm alone received nearly 200,000 visitors a year indicating that potential of such farms to infect large numbers of individuals.

### 1.1.7e Person-to-person faecal–oral transmission

*E. coli* O157:H7 can cause infection in very low doses (Nataro & Kaper, 1998). Person-to-person faecal–oral transmission can easily occur in poor hygiene conditions and with close contact (Karch *et al.*, 1999). This mode of transmission is responsible for the spread of infection within families (Ludwig *et al.*, 1997) and hospitals (Karmali *et al.*, 1988). Person-to-person transmission was the predominant

route of infection in outbreaks within day-care settings (Reida *et al.*, 1994) and mental institutions (Pavia *et al.*, 1990).

### 1.1.7f Epidemiology of non-O157 EHEC

The significance of non-O157 EHEC in human diseases is less well understood than that of EHEC O157:H7 (Karch *et al.*, 2005). *E. coli* O157:H7 is simply differentiated from other *E. coli* by its inability to rapidly ferment sorbitol. However, non-O157 STEC are phenotypically the same as commensal non pathogenic *E. coli* and are not detected with sorbitol MacConkey agar. To detect non-O157 STEC, various methods are used, such as enzyme immunoassays or PCR, which are classically only performed in reference laboratories (Fey *et al.*, 2000). The prevalence of non-O157 STEC in faecal samples from the Northern USA, in which cattle and other animal reservoirs of STEC are plentiful, was determined to be 4.2%. This prevalence was elevated than earlier reported in the USA. Five different non-O157 STEC serotypes were isolated: O111:NM, O26:H11, O145:NM, O103:H2, and O rough:H2. Four of these have been associated with HUS (O111:NM, O26:H11, O145:NM, and O103:H2; Griffin *et al.*, 1990; Fey *et al.*, 2000).

Outbreaks by non O157 STEC have been associated with food, including an outbreak of bloody diarrhoea in the USA by STEC O4:H21 in 1994 (pasteurized milk) (CDC, 1995), and an outbreak in Australia caused STEC O111:H- in 1995 (dry fermented sausages) (Paton *et al.*, 1996).

Many non-O157:H7 serotypes of EHEC have also been implicated with sporadic or large-scale human outbreaks. EHEC O26:H11 is the most common non-O157 EHEC serogroup associated with human diseases throughout Europe. On the other hand, the second most common non-O157 EHEC serogroup associated with HUS in Germany is O145 (Karch *et al.*, 2005). The non-O157:H7 EHEC strains caused the majority of HUS cases in many countries like Argentina (Bentancor *et al.*, 2007) and Australia (Pearce *et al.*, 2010), but worldwide O157:H7 is the most common. In general, EHEC O145 appears to be shed by cattle less frequently than EHEC O26. Most bovine EHEC O26 and O145 isolates possess virulence characteristics identical to those isolated from human patients, suggesting that strains shed by cattle can cause human disease (Geue *et al.*, 2002; Jenkins *et al.*, 2003). The Stx2 type causes more severe HUS disease than that of Stx1, but Stx1 was more common in bovine EHEC

O26 isolates (Karch *et al.*, 2005), and was also the most prevalent one in human isolates from patients with HUS or diarrhoea (Karch *et al.*, 2005), thereby providing evidence for cattle being major reservoirs for such organisms. In 2011 a sever outbreak of HUS and bloody diarrhoea has occurred in Germany. It was caused by enteroaggregative *E. coli* strain O104:H4. This strain acquired the ability to express Stx2a by integration of phage (Scheutz *et al.*, 2011). Fresh sprouts produced by a farm in Lower Saxony are accountable for the present outbreak in Germany (CDC, 2011).

### 1.1.8 Diseases caused by EHEC

#### 1.1.8a Disease in cattle

Some STEC isolates can induce diarrhoea in young calves including strain O157:H7. However, this strain is avirulent in older cattle (Pearson *et al.*, 1999. Stordeur *et al.*, 2000), implying that *E. coli* O157:H7 lacks certain factors required for virulence in such animals.

Serogroups O26, O111, and O118 are virulent to cattle, particularly young calves. *E. coli* O26 has been isolated from clinical cases of haemorrhagic diarrhoea (Pearson *et al.*, 1999; Gunning *et al.*, 2001). However, an *E. coli* O26:H-strain, originally isolated from a calf with diarrhoea in 1968, was inoculated into calves and did not produce diarrhoea (Mainil *et al.*, 1987), but has subsequently been used to induce transient diarrhoea in 4-day old calves (Dziva *et al.*, 2004). Hall and co-workers (Hall *et al.*, 1985) experimentally-infected 3 calves with between $10^9$ and $10^{10}$ CFU of *E. coli* serogroup O5, inducing diarrhoea for 2-4 days. *E. coli* O118:H16 is frequently associated with calf diarrhoea, and virulence has been confirmed in experimentally-challenged new-born calves (Stordeur *et al.*, 2000).

#### 1.1.8b Disease associated with EHEC in human

Haemolytic uremic syndrome (HUS) is the most serious complication of *E. coli* O157:H7 infection. Clinically, it is associated with microangiopathic haemolytic anaemia, thrombocytopaenia, and acute renal failure. It occurs 5-10 days after the initial diarrhoeal illness is resolved (Advisory committee on the microbiological safety of food, 1995).

Haemorrhagic colitis (HC) is characterized by severe bloody diarrhoea indicating gastrointestinal haemorrhage (Riley *et al.*, 1983). However, bloody diarrhoea may alternate between non-bloody and blood-streaked faeces (Swerdlow *et al.*, 1992; Bell *et al.*, 1994). The main pathological feature of the infected colon includes oedema, congestion of colonic mucosa, sometimes with ulcers (Griffin *et al.*, 1990).

Other complications following EHEC O157:H7 infection include: cholecystitis, colonic perforation, intussusceptions, pancreatitis, post hemolytic biliary thiasis, post infection colonic stricture, rectal prolapse, appendicitis, hepatitis, haemorrhagic cystitis, pulmonary oedema, myocardial dysfunction, and neurological abnormalities (Griffin, 1995; Tarr, 1995).

### 1.1.9 EHEC and EPEC colonization and virulence factors

#### 1.1.9a Shiga toxins

Shiga toxins are encoded on lambdoid bacteriophages that integrate into the bacterial chromosome. This is the major virulence factor that plays a key role in pathogenesis of EHEC infections. It is reported to mediate diarrhoea as a result of compromised intestinal epithelium integrity; haemorrhagic colitis due to damage to intestinal mucosal vasculature and damage to kidneys, circulatory and the central nervous system when disseminated systemically leading to life-threatening syndromes such as HUS and thrombotisch-thrombozytopenische Purpura TTP (O'Brien & Holmes, 1987). The role of Stx in pathogenesis of these EHEC diseases has been modelled in various experimental studies (Marcato *et al.*, 2005). Two main groups of Stx exist: Stx1 and Stx2. Stx1 has a several variants, which are identical to the toxin of *Shigella dysenteriae* (Jackson *et al.*, 1987; Smith *et al.*, 2002). Stx2 is divided into different variants e.g. Stx2, Stx2c, Stx2d, Stx2e, Stx2f, and Stx2g. The two subgroups of Stx vary in their toxicity and capacity to bind to selected human cells (Jacewicz *et al.*, 1999). Stx2 and Stx2c are most associated with human diseases but also found in *E. coli* isolated from animals (Boerlin *et al.*, 1999; Bidet *et al.*, 2005; Schmidt *et al.*, 2000). The toxin consists of two subunits, A and B. The B subunit binds to the host cell membrane via the glycolipid receptor Gb3 (Lingwood *et al.*, 1987; Lingwood, 1993; Lingwood, 1996). It was considered that cattle lack the vascular receptors for Stx, so this host does not suffer the more severe effects of EHEC infection

(Pruimboom-Brees *et al.*, 2000). Gb3 is considered to be present in bovine intestinal crypt cells (Hoey *et al.*, 2002). Intracellular trafficking in primary bovine intestinal cells excludes Stx from endoplasmic reticulum and instead localises it to lysosomes leading to its inactivation (Hoey *et al.*, 2003). The A subunit is cleaved to produce an A1 peptide with N-glycosidase activity that inhibits protein synthesis through cleavage of the 28s ribosomal RNA (Pruimboom-Brees *et al.*, 2000; Hoey *et al.*, 2003). The mechanisms by which Stx result in HUS are complicated and may be due to direct toxicity to endothelium, interference of haemostatic pathways and release of chemokines. Alteration of haemostatic pathways results in thrombotic microangiopathy (Richardson *et al.*, 1988). Clinical studies have indicated that the production of a proinflammatory cytokines (Nataro & Kaper, 1998) sensitize the endothelial cells to the action of VT and induce further Gb3 expression (Richardson *et al.*, 1988; Van De Kar *et al.*, 1992). Stx increases bacterial adherence and intimin attachment. It induces eukaryotic cells to express more receptors for the EHEC O157:H7 attachment factor intimin (Robinson *et al.*, 2006). Other additional cell cytotoxicity mechanisms have also been proposed, for instance Stx1 and Stx2 induced apoptosis via inhibiting expression of anti-apoptotic Bcl-2 family member, Mcl-1 in endothelial cells (Erwert *et al.*, 2003). Stx1 also triggered apoptosis via activating caspase- and mitochondria-dependent pathways (Tétaud *et al.*, 2003).

### 1.1.9b Virulence factors encoded on pO157

EHEC O157:H7 contains the pO157 plasmid, which encodes the virulence factors StcE, enterohaemoloysin, EspP, and KatP (Bauer & Welch, 1996; Brunder *et al.*, 1997; Lathem *et al.*, 2002; Grys *et al.*, 2005). These are described below. pO157 or related plasmids are also present in strain O26:H11 and are also found in some human STEC strains (Nataro & Kaper, 1998). The plasmid contributes to STEC survival (Boerlin *et al.*, 1999). However, there is conflicting evidence regarding its contribution to the development of HC or HUS (Schmidt *et al.*, 1996). The plasmid encodes the following factors:

### 1.1.9c Enterohaemolysins

Ehx belongs to the RTX toxin family, members of which are expressed by uropathogenic *E. coli*, *Pasteurella haemolytica*, and other human and animal pathogens (Bauer & Welch, 1996). Ehx is highly conserved amongst STEC strains, suggesting that it contributes to survival in someway (Boerlin *et al.*, 1999). However, there is conflicting evidence regarding its contribution to the development of HC or HUS (Schmidt *et al.*, 1996; Boerlin *et al.*, 1999).There are three types of *E. coli* haemolysins: β, a cell bound haemolytic factor; α, a cell free factor, and γ haemolysins (Smith, 1963; Walton & Smith, 1969). Ehx is encoded by the 92Kbp virulence plasmid (pO157) (Schmidt *et al.*, 1994). Ehx causes β haemolysis when cultured on blood agar plates (γ haemolysin does not haemolyse human or rabbit RBCs but RBCs of other species). α factor is cytotoxic for human leukocytes and fibroblasts *in vitro*. RBCs haemolysis results in release of iron which is potentially important factor for bacterial growth (Nataro & Kaper, 1998). The cytotoxic effect and iron released from RBCs are the most important function of α haemolysin in *E. coli* pathogenesis (Cavalieri *et al.*, 1984)

### 1.1.9d EspP

The EspP (*E. coli* secreted protein P) is a protein secreted by EHEC O157 and is one member of the serine protease autotransporter of Enterobacteriaceae (SPATE) family (Leyton *et al.*, 2003). It cleaves pepsin A and human coagulation factor 5 (Brunder *et al.*, 1997). EspP has been reported to be cytotoxic for Vero cells. The protease cleaved human coagulation factor V and hence could result in exacerbation of haemorrhagic disease in EHEC-infected patients. A role of EspP in pathogenesis is consistent with presence of antibodies to the protease in sera from EHEC-infected patients (Brunder *et al.*, 1997).

### 1.1.9e StcE

StcE (secreted protease of C1 inhibitor from EHEC) is a secreted protein and a C1 esterase inhibitor. It is secreted from EHEC via a type 2 secretion system and is a zinc metalloprotease Cl-esterase inhibitor that regulates complement and other mechanisms of inflammation (Grys *et al.*, 2005). StcE has been shown to be regulated by the LEE-encoded regulator Ler, and its action results in many

pathological effects which may include localized proinflammatory and coagulation responses, leading to tissue damage, intestinal oedema and thrombotic abnormalities (Lathem *et al.*, 2002). StcE cleavage of C1 esterase inhibitor provides a significant protection system against complement for bacteria and infected host cells (Lathem *et al.*, 2004).

### 1.1.9f KatP

KatP is catalase peroxidase that is considered to protect EHEC from reactive oxygen intermediates found in host cells (Burland *et al.*, 1998).

### 1.1.9g H7 flagella

Flagella are multi-functional organelles that play different roles in the biology of bacteria. The motelity functions give specific advantages upon host-adapted prokaryotes. Possible benefits of motility include increased ability of nutrient gaining, escaping of toxic substances, to access optimal colonization sites within hosts and to spreading in the environment during the course of transmission between hosts (Girón, 2005). The flagellum also activates the host immune system through activation of TLR5 receptors. Once the flagellin monomer is bound to TLR5 receptors, this activates the NF-κB cell signalling pathway resulting in activation of inflammatory responses including IL-8 secretion that stimulates the migration of neutrophils and dendritic cells to the infection site (Dahan *et al.*, 2002). The bacterial flagellum can be described in three parts (Charlier & Glansdorff, 2004): (1) the basal body, which is composed of a sequence of rings and a middle rod. The basal body is anchored in the inner and outer membranes of the bacteria and provides the engine for the chemotactic apparatus; (2) the hook that is located external to the cell and provides the link between the basal body and the filament; and (3) the flagellar filament, which is the biggest part of the flagellum. It is formed of repeating sub-units of the protein flagellin in a helical arrangement and often extends many times the length of the cell.

Rotation of this filament (clockwise or counter-clockwise), which is controlled by the motor, ultimately determines the swimming or tumbling phenotype that defines

whether a bacterium will stay in a particular microenvironment or move away from it (Macnab, 1992; Aizawa, 1996).

The H7 flagellum plays an important role in the initial adhesion of EHEC O157 to the bovine terminal rectal epithelium cells (McNeilly *et al.*, 2008). Purified IgA or IgG from cattle vaccinated with H7 flagella was able to inhibit bacterial binding to rectal primary epithelial (Mahajan *et al.*, 2009).

### 1.1.9h Type III secretion (TTSS) system and associated effector proteins

The bacteria must defend themselves against various challenges in their environment to continue growth and multiply. To deal with these challenges bacteria secrete enzymes, proteins and/or toxins. These factors to be secreted from the cytoplasm of bacteria to the external environment or directly into the host cells need dedicated 'machinery' to do this, known as secretion systems. The Gram negative bacteria possess at least 6 different secretion systems (Gerlach & Hensel, 2007).

The type I secretion system is expressed by a wide range of Gram negative bacteria for secretion of toxins, proteases, and lipases directly into host cell for example α-hemolysin of *E. coli* represents the prototypical type I exported factor (Thanabalu *et al.*, 1998).

The type II system is used by different Gram negative bacteria for secretion of extracellular enzymes and toxins (exotoxins), for example pullulanase (PulA) secretion from *Klebsiella oxytoca* (Takizawa & Murooka, 1985) and StcE from EHEC O157 (Grys *et al.*, 2005).

Type III secretion will be discussed below.

Concerning Type IV, this secretion system mediates inter-bacterial DNA transfer, for example to protect itself from antibiotic challenge for example the *E. coli* F plasmids (IncF), RP4 (IncP) Trb, R388 (IncW), and Trw genetic exchanges. Type IV system can also translocate virulence factors into eukaryotic host cells (Lawley *et al.*, 2003).

Type V is the simplest protein secretion pathway. Proteins are secreted via an autotransporter system (type Va or AT-1), the two-partner secretion pathway (type Vb), and the recently described type Vc system (also termed AT-2). Proteins secreted via these pathways have similarities in their primary structures as well as striking similarities in their modes of biogenesis. IgA1 protease is an example of a protein

secreted via this secretion system ( Pohlner *et al.*, 1987; Desvaux *et al.*, 2003). EspP, EhaA, and EhaB are also secreted by type V secretion system (Dziva *et al.*, 2007).
Type VI secretion system has been shown to be important for Gram-negative bacterial pathogens virulence in host cell. For example it is required for cytotoxicity caused by of V. cholerae toward Dictyostelium amoebae and mammalian J774 macrophages by a contact-dependent mechanism (Pukatzki *et al.*, 2006).

TTSS are found in many different Gram negative bacteria and are complex macromolecular structures used to translocate effector proteins directly from the bacterial cytosol into the cytoplasm of eukaryotic cells (Hueck, 1998). The TTSS apparatus spans the bacterial inner and outer membranes and exports proteins past the cell wall. Two classes of proteins are exported by TTSS: translocon and effector proteins. Translocon proteins are required for translocation of effectors across the eukaryotic plasma membrane and some may also function as effectors themselves. Effector proteins alter host cell physiology and promote bacterial survival in host tissues (Mecsas & Strauss, 1996). TTSS is triggered when a pathogen comes in contact with host cells (Ginocchio *et al.*, 1994; Menard *et al.*, 1994; Watarai *et al.*, 1995). The TTSS consist of a basal apparatus with proteins present in the outer and inner membranes, and a needle complex that leads to the formation of a pore in the host cell membrane. This creates a conduit from the bacterium to the host cell through which effector proteins can be translocated (Roe *et al.*, 2003).

### 1.1.9h.1 TTSS system of EPEC and EHEC

In EHEC and EPEC all of the genes encoding this protein secretion system are expressed from the locus of enterocyte effacement (LEE) *(*Jarvis *et al.*, 1995; Hueck, 1998). The LEE regions of EPEC and EHEC have been sequenced and consist of 41 and 54 open reading frames (ORFs), respectively. The additional 13 ORFs found in the EHEC LEE fall within a putative P4 family prophage designated 933L; the prophage is not present in the prototype EPEC O127:H6 strain E2348/69 but is found in a closely related EPEC O55:H7 serotype and other O157:H7 isolates. LEE genes are arranged in five large, polycistronic operons, LEE1 to LEE5 of which the first three are identical between EPEC O127 and EHEC O157, and code for the type III

secretory apparatus (Elliott *et al.*, 1998; Nataro & Kaper, 1998; Perna *et al.*, 1998; Clarke *et al.*, 2003). This is a contact-dependent mechanism in which proteins are secreted across bacterial and host cell membranes and inserted directly into the host cell cytoplasm (Hueck, 1998). LEE4 encodes the secreted proteins EspA, B, and D which form the translocon and a pore required to deliver effector proteins into the cytoplasm of host cell (Shaw *et al.*, 2001; Roe *et al.*, 2003; Gauthier *et al.*, 2003). LEE 5 encodes an effector protein chaperone, CesT, as well as intimin and the translocated intimin receptor (Hartland *et al.*, 1999) whic is inserted into the host cell membrane by the TTSS. Other smaller operons and ORFs are also present in the LEE that code for various effector proteins including EspF (Crane *et al.*, 2001), EspG (Elliott *et al.*, 2001), EspH (Tu *et al.*, 2003), mitochondrial-associated protein, (Kenny & Jepson, 2000), Tir (Kenny *et al.*, 1997), and a chaperone protein CesT for Tir (Abe *et al.*, 1999). Many effector proteins are involved in modulating the host cytoskeleton.

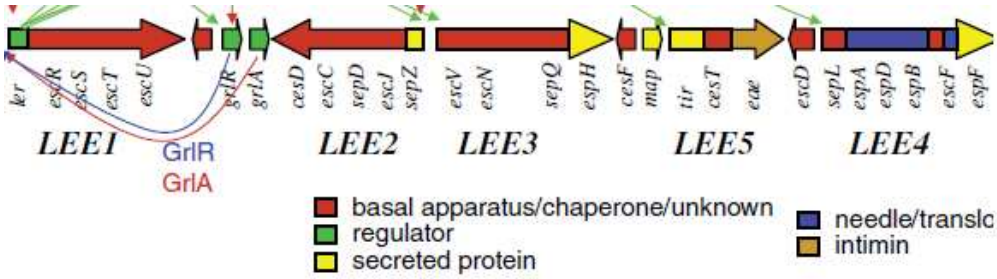


**Fig. 1.1 The LEE regions** consist of 5 operons from LEE1to LEE5. This figure is taken directly from (Spears *et al.*, 2006)**.**

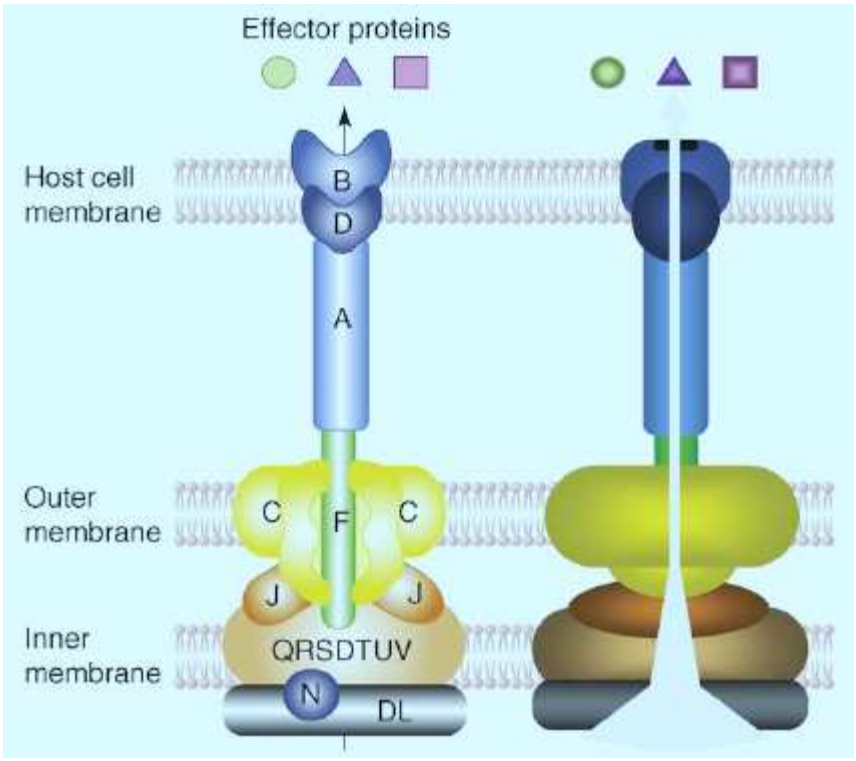


**Fig. 1.2 The TTSS.** This figure shows the translocation of bacterial effector protein from its production site, through the structural needle, directly into the host cell cytoplasm. EspA the sytem needle is formed through the collection of TTSS structural proteins, and is approx. 80 nm long. This molecular needle is directly able to inject the bacterial effectors directly into the host cell. This figure is taken directly from (Tree *et al.*, 2009).

### 1.1.9h.2 Initial attachment

EPEC adhere to intestinal epithelial cells depending on the presence of a plasmid that encodes 7nm diameter type 4 and 5 fimbriae that are anchored to the outer membrane and combined to form bundles known as bundle forming pili (Cravioto *et al.*, 1979; Giron *et al.*, 1991; Bieber *et al.*, 1998). These pili are not encoded by EHEC O157:H7, so other initial binding factors may be important. The OmpA outer membrane protein of *E. coli* acts as an adhesion and invasion factor as well as it helps in boilfilm formation. These functions are due to four short protein loops that come from the protein outside of the cell (Smith *et al.*, 2007). H7 flagellum plays an important role in EHEC O157:H7 initial colonization to bovine terminal rectum (McNeilly *et al.*, 2008). F9 fimbriae enhance the initial adherence of EHEC strains O26:H- and O157:H7 to bovine epithelial cells and bovine gastrointestinal tissue (Naylor *et al.*, 2005b) and were identified as important for cattle colonisation using signature-tagged mutagenesis studies (Low *et al.*, 2006). Curli are heteropolymeric proteinaceous filamentous appendages that are composed of a large (CsgA) and small (CsgB) subunits that affect on the binding activities of several biofilm-forming *E. coli* strains, including STEC (Van Houdt & Michiels, 2005). Although these gene clusters are very preserved among *E. coli* strains, many laboratory strains do not create curli due to the silencing of the csgD promoter (Van Houdt & Michiels, 2005). It has been shown that among clinical *E. coli* isolates, curli are expressed in most EHEC and ETEC strains, but not in EIEC or EPEC strains, signifying a specific role in pathogenicity (Van Houdt & Michiels, 2005).

### 1.1.9h.3 Attaching and effacing (A/E) lesions and intimate attachment

EHEC and EPEC intimately adhere to host cells forming characteristic histopathological lesions in the intestine, known as attaching and effacing (A/E) lesions (Andrade & DaVeiga, 1989; McDaniel & Kaper, 1997; Frankel *et al.*, 1998; Jerse *et al.*, 1990). Adherence of EHEC and EPEC to epithelial cells causes a variety of signal transduction pathways in epithelial cells. Both EHEC and EPEC can express a molecular syringe that can export proteins from the bacteria into the host cell. This is called a type III secretion system (TTSS) (Fig. 1.5). ‘Effector’ proteins are injected. One of the first to be characterized was the translocated intimin receptor

(Tir) (Kenny *et al.*, 1997). When Tir is translocated into host cells, it acts as a receptor for the bacterial surface protein intimin (Fivaz & van der Goot, 1999). Intimin can also bind to an eukaryotic cell receptor called β1 integrin, which travels from the basolateral surface to the apical cell surface, to act as receptor to intimin (Gullberg & Söderholm, 2006). Nucleolin, a protein included in cell growth regulation that can be cell surface-expressed, can also act as eukaryotic intimin receptor (Sinclair & O'Brien, 2002; Humphries & Armstrong, 2010). Once the bacteria intimately attach to intestinal epithelial cells, they cause marked cytoskeletal changes including the accumulation of polymerized actin directly beneath the adherent bacteria (pedestal formation). The intestinal microvilli are effaced (lost) and bacteria are attached on pedestal-like structure of actin (Moon *et al.*, 1983). Furthermore, other translocated effectors activate host signaling pathways leading to increases in intracellular calcium levels, chloride ion secretion, disruption of tight junctions (Simonovic *et al.*, 2000), and interference with phagocytosis (Vallance & Finlay, 2000). It is interesting that several intimin-negative EHEC serotypes, such as O91:H21, O113:H21, and O121:H19 can also cause HUS or bloody diarrhoea as well as outbreaks of intestinal disease (Paton *et al.*, 2001; Jelacic *et al.*, 2003). The pathomechanism by which these atypical EHEC strains cause disease is not well known. Such strains appear to be more frequent outside Europe, specifically in the United States (O121:H19) (Jelacic *et al.*, 2003) and Australia (O91:H21 and O113:H21) (Paton *et al.*, 2001). In Germany, intimin-negative strains of the serotypes O91:H21 and O113:H21 also cause HUS.

### 1.1.9h.4 Type III secreted effector proteins

EPEC and EHEC secrete many effector proteins using TTSS; some of them are encoded within LEE but a others are not, for example EspC and the glycolytic pathway enzyme glyceraldehyde-3-phosphate dehydrogenase. The *espC* gene that is located within a second PAI, encodes immunoglobulin A protease-like protein. This protein has no role in adherence or signal transduction. It can be secreted independently from TTSS (Clarke *et al.*, 2003).The most important LEE encoded proteins are the translocated intimin receptor (Tir), EspB, Map, EspF, EspH, and EspG. These proteins are injected directly into host cells. Tir and EspB play crucial

role for A/E lesion formation in EPEC (Kenny & Jepson, 2000; Elliott *et al.*, 2001; Tu *et al.*, 2003). Tir also links extracellular EPEC/EHEC to the cell cytoskeleton, where the extracellular domain of Tir binds intimin (an outer-membrane bacterial adhesin protein) ( Kenny *et al.*, 1997; Frankel *et al.*, 2001), while the intracellular domain interacts with a number of cytoskeletal proteins (Hartland *et al.*, 1999; Kenny, 1999). Inside the eukaryotic cell EPEC tir is phosphotyrosonated by phosphotyrosin and binds to the NCK recruit it to polymerize the actin beneath the attached bacteria and the formation of A/E lesions. EHEC tir is unable to recruit NCK pathway for actin assembly. However, EspFU, a TTSS effector associate with tir binds NWASP and stimulates NCK-independent actin assembly pathway (Campellone *et al.*, 2004).

EspB acts as a component of TTSS translocon; it forms the pore of the TTSS needle so it is important for translocation of other effectors. It also functions as an effector protein that is involved in actin modulation and pedestal formation (Taylor *et al.*, 1998; Wolff *et al.*, 1998). EspB binds to the actin-interacting region of myosin-1c and inhibits the interaction between myosin-1c and actin. This leads to the inhibition of phagocytosis (Iizumi *et al.*, 2007). EspJ targets an essential host molecule or complex normally involved downstream of these two phagocytic receptors. It inhibits both FcγR- and complement receptor type 3 (CR3) mediated phagocytosis (Marchès *et al.*, 2008). Map is targeted to and interferes with mitochondrial function and also promotes rapid filopodia formation ( Kenny & Jepson, 2000; Kenny *et al.*, 2002). The function of EspG is not known, although homology with *Shigella* VirA, which interacts with tubulin and causes microtubule instability, suggests EspG may play a similar role in EPEC/EHEC infection (Elliott *et al.*, 2001; Yoshida *et al.*, 2002). EspH is a modulator of the host actin cytoskeleton affecting filopodia and pedestal formation (Tu *et al.*, 2003). Thus, a number of EPEC/EHEC effectors have been shown to modulate the host cytoskeleton and may be important in the events leading to A/E lesion formation.

### 1.1.9h.4a EspF

EspF is a focus of this PhD research. It is a multi-functional effector protein encoded by the LEE Pathogencity Island and injected into the host cell through the TTSS.

EspF is a proline-rich protein. Based on the presence of proline-rich repeats (PRRs) and SH3 binding motifs, it was anticipated that EspF could be an effector protein and subsequent secretion, and translocation assays revealed this to be the case (McNamara *et al.*, 2001).

EspF requires a chaperone, CesF to be translocated into host cells (Clarke *et al.*, 2003). It is involved in disruption of tight junctions (TJ), possibly through manipulation of the actin cytoskeleton (McNamara *et al.*, 2001). It was discovered that EspF does not disrupt TJ in intimin negative bacteria, this indicates that the intimin-Tir interaction is necessary to activate TJ disruption by EspF (Dean & Kenny, 2004; Alto *et al.*, 2007). EspF plays a crucial role in the loss of transepithelial electrical resistance, and loss of epithelial barrier function, resulting in increased monolayer permeability by its role in redistribution of tight junction proteins, including occludins ( Guttman *et al.*, 2006; Nougayrède *et al.*, 2007). Loss of TER induced by EHEC was shown to be slower and more modest than that induced by EPEC, and this is considered to be due to variation in the EspF protein between the *E. coli* pathotypes (Viswanathan *et al.*, 2004a).

It has also been shown that EspF causes elongation of the intestinal brush border microvilli possibly by inducing actin assembly (Shaw *et al.*, 2005; Marchès *et al.*, 2008). EPEC EspF plays a role in effacement of microvilli around the attached bacteria (Dean *et al.*, 2006). EspF interferes with water and ion re-absorption from the intestine (by binding) and inhibits the action of the sodium hydrogen exchanger NHE3 or the sodium glucose co-transporter SGLT1 (Hodges *et al.*, 2008; Dean *et al.*, 2006).

EPEC EspF is targeted to mitochondria, the N-terminal region of EspF acts as a mitochondrial import signal; EspF causes an increase in mitochondrial membrane permeabilization in addition to release of cytochrome C from mitochondria into the cytoplasm and with caspase-9 and caspase-3 cleavage. This process is mediated by EspF binding and disruption of the anti apoptosis host protein Abcf2 within the mitochondria leading to mitochondria and apoptotic cell death (Nougayrède & Donnenberg, 2004; Nagai *et al.*, 2005; Nougayrède *et al.*, 2007). The amino acid leucine at position 16 is the key amino acid residue in mitochondrial targeting (Nagai *et al.*, 2005). EspF is cleaved within the mitochondrial fraction of EPEC-infected

cells, resulting in two distinct forms with the approximate sizes ~28 kDa and ~17 kDa (Nougayrède & Donnenberg, 2004; Dean *et al.*, 2010). Processing of EspF is entirely dependent on an intact mitochondrial membrane potential as the smaller cleaved form is not present in cells treated with the mitochondrial inhibitor valinomycin (Dean *et al.*, 2010).

EspF is targeted to the nucleolus, the site for ribosome biogenesis, and disrupts its function by relocating nucleolin from the nucleolus to the cytoplasm (Dean *et al.*, 2010). EspF disrupts cell integrity by interacting with Cytokeratin 18 a eukaryotic intermediate filament, making it more soluble (Viswanathan *et al.*, 2004b).

The PRRs of EspF bind to Sorting Nexin 9 (SNX9) via its SH3 amino terminal region (Marchès *et al.*, 2006; Alto *et al.*, 2007). Each PRR contains two putative overlapping SH3 binding domains with the consensus PxxP motif (Mayer, 2001). SNX9 is essential for formation of clathrin-coated pits (CCPs) by interacting with β2-appendages of adaptor protein complex 2 (AP-2), at the late stages of vesicle formation. SNX9 also interacts with clathrin and dynamin-2, two other vital molecules in the endocytic process (Lundmark & Carlsson, 2003; Soulet *et al.*, 2005). SNX9 has two lipid contact domains; a phospholipid-binding region termed the phox (PX) domain, the second is a putative Bin/Amphiphysin/Rvs (BAR). The BAR domain can reconfigure lipid vesicles or sheets into membrane tubules (Peter *et al.*, 2004). SNX9 also has an N-terminal Src homology-3 (SH3) protein active site that was recently shown to bind WASP (Badour *et al.*, 2007) and to the activate dynamin at clathrin-coated pits (CCPs). Therefore, SNX9 is a vital factor in re-modelling the membrane and cytoskeletal apparatus during endocytosis (Soulet *et al.*, 2005; Alto *et al.*, 2007).

N-WASP plays an important role in endocytosis, by remodelling membranes through the spatiotemporal control of several phospholipids and F-actin binding proteins (Merrifield *et al.*, 2002; Kaksonen *et al.*, 2006; **Holmes *et al.*, 2010**). The PRRs also contain a functional N-WASP binding motif towards the C-terminal end of each repeat (Alto *et al.*, 2007). A direct interaction between actin and EspF from the rabbit EPEC strain E22 (Peralta-Ramirez *et al.*, 2008) was revealed to contribute to the extent and size of actin-based pedestals in EPEC-infected cells most probably

through its direct alteration of the cytoskeleton, even though no role for N-WASP was demonstrated (Peralta-Ramirez *et al.*, 2008).

EPEC EspF has been shown to play an important role in inhibition of bacterial uptake by macrophages (Quitard *et al.*, 2006; Martinez-Argudo *et al.*, 2007; Marchès *et al.*, 2008). EPEC prevents macrophage phagocytosis via inhibition of the phosphatidyl inositol-3 (PI3) kinase dependent pathway of bacterial uptake using TTS protein EspF (Celli *et al.*, 2001; Quitard *et al.*, 2006).

The intestinal mucosa is generally impermeable to macromolecules and microorganisms, except at Peyer's patches, where the lymphoid follicle-associated epithelium (FAE) contains M cells that transport antigens and microorganisms to antigen presenting cells in the sub-mucosa (Kerneis *et al.*, 1997). Potentially as gastrointestinal FAE is less well protected in terms of a mucus barrier, these sites are often targeted by bacteria for colonization and invasion. Some EPEC strains exhibit a tropism for the specialized follicle-associated epithelium (FAE) overlying lymphofollicles in the gut, but resist translocation through the intestinal lumen. This inhibition of translocation resembles the capacity of EPEC to inhibit phagocytosis by macrophages. This inhibition is dependent on the expression of a bacterial type III secretion system (TTSS) by EPEC and translocation of EspF (Martinez-Argudo *et al.*, 2007).

**Table 1.1 Bacterial effectors implicated in epithelial cell function** (taken directly from Prof. David Gally Teaching lectures).

| Host cellular processes | Type III Secreted effector proteins |
|---|---|
| **Intracellular trafficking** | 1. Inhibition of vesicle trafficking and protein/peptide export (NleA) (Kim *et al.*, 2007).<br>2. G protein antagonist vacuole positioning (SifA, SopE) (Jackson *et al.*, 2008) |
| **Cytoskeleton** | 1. Actin polymerization for intimate attachment (Tir/EspFu) (Campellone *et al.*, 2004).<br>2. Microvillus effacement and inhibition of phagocytosis by blocking myosin interactions with actin (EspB) (Iizumi *et al.*, 2007).<br>3. Inhibition of endocytosis via SNX9 and N-WASP binding (EspF) (this study).<br>4. Prevent actin depolymerization, binding to F-actin to block protein function (SipA) (Zhou *et al.*, 1999). SipC and SopB in *Salmonella* invasion (McGhie *et al.*, 2004)<br>5. Actin-based motility (VirG/ActA) (Suzuki *et al.*, 1996) |
| **Protein stability** | Deubiquitinating cysteine Proteases (SseL) (Rytkönen *et al.*, 2007) Cysteine peptidase-like activity degrades α-tubulin (VirA) Ubiquitin ligases (SopA, IpaH) (Rohde *et al.*, 2007) |
| **Inflammation** | 1. Kinase manipulation Acetylation of specific kinasesat phosphorylation sites (YopH, AvrA). (Orth, 2007); Du,and Galán 2009 (Du & Galán, 2009); NleE/NleB, (OspZ and OspF/G) on NFkB activation pathways (Newton *et al.*2010)<br>2. Shielding of an inflammatory<br>Factor (YopB) by another (YopK) (Viboud *et al.*, 2003) |
| **Cell turnover & apoptosis** | 1. OspE interaction with ILKinhibits turnover of focal adhesion points to ECM; EPEC Cif (Nougayrede *et al.*, 2001; Kim *et al.*, 2009)<br>2. NleH inhibits apoptosis working with Bax inhibitor-1 & so inhibit caspase-3 activation (Hemrajani *et al.*2010).<br>SopB on Akt (Knodler *et al.*, 2005)<br>3. IpaB acts on Mad2L2 increases APC activity to block the cell cycle (Iwai *et al.*, 2007). |
| **G protein signalling** | Activity on guanine nucleotide-binding proteins, such as Rho and Rac (YopE, SopE, SptP, SopB) (Galán & Zhou, 2000; Murli *et al.*, 2001; Buchwald *et al.*, 2002; Aepfelbacher *et al.*, 2004) this activate actin dependent bacterial internanalization |

## 1.2 The genus *Salmonella*

*Salmonella* represents an important group of food borne pathogens. They are Gram negative facultative anaerobic bacilli, ferment glucose, and are oxidase negative. The

majority of *Salmonella* species produce hydrogen sulfide that can readily be detected by growing the bacteria on media containing ferrous sulfate, such as triple sugar iron. The nomenclature of *Salmonella* is complex (Tindall *et al.*, 2005) and is currently considered to contain 2 species: *enterica* and *bongori*. *Salmonella enterica* is divided into 6 subspecies: *enterica* (I), *salamae* (II), *arizonae* (III), *diarizonae* (IV), *houtenae* (V), and *indica* (VI). Each of these sub-species have multiple serovars (Table 1.2), defined by the presence of three major antigens; the "H" or flagellar antigen (some time it is two phases, phase 1 and 2 especially for *S.* Typhimurium), the "O" or somatic antigen, and the "Vi" or capsular antigen (referred to as "K" in other *Enterobacteriaceae*), (Lin *et al.*, 2001).

**Table 1.2 *S. enterica* subspecies and their serovars (**taken directly from Grimont, 2007).

| **Species** | **Subspecies** | **Number of Serovars** |
|---|---|---|
| *S. enteric* | | *2557* |
| | *Enterica* | 1531 |
| | *Salamae* | 505 |
| | *Arizonae* | 99 |
| | *Diarizonae* | 336 |
| | *Houtenae* | 73 |
| | *Indica* | 13 |
| *S. bongori* | | *22* |
| Total number of serovars | | 2579 |

### 1.2.1 Epidemiology of *Salmonella:* disease transmission

The majority of cases of human infections by *Salmonella* resulted from the ingestion of contaminated foods of animal origin (Mead *et al.*, 1999). Wild-living birds may play an important role in spreading *Salmonella* to humans and to various animal species, via contamination of the surrounding environments, such as water, fresh fruits, and vegetables (Bodey *et al.*, 1986; Mahon et al., 1993). Direct person-to-person transmission through faecal-oral contamination is also possible (Stone *et al.*, 1993).

### 1.2.2 Diseases caused by *Salmonella*

*Salmonella* infections can simplistically be divided into two categories: (1) those that are relatively host specific and often quite severe leading to systemic spread and sometimes the death of the animal or human; and (2) those that can cause gastrointestinal infections in multiple hosts. The first category includes *Salmonella* Typhi in humans and *S.* Dublin in cattle, whereas the second category includes the significant public health pathogens *S.* Typhimurium and *S.* Enteritidis. These can be both transmitted to humans from contaminated meat products, with *S.* Enteritidis being more commonly associated with poultry products and *S.* Typhimurium with pig and cattle products (Wallis, 2005).

### 1.2.2a Disease in human

The disease caused by *Salmonella* is called Salmonellosis. Salmonellosis has been defined as one of the main public-health problems worldwide. *Salmonella enterica* serovar Typhimurium is one of the serotypes most widely associated with human infections. *Salmonella* Typhimurium is transmitted from contaminated meat products. Salmonellosis symptoms are usally associated with fever, abdominal pain, diarrhoea, nausea, and sometimes vomiting. Furthermore, severe dehydration caused by the gastrointestinal infection can be life-threatening in young children and the elderly (Bodey *et al.,* 1986; Kapperud *et al.,* 1998).

### 1.2.2b Disease in birds

Poultry are generally infected with a wide range of *Salmonella* serovars. *S.* Gallinarum and *S.* Pullorum are the most widespread serotypes concerned with poultry diseases. These serovars cause fowl typhoid and pullorum disease respectively. The disease affects chicken and turkeys and it can also affect game birds. *S.* Pullorum usually infects young birds that show different clinical signs including white diarrhoea, lameness, laboured breathing, and occasional blindness. It causes mortality and morbidity that could reach 100%. Adult birds are usually infected subclinically, and a drop in egg production, fertility or hatchability may occur (Kapperud *et al.*, 1998). Fowl typhoid characterised clinically by elevated temperature 44-45$^{o}$C, pale and shrunken combs, greenish-yellow diarrhoea, depression, and/or anaemia (Pattison *et al.*, 2008).

*Salmonella* enteritidis may accidentally infect poultry. The infection is usually asymptomatic and the chicken become chronic carrier (Lister, 1988). However, it may produce clinical disease in chicks up to six weeks of age and occasionally in adult laying birds. Affected birds are depressed, disinclined to move, and usually have diarrhoea (Wray et al., 1996). Mortality may be high in chicks under one week of age. Older chicks may show uneven growth and stunting. The diseased birds may show lesions of pericarditis and septicaemia (Lister, 1988).

The symptoms of *S.* Typhimurium infection in poultry are nearly the same as to those caused by *S. enteritidis.* However, clinical disease due to *S.* Typhimurium is rarely seen in birds over month of age. Infection of adult chickens with *S.* Typhimurium is usally without clinical manifestation ( Wray et al., 1996).

### 1.2.2c Disease in Cattle

Cattle are primarily affected by serovars *S.* Dublin and *S.* Typhimurium. Salmonellosis in cattle is characterised by pyrexia, anorexia, and reduced milk yield followed by diarrhoea. *S.* Dublin could result in a systemic disease and generally results in abortion in pregnant cows. Most cases of *S.* Dublin infection have been encountered in young calves (Sojka *et al.*, 1977). Cattle can be infected with *Salmonella* without displaying the clinical symptoms. These animals are referred to as active carriers. The active carriage of *Salmonella* occurs before clinical enteritis or

systemic infection. Infected animals may secrete *Salmonella* for years or even life. Active carriers may excrete *Salmonella* continuously in concentrations greater than $10^5$ CFU/g of faeces and thus can be detected by routine bacteriological examination (Pullinger *et al.*, 2007).

Cattle infected with *Salmonella* exhibiting clinical symptoms can excrete up to $10^8$ CFU/g of faeces. These animals contaminate the surrounding environment potentially causing infection of other livestock species in the area. Cattle are most likely to be infected by the oral route but infection may also occur via the respiratory tract or conjunctiva (Pullinger *et al.*, 2007)..

### 1.2.2d Disease in pigs

Pigs are affected by a host-restricted serovar of *Salmonella*, *S.* Choleraesuis which results in systemic disease. In the 1950's and 1960's *S.* Choleraesuis was the predominant serovar isolated from pigs (Sojka *et al.*, 1977). In a European Food Safety Authority (EFSA) survey between 2006 and 2007, 87 different serotypes of *Salmonella* were found to affect pigs across Europe. *S.* Typhimurium was the most common serotype of *Salmonella* isolated with ~40% of the positive pigs infected with *S.* Typhimurium according to EFSA.

*S.* Typhimurium typically causes disease in pigs aged between 6 and 12 weeks and rarely in adult pigs. Older animals frequently have subclinical infections which result in high transmission rates to surrounding animals if not detected. The initial clinical signs of infection include watery diarrhoea. Pigs become anorexic, lethargic, and febrile following infection; however mortality is usually low. The mesenteric lymph nodes are usually swollen and intestinal necrosis is often seen as distinct button ulcers (Pullinger *et al.*, 2007).

### 1.2.3 Factors affecting *S.* Typhimurium adherence and colonisation

#### 1.2.3a Fimbria

Fimbriae are proteinaceous appendages in many Gram-negative bacteria. These appendages range from 3-10 nanometers in diameter and can be up to several micrometers long. It is also thinner and shorter than a flagellum. Bacteria use the fimbriae to help in adherence to one another and to the host cells and some inanimate objects. A bacterium can have up to 1,000 fimbriae. The fimbriae are only visible with the use of an electron microscope. Fimbrial adhesins of *S*. Typhimurium play an important role during bacterial attachment to and invasion of the intestinal mucosa (Baumler *et al.,* 1997). Type 1 fimbriae are essential for *Salmonella* adhesion to various kinds of epithelial cells (Baumler *et al.,* 1996). Mutation of in lpfC, the gene encoding the PE fimbriae outer membrane usher reduce the ability of *S.* Typhimurium to attach to mice peyer's patches. This mutant also lost the ability to destroy M cells of the follicle-associated epithelium. (Bäumler *et al.,* 1996)**.**

#### 1.2.3b Type III secretion system

*Salmonellae* encode two distinct virulence-associated TTSS within *Salmonella* pathogenicity islands 1 and 2 (SPI-1 and SPI-2) that are measured to be required in different aspects of *Salmonella*'s pathogenicity, although there are many reports of overlap in their function. These TTSS inject various *Salmonella* effectors directly into the host cell (Hernandez *et al.*, 2004). The SPI-1 TTSS is active on contact with host cells. SPI-1 is required for bacterial invasion of epithelial cells. SPI-1 TTSS translocates at least eight effector proteins that mediate several effects, including membrane ruffling, bacterial invasion, cell death, and trans-epithelial migration of neutrophils (Miao & Miller, 2000). SPI-1 is expressed in growth conditions that are consistent with the intestinal environment. This regulation depends on a variety of transcription factors encoded within SPI-1 (Miao & Miller, 2000).

In contrast, SPI-2 expression is induced in phagocytosed bacteria to defend the *Salmonella*-containing vacuole (SCV) from lysosome fusion and the anti-bacterial factors present in lysosomes. This TTSS helps in the spread of infection and causes

apoptosis of infected cells so it is essential for survival/replication in phagocytic cells (Miao & Miller, 2000; Galán, 2001; Waterman & Holden, 2003).

### 1.2.3c *Salmonella* secreted effector proteins

The initial site of *S.* Typhimurium infection occurs in the distal small intestine. Here the bacteria induce their own uptake, invading non-phagocytic cells by manipulating the host actin cytoskeleton resulting in intense membrane ruffling and the formation large macropinosomes. The bacteria do this using a range of effector proteins injected via the TTSS encoded by *Salmonella* pathogenicity island 1 (SPI-1) (Hernandez *et al.*, 2004; Ly & Casanova, 2007).

The specialized antigen-sampling intestinal M cells are an important route of *Salmonella* invasion during the early stages of infection through inducing ruffle-like entry foci in M cell membrane and cytoskeletal rearrangements (Jepson & Clark, 2001). *S.* Typhimurium efficient M-cell invasion correlates with the induction of cytotoxicity and are accompanied by M-cell destruction and loss of adjacent regions of the FAE (Autenrieth et al. 1996; Daniels *et al.*, 1996). The mechanisms by which *Salmonella* invade the intestinal epithelium are being clarified; *in vitro* studies have identified several genes which are required to optimize *S.* Typhimurium invasion of epithelial cells. SPI-1- encoded genes include a type III protein secretion system together with several of its target proteins that are required for bacterial invasion (Clark *et al.*, 1998). This including SipA, SipC, SopB/SigD, SopE, SopE2, and SptP that are known to alter the host cell actin cytoskeleton to drive internalization of *Salmonella* (Ly & Casanova, 2007).

The *Salmonella* invasion protein A (SipA), a component of *Salmonella* Pathogenicity Island 1 (SPI-1), is vital for efficient invasion of cultured cells. It binds host F actin, enhances its polymerization near the adherent extracellular bacteria, and contributes to cytoskeletal rearrangements that internalize the pathogen (Bourdet-Sicard & Van Nhieu, 1999; Lilic *et al.*, 2003). Once *Salmonella* passe through M cells, it binds to the surface of macrophages, induces ruffle-like entry foci in the macrophage surface and utilize TSS to inject *Salmonella* outer proteins E (SopE) and SopE2 (Stender, Friebel et al. 2001). SopE has GDP/GTP exchange activity specific for Rac and Cdc42 (Friebel et al. 2000). SopE is required for invasion of macrophages and

capable entry into epithelial cells by alteration of the actin network. It also regulates nascent *Salmonella*-containing vacuoles (SCV). SigD/SopB is an effector protein that is encoded by SPI5 and is injected directly into the host cell using SPI-1. SopB needs the SigE chaperone for its translocation. It is a phosphatidylinositol phosphatase that hydrolyzes phosphatidylinositol 3, 4, and 5 (Norris *et al.*, 1998). This manipulates the level of endosomal phosphoinositides leading to recruitment of sorting nexin 1(SNX1) to SCV and results in the formation of extensive, long-range tubules termed 'spacious vacuole-associated tubules (Bujny *et al.*, 2008; Braun *et al.*, 2010). These tubules enhance bacterial replication within SCVs (Bujny et al., 2008). SigD/SopB is also concerned with invasion of the host cell. It binds to the rab5 GTPase, a central regulator of membrane fusion in early endocytosis. SigD is essential for Akt activation as well. Akt stimulates cellular signals that are essential and sufficient for host cell survival. Thus, it is possible that *Salmonella*-induced activation of Akt in epithelial cells would increase host cell survival, perhaps allowing the pathogen a greater intracellular time frame within which to replicate. SigD activates AKT by its phosphorylation at two sites, Ser473 and Thr308 (Steele-Mortimer *et al.*, 2000). Activation of AKT has no function in cell invasion (Steele-Mortimer *et al.*, 2000). Following translocation, the host cell membrane returns to normal and the bacteria reside within SCV. The nascent vacuole is enormous relative to the size of the bacterium and has been called a "spacious phagosome" that undergoes a maturation process which allows the bacteria to multiply (Ly & Casanova, 2007). The SCV is modified by the bacteria to prevent maturation into, or fusion with lysosomal compartments, thus providing them with protection from lysosomes. During a lag period of 2–3 h, the bacteria modulate their vacuolar environment before they start to replicate (Hernandez *et al.*, 2004b). *Salmonella*-induced apoptosis contributes to the break out of intracellular bacteria from exhausted host cells following nutrient deprivation and the stop of bacterial replication. Induction of apoptosis is performed via SPI-1 TTSS effector SipB that binds to and activates caspase-1 which mediates apoptosis, and induces the release of IL-1β which is required for inflammation (Hersh *et al.*, 1999a; Coburn *et al.*, 2007).

TTSS-2 effectors modulate host cell functions, especially cytoskeletal organization and membrane trafficking, including inhibition of various aspects of endocytic

trafficking, an avoidance of NADPH oxidase-dependent killing, the induction of a delayed apoptosis-like host cell death (Kuhle & Hensel, 2004). SPI-2 mutants fail to replicate within macrophages and epithelial cells (Cirillo *et al.*, 1998).

The mechanism by which SPI-2 promotes bacterial virulence is unknown. Although a number of translocated effectors of SPI-2 TTSS have been identified, it has been shown to promote intracellular survival by inhibition of phagosome–lysosome fusion (Brumell & Grinstein, 2004).

*Salmonella*-induced filaments (Sifs), are intracellular projections that extend from the SCV to the cytoplasm of infected epithelial cells or macrophages (Knodler *et al.*, 2002). These structures are associated with cell organelle trafficking (Brumell *et al.*, 2002). They are also necessary for the binding of SCV with the late endosomal compartments. Although the advantages of doing this function are unclear, but are all potentially about controlling trafficking, position, and maturation of the SCV (Brumell *et al.*, 2001).

SpiC and SifA are *Salmonella* Pathogenicity Island 2 (SPI-2) TTSS effectors that are injected from the bacteria in the vacuole into the cytosol outside the vacuole. SpiC inhibits fusion between the SCV and lysosomes (Shotland *et al.*, 2003). It stimulates actin polymerisation that results in cytoskeletal rearrangements (Hayward & Koronakis, 1999). SifA controls the maintenance of the SCV binds F-actin and inhibits its polymerisation, enhances the efficiency of SipC and is necessary for the formation of Sifs (Gorvel & Meresse, 2001; Boucrot *et al.*, 2005; Waterman & Holden, 2003).

**Table 1.3 Role of SPI-1 and SPI-2 effectors in *Salmonella* pathogenesis**

| SPI-1 effector protein | Function | References |
|---|---|---|
| SipA | It enhances actin polymerization near the adherent extracellular bacteria, and contributes to cytoskeletal rearrangements that internalize the pathogen. | (Bourdet-Sicard & Van Nhieu, 1999; Lilic *et al.*, 2003a). |
| SipB | Induction of apoptosis via activation of caspase-1 | (Hersh *et al.*, 1999b) |
| SopE + E2 | It plays a role in recruitment of Arp2/3 to membrane ruffles. Required for optimal invasion of cultured epithelial cells. | (Stender *et al.*, 2000) |
| SopB | It is encoded by SPI-5, dependent on SirA, inositol phosphate phosphatase that hydrolyzes phosphatidylinositol 3,4,5-triphosphate, an inhibitor of chloride secretion. It mediates fluid secretion by increasing chloride secretion. it also affects host cell signalling pathways that may be concerned in regulation of cytokine expression such as activation of the serine-threonine kinase Akt. | (Eckmann *et al.*, 1997; Steele Mortimer *et al.*, 2000b) |
| SopD | It has additive effect to SopB in the induction of enteritis | (Jones *et al.*, 1998) |
| SopA | Involved in the induction of neutrophil transepithelial migration | (Wood *et al.*, 2000) |
| **SPI-2 effector protein** | **Function** | **References** |
| SipC | It nucleates actin polymerisation which results in cytoskeletal rearrangements. It also prevents the fusion of SCV with endosomes and lysosomes. | (Hayward & Koronakis, 1999; Uchiya *et al.*, 1999) |
| SifB, SseJ, SseF, SseG and PipB | are localized to Sifs and are required for their formation | (Guy *et al.*, 2000; Waterman & Holden, 2003) |
| SopD2 | required for Sifs formation | (Jiang *et al.*, 2004) |
| SifA | It prevent binding of SCV with lysosomes and is important for binding of SCV with the late endosomal compartments by inducing expression of other sifs | (Brumell *et al.*, 2001) |

## 1.3 Intestinal epithelium and M cells

The intestinal immune system must cope with many infectious and toxic assaults that may damage the epithelium. The immune system must also recognize epithelial cell transformation. Simultaneously, the intestinal immune system must ignore the collection of commensal organisms and dietary antigens that do not harm the host. To deal with this challenge, the gut-associated lymphoid tissue has developed several important modifications in relation to antigen processing to manage its organ-

specific responsibilities. These include flattened epithelial microfold cells that are a kind of intestinal epithelial cells in the follicle-associated epithelium of Peyer's patches (Gebert *et al.*, 1999).

The epithelium lining the intestinal mucosa is composed of multiple cell types including enterocytes, enteroendocrine, goblet, and Paneth cells. These cells derive through asymmetrical division, migration, and differentiation from pluripotent stem cells. An additional specialised epithelial cell type, termed M-cells, "membranous" or "microfold" cells, can transport antigens and micro-organisms into underlying lymphoid tissues and are associated particularly with epithelium overlying the gut-associated lymphoid tissue Peyer's patches (Gebert *et al.*, 1999). This is referred to as follicle-associated epithelium (FAE), the site of active immunological function. In contrast to villous epithelium, FAE contains very few or no goblet or enteroendocrine cells, fewer defensin- and lysozyme-producing Paneth cells, and lower membrane-associated hydrolases. FAE is devoid of polymeric immunoglobulin receptor that mediate the transcellular transport of immunoglobulin A & M and therefore not able to transport protective IgA from the interstitium to the lumen. The glycocalyx (glycoprotein secteted by the epithelial cell to coat the epithelial surface) of FAE presents array of glycoconjugates different from the surrounding villus epithelium (Gebert *et al.*, 1994; Giannasca *et al.*, 1994; Giannasca *et al.*, 1999; Owen, 1999)**.** All these features tend to promote local contact of pathogens and intact antigens with FAE, one of its functions.

The FAE M-cells sample antigens from the lumen directly to the intraepithelial lymphoid cells and to sub-epithelial organised lymphoid tissue, initiating a protective immune response (Giannasca *et al.*, 1994). M-cells generally lack an organised brush border and a well-defined filamentous brush border glycocalyx (FBBG). Their apical surfaces have only a thin (20-30nm) glycoprotein coat (Frey *et al.*, 1996) in comparison to villus enterocytes which have a highly differentiated structure consisting of rigid, closely packed microvilli coated with a 400-500nm thick FBBG composed of highly glycosylated transmembrane mucins. Therefore, though the M-cells are crucial for induction of protective mucosal immune responses, they can be

considered as potentially less well-protected sites that act as entry portals to many enteropathogens.

Human M-cells covered by small microfolds over their apical surface, instead of microvilli. These microfolds are similar to ruffles on the edges of a macrophage. They can enfold a wide range of particles that reach their surfaces. However, in mice M-cells are characterized by the presence of small, irregular microvilli instead of microfolds. The ultrastructural morphology by transmission electron microscopy, revealed that murine and human M-cells, are invaginated to form pockets for lymphocytes that directly engulf the antigen transcytosed across M cell (Ogra, 1994). Murine and human M-cells expressed glycoprotein 2 (GP2) receptors on the surface plasma membrane and it is consider to be a specific marker for that cell in those species (Hase *et al.*, 2009).

M cells bind to adjacent cells by tight junctions. M cells have lateral inter-digitations, oriented along a basal lamina, and are epithelial cells by all characteristics and not posses any features of connective or mesenchymal cells. In fact, M cells have some prelysosomes and a few lysosomes may be able to digest some of the material they engulf (Owen, 1994).

### 1.3.1 Development of M cells

The origin of both villous epithelium and FAE is from crypt stem cells. The intestinal crypts can be simplistically considered as formed from a unit of pluripotent stem cells that are anchored together as a ring, which gives rise to multiple cell types that migrate upward in columns onto several adjacent villi (Bye *et al.*, 1984). FAE is formed from a mixture of migrating cells from 12 or more follicle-associated crypts (Savidge and Smith, 1995). The follicle-associated crypts can be differentiated through two directions. Cells located on one side of the crypt migrate and differentiate into villous epithelium with absorptive enterocytes, goblet, and enteroendocrine cells, whereas cells on the follicle side of the crypt move onto the dome and differentiate into FAE and M-cells (Bye *et al.*, 1984; Gebert *et al.*, 1999; Kraehenbuhl & Neutra 2000).

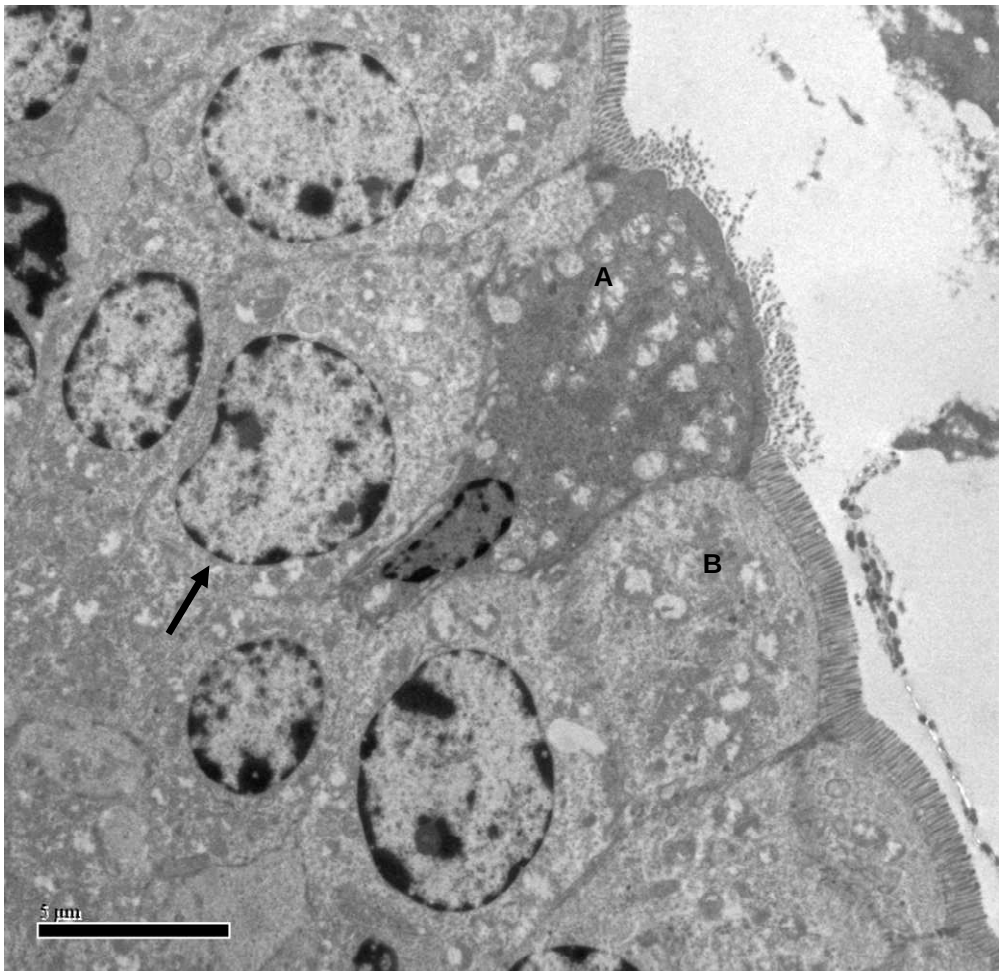


**Fig. 1.3 The relation of M cell to enterocyte and antigen presenting cells in the follicle associated epithelium (FAE).** M cell (A) is charcterized by presence of fewer and shorter microvilli. The antigen presenting cells are invaginated in middle hollow of M cell (Arrow). The absorbtive enterocte (B) is charcterized by long and thick brush border.

The mouse FAE specimens were viewed in a CM 120 transmission electron microscope (TEM).

There are various hypotheses regarding the ontogeny of FAE and M-cells: (i) M-cells may originate in the crypts as a distinct cell lineage from specific progenitor stem cells via an independent differentiation programme (Bye *et al.*, 1984; Gebert *et al.*, 1999); (ii) they may derive from enterocytes under the influence of surrounding lymphoid micro-environment as enterocytes are subject to different signals from lymphocytes (T&B cells) that can play important roles in M cell differentiation in these follicles leading to production of M-like cells (Smith & Peacock, 1980; Freeman *et al.*, 1995; Savidge, 1996; Kerneis *et al.*, 1997); (iii) exposure to microbial pathogens in the lumen may induce enterocytes to specialise into M-cells (Savidge *et al.*, 1991; Borghesi *et al.*, 1996; Borghesi *et al.*, 1999; Meynell *et al.*, 1999); or (iv) they represent a transient phenotype of FAE enterocytes as they migrate from the crypts to the apex of the dome. Certain microbes appear to exploit the innate plasticity of epithelial cells to trigger their transformation into a M cell phenotype that suits their habitat or life style, for example, Campylobacter *jejuni* that increased cells expressing the M cell-specific marker, galectin-9, in Caco-2 monolayers. This coincided with decreased numbers of normal epithelial cells that stained enterocyte marker, Ulex europaeus agglutinin-1. Also, this culture was charcterised by reduced activities of enzymes classically related to absorptive enterocytes such as alkaline phosphatase, lactase, and sucrase. These cells are functionally similar to M cells (Kalischuk *et al.*, 2010).

Whatever the basis for their ontogeny, M-cells represent an important interface between immunogens in the gut lumen and the immune system. This is an intimate relationship as evidenced from work showing that FAE secrete certain homing chemokines that are involved in function and maintenance of organised mucosal associated lymphoid tissue.

### 1.3.2 Production of M cells *in vitro*

M cells arise from pluripotential epithelial stem cells in adjacent crypts and migrate to the FAE, where they differentiate into their distinctive phenotypes under the influence of the lymphotoxin-β receptor ( Kraehenbuhl & Neutra, 2000; Debard *et al.*, 2001). However, M cells develop in mice, which lack T and B lymphocytes,

indicating that lymphotoxin can arise from other sources (Debard *et al.*, 2001). Co-culture of Murine intestinal epithelial cell line (MIE) or Caco-2 cells with Peyer's patches or Raji B cells lead to differentiation of these epithelial cells to cells phenotypically and functionally resemble to M cells (Kerneis *et al.*, 1997; Verbrugghe *et al.*, 2006; Kanaya *et al.*, 2008).The receptor activator of NF-κB ligand (RANKL) is a cytokine expressed by subepithelial stromal cells (B lymphocyte) in peyer's patches immediately beneath FAE and received by its receptor RANK that is expressed throughout intestinal epithelial cells promoting differentiation of some intestinal epithelial to M cells (Knoop *et al.*, 2009).

### 1.3.3 Interaction of various enteric bacterial pathogens with M-cells

As gastrointestinal FAE is less well protected in terms of a mucus barrier, these sites are often targeted by bacteria for colonization and invasion. However, many virulent bacteria that do not function as intracellular pathogens use different mechanisms included secreted proteins to protect themselves from phagocytosis; some of these mechanisms require type III secreted effector proteins (Celli & Finlay, 2002).

The availability and accessibility of different glycoconjugates, potential ligands for the bacterial lectin-like adhesions, facilitates adherence of many enteric pathogens to FAE. The diversity of glycoconjugates and apical membrane proteins including β-1 integrins may allow M-cells in the FAE to sample a wide variety of microbes in the gut to induce protective immune responses (Gebert, 1997; Giannasca *et al.*, 1999). Although intact commensal bacteria are rarely taken up, numerous pathogens, including human immunodeficiency virus, reoviruses, *Vibrio cholerae*, and *Shigella* species, selectively adhere to M cells, possibly through specialized carbohydrate-binding mechanisms, and are transported without degradation (Owen *et al.*, 1986a; Owen *et al.*, 1986b; Owen, 1999). It is likely that adherence and uptake of micro-organisms by M-cells results from selective adherence to FAE and M-cells that involves a sequence of molecular interactions including initial recognition of M-cell surface oligosaccharides by bacterial lectin-like adhesins, followed by more intimate association that would require expression of other additional virulence determinants. Processing of M-cell surface molecules and recruitment of integral membrane proteins of M-cells to the site of attachment (Corr *et al.*, 2008).

Transcytosis by M cells is a highly specialized process. It depends upon the interaction of a specific receptor from the bacterial side with its target receptor on the host M cell. For example, *E. coli* or *Salmonella* Typhimurium were selectively co-localized with apical and endogenous GP2, while FimH mutants failed to be transcytosed across M cells. Transcytosis of naturally FimH- bacteria such as Yersinia was not affected in GP2 mutant mice as *Yersinia* use β-integrin as a receptor for interactions with M cells (Hase *et al.*, 2009).

Once a particle/antigen is translocated through an M-cell, it is immediately exposed to phagocytes and antigen-presenting cells. It was shown that the soluble GP2 opsonized the luminal FimH+ bacteria and increases antibody responses against these bacteria (Ermak *et al.*, 1990; Ermak *et al.*, 1994; Farstad *et al.*, 1994; Ermak *et al.*, 1995; Hase *et al.*, 2009).

### 1.3.3a *Yersinia*

*Yersinia* goes through the FAE, in the Peyer's patches of the ileum where M cells express β1 integrins on their apical surface. The β1 integrins act as a receptor for enteropathogenic *Yersinia* species (Clark *et al.*, 1998).

### 1.3.3b *Shigella*

The pathogenesis of *Shigella* depends on the ability of the bacteria to cross the colonic mucosa via M cells associated with Peyer's patches. Although there is no identified receptor for *Shigella* on M cells, it selectively translocates through M cells. Invasive *Shigella* translocate more significantly across M cells than a non-invasive mutant, demonstrating that expression of an invasive phenotype plays a main role in *Shigella*–M cell interactions (Hersh *et al.*, 1999). *Shigella* induces ruffle-like entry foci in M cells. *Shigella flexneri* is dependent on TTSS effector proteins.

It translocates the plasmid-encoded invasion antigen B (IpaB) via a TTSS. IpaB directly binds and activates caspase-1, results in apoptosis, and initiates inflammation by causing the maturation of two inflammatory cytokines interleukin 1β and interleukin 18. This early inflammatory process leads to quick disruption of the epithelial barrier, thereby facilitating further *Shigella* invasion (Grassme *et al.*, 2001; Sansonetti, 2002).

### 1.3.3c *Salmonella*

The specialized antigen-sampling intestinal M cells are a route of *Salmonella* invasion by inducing ruffle-like entry foci in M cell membrane and cytoskeletal rearrangements (Jepson & Clark, 2001). *S.* Typhimurium efficient M-cell invasion correlates with the induction of cytotoxicity and are accompanied by M-cell destruction and loss of adjacent regions of the FAE (Daniels *et al.,* 1996; Jepson & Ann Clark, 1998; Bolton *et al.,* 1999). The mechanisms by which *Salmonella* interact with M-cells are considered to be very similar to those described above in sections for *Salmonella* secreted effector proteins.

### 1.3.3d EHEC O157:H7

*E. coli* O157:H7 colonizes the epithelium of terminal rectum of the cattle (Naylor *et al.,* 2003). The majority of *E. coli* O157:H7 shed from the calves persisted in the final 5 cm of the rectum adjacent to the recto-anal junction (Naylor *et al.,* 2003). It has been shown that this region of the cattle rectum is characterised by the presence of a high density of lymphoid follicles. It is reasonable to postulate that epithelium associated with lymphoid dense tissue in the terminal rectum may be crucial for *E. coli* O157:H7 colonisation. The local environment at the site may be conducive for the expression of certain bacterial adhesins, and the availability and accessibility of distinct glycoconjugates and other integral membrane proteins on FAE or M-cells may be the determining factors in *E. coli* O157 colonization in the terminal rectum of the cattle. It is clear that EHEC interaction with intestinal epithelium is a complex phenomenon and involves multiple bacterial and host determinants. If M-cells are targeted then the bacterium would need a mechanism to prevent transcytosis, with EspF activity being a prime candidate. This function has been demonstrated for EPEC O127 EspF (Martinez-Argudo *et al.,* 2007). The aim of the current investigation was to gain further understanding of the molecular basis of terminal rectum-specific tropism of *E. coli* O157:H7 in cattle with a particular focus on host and bacterial determinants.

Many bacterial pathogens including, *S.* Typhimurium (Savidge *et al.,* 1991), *Streptococcus pneumoniae* (Borghesi *et al.,* 1999; Meynell *et al.,* 1999b), *Y.*

*pseudotuberculosis* (Ragnarsson *et al.*, 2008), and *Campylobacter jejuni* (Kalischuk *et al.*, 2010) have earlier been reported to enhance the number of M cells in the FAE. Part of my research focused to determine molecular basis of bacterial mediated increase in M cell number using *S.* Typhimurium as a model pathogen.

### 1.4 Objectives:

1. To compare the capacity of different *espF* alleles to inhibit: (i) bacterial phagocytosis by macrophages; (ii) translocation through an M-cell co-culture system; (iii) uptake by and translocation through cultured bovine epithelial cells.
2. Yeast-2-hybrid screening for different EspF variants to identify additional host proteins that may interact with EspF and confirmation of interactions using localization studies.
3. To address whether bacterial interactions can induce M-cell differentiation from follicle-associated epithelium. The work will focus on bovine rectal primary cell cultures interacting with *Salmonella enterica* serovar Typhimurium and study the molecular basis of any differentiation.

# *Chapter 2*

## *Materials and Methods*

# 2. Materials and Methods

## 2.1 Bacterial strain and media

EPEC, EHEC and other *E. coli* strains used in this study are described in Table 1. Bacteria were cultured in Luria Bertani (LB) broth, MEM-HEPES or DMEM culture media with antibiotics included when required at the following concentrations: chloramphenicol (cam) 20 µg /ml, kanamycin (kan) 50 µg /ml and ampicillin (amp) 100 µg/ml. 1 litre of M9 growth medium consists of 200 ml of M9 minimal salts X5 (Sigma), 20 ml 20% Glucose, 2 ml of 1M $MgSO_4$ and 5 ml of 10% Casamino Acids

**Table 2.1. Bacterial strains used in the study**

| Strain | Serotype | Source |
|---|---|---|
| *E. coli* ZAP 1139 | O26:H11 | Cattle isolate, Stx negative; Dr. Chris Low, Scottish Agricultural College, Penicuik, Scotland. |
| *E. coli* TUV93-0 (ZAP 1163) | O157 :H7 | Stx phage negative derivative of the sequenced human strain EDL933. Provided by Prof. John Leong, Massachusetts Medical School, Boston, USA (Campellone *et al.*, 2007) |
| *E. coli* TUV93-0Δ*espF* | O157:H7 | This study |
| *E. coli* ZAP 198 Walla3 | O157:H7 | Shiga toxin phage negative human isolate from an outbreak in Walla Walla, USA. Supplied by Dr. Mary Reynolds originally at the University of Atlanta, USA (Ostroff *et al.*, 1990) |
| *E. coli* EPEC E2348/69 (Δ*espF*) | O127:H6 | Derivative of human isolate supplied by Prof. Brendan Kenny, University of Newcastle, UK. (Shaw *et al.*, 2005) |
| ZAP 120 | O5: H- | Prof. Tom Besser |
| ZAP 1076 | O103:H- | SAC Laboratory stock |
| ZAP 286 | O127:H6 | Prof. Mark Stevens Laboratory stock |
| ZAP 124 | O26:H2 | Prof. Tom Besser Laboratory stock |
| ZAP 125 | O111:H12 | Prof. Tom Besser Laboratory stock |
| ZAP 268 | O103:H2 | Prof. Mark Stevens, IAH, Newbury Laboratory stock |
| *E. coli* AAEC 185 | O148 | Laboratory stock |
| *E. coli* DH5α | Rough | Laboratory stock |
| *E. coli* strain BL21 (DE3). | | Laboratory stock |
| *E. coli* strain K12 | Zap 1167 | Laboratory stock |
| *S.* Typhimurium | SL1334 strain | Prof. Mark Japson University of Bristol, UK. |
| Δ *spI*1 *S.* Typhimurium | | Dr Andrew Roe University of Glasgow, UK |
| Δ *sopB S.* Typhimurium | | Prof. Mark Japson University of Bristol, UK. |
| Δ *sipA S.* Typhimurium | | Prof. Mark Japson University of Bristol, UK. |
| Δ *sopE S.* Ttyphimurium | | Prof. Mark Japson University of Bristol, UK. |
| Δ *sopE2 S.* Typhimurium | | Prof. Mark Japson University of Bristol, UK. |

**Table 2.2 Antibiotics used in the study**

| **Antibiotic** | Source | Stock concentration | Final concentration |
|---|---|---|---|
| Gentamycin | Sigma-Aldrich (fluid) | 50mg/ml | 15 µg/ml in the medium |
| Ampicillin | Sigma-Aldrich (powder) | 100 mg/ml distilled water | 100 µg/ml in the medium |
| Chloramphenicol | Sigma-Aldrich (powder) | 20 mg/ml of ethanol | 20 µg/ml of medium |
| Kanamycin | Sigma-Aldrich, (powder) | 50 mg/ml of sterile distilled water | 50 µg/ml of medium |
| Spectinomycin | Sigma-Aldrich (powder) | 50 mg/ml of sterile distilled water | 50 µg/ml of medium |

## 2.2 Nucleic acid production and manipulation

### 2.2.1 Materials used in DNA techniques

### 2.2.1a Molecular weight markers:

1. Gene Ruler 100 bp DNA ladder: Purchased from MBI Fermentas.
2. Gene Ruler DNA 1 kbp ladder: Purchased from MBI Fermentas.
3. 1 kbp DNA ladder: Purchased from New England Biolabs.

### 2.2.1b Kits:

1. Gel and PCR clean up system: Purchased from Promega
2. Plasmid DNA Purification Gene Jet Kit: Purchased from Fermentas life Science.
3. QIAprep Spin Miniprep Kit: Purchased from Qiagen.
4. PureYield TM Plasmid Midiprep System Kit: Purchased from Promega.

### 2.2.1c Enzymes:

1. Gateway® BP clonase enzyme mixture: Purchased from Invitrogen.
2. Gateway® LR clonase enzyme mixture: Purchased from Invitrogen.
3. Restriction enzymes: *Bam*HI, *Xba*I, *Xho*I, *Nhe*I, *Hind*III, *Eco*RI and *Ban*II: Purchased from New England, Biolabs.

### 2.2.1d Buffers and solutions used in DNA techniques

1x TAE: Composed of 40 mM Tris base, 20 mM Acetic acid and 2 mM EDTA. Distilled water was added up to 1 litre.

1x TBE: Tris-BORATE-EDTA consists of 0.09 M Tris base, 0.09 M boric acid and 0.002 M EDTA disodium salt dissolved in distilled water.

### 2.2.1e Preparation of whole cell lysates for PCR

Whole cell lysates for PCR were prepared by taking a single colony from an LB plate and mixing with 100 µl of molecular grade (MG) water (Sigma) before heating for 15min at 100°C in a heated block.

(b) The PCR primer pairs used in this research study are listed in Table 2. Each primer pair was used to amplify *espF* from different serotypes for both detection and for sequencing.

A- Primer set1 was used for amplification of *espF* from

1. O26:H11 2. O5:H- 3. O26:H2 4. O111:H12 5. O103:H2 6. O118:H16

B- Primer set2 was used for amplification of *espF* from

7. O103:H- 8. O157:H7 9. O127:H6

PCR products were gel purified and sent for sequencing by MWG Biotech.

### 2.2.2 Techniques for nucleic acids manipulation

### 2.2.2a RNA extraction and purification

RNA was isolated according to the manufacturer's instructions using RNeasy Mini Kit50®, and Qiashredder protocol (Qiagen). In brief, bovine rectal epithelial cells were kept in RNA cell protect at -80$^{o}$C until use. The cells were centrifuged at 300 *g* for 5 m, the supernatants were removed and the pellets were suspended and disrupted in 350-600 µl of a high-salt denaturing lysis buffer containing guanidine isothiocyanate solution (RLT buffer). 10 µl of β-Mercaptoethanol per 1 ml of supplied RLT buffer. The cell lysates were homogenised on a QIA shredder column (Qiagen, UK) by centrifuging at 13000 rpm for 2 m. The cell lysates were transferred to gDNA eliminator spin column and centrifuged at 10000 rpm for 30 sec. The flow-throughs were added to the RNA-binding columns (RNeasy spin column) after the addition of one volume of 70% ethanol, to create correct resin-binding conditions, and centrifuged at 10,000 rpm for 15 sec. Flow-throughs were discarded and the

bound RNA was washed once in 350 µl buffer RW1. 80µl of DNase I mix (10 µl of DNase I added to 70 µl of buffer RDD and mixed gently) was added directly to RNeasy spin column membrane and incubated at RT for 15 min. The RNeasy spin column was then washed once in 350µl buffer RW1 and twice in 500 µl buffer RPE by centrifuging for 15 sec at 10000 rpm. RNA was eluted in 30 µl of RNase-free water. RNA concentration and quality was determined by means of Nanodrop Spectrophotometer supplied by British Labtech® International; these values are converted accordingly to cDNA Protocol.

### 2.2.2b Reverse transcription

RNA was reverse transcribed to cDNA using a Promega® protocol. Reverse transcription was performed according to the manufacturer's instructions, by adding 4 µl of MgCl2, 2 µl of 10 X buffer, 2 µl of dNTPs,0.5 µl of RNase inhibitor, 0.6 µl of Reverse Transcriptase, 1 µl of random primers, 1 µg of RNA and RNase free water to make a total volume of 20 µl. This mixture was kept at room temperature for 10m before the mixture was transferred to water bath and incubated at 42˚C for 15 m, then to an incubator at 95˚C for 5 m. The mixture was left on ice for 5min. The resulting cDNA was incubated with 1 µl of *E. coli* RNase H at 37°C for 20min for removal of the RNA complementary to the cDNA.

### 2.2.2c Semi quantitative real time-PCR (SQRT-PCR)

To detect the relative gene expression levels or DNA copy number in tested samples, PCR was performed using Expand Long Template PCR System (Roche Diagnostics). The reaction was carried out by making a Master Mix with following volumes: 5 µl 10X Expand Long Template buffer, 30 µl nuclease-free molecular water, 2.2 µl 10mM dNTP, 1 µl of forward and reverse gene-specific primers, 0.75 µl DNA polymerase with proofreading activity. 50 µl of Master Mix was transferred to individual PCR tubes, and 10 µl of different template cDNA were added individually. PCR tubes were centrifuged for 10 sec before running the PCR with an initial denaturing step of 94˚C for 2 min followed by 35 three-step cycles of 94˚C for 10 sec, 56˚C for 45 sec and 68˚C for 5 min.

**Table 2.3 Primers for EspF in the study**

| ***Primer name*** | ***Sequence*** | ***Primer use*** |
|---|---|---|
| 1. espF 1f1 ( BamHIsite)<br>2. espF ra (HindIII site) | 1.caggatccaccacacaatttgatatcggt ( o157 )<br>2.ctaagcttgatataaagaggcataaattatgc ( o 157) | O157 *espF* PTS 1 Cloning |
| 3. R2 BamHI site (O 127 espF )<br>4. O127 espF (EcoRV 5 site) | 3.caggatccaccacgcaatttgatatcggt<br>4. ctgatatcgatataaagaggcataaattatgc | EPEC *espF* PTS 1 Cloning |
| 5. espF 3 f3 ( BamHI site)<br>6. espFb ( HindIII ) | 5. caggatcctcacaatgcaacttaatatcgg<br>6. ctaagcttgatataaaaaggcatgaattatgc | O26 *espF* PTS 1 Cloning |
| 7. Primer set 2f<br>8. Primer set 2r | 7. aactgcagttcattagcgtacagactgg.<br>18. aaggtacccgactcaacctgtatcaagt | *espF* amplification and sequencing |
| 9. Primer set1f<br>10. Primer set 1r | 9. ccatgtgtgtttgatttaatag<br>10. cgatattaccaatcacatttat | *espF* amplification and sequencing |
| 11. espF allelic exchange sacI<br>12. espF allelic exchange sacI BamHI<br><br>13. espF allelic exchange pstI<br>14. .espF allelic exchange pstI BamHI | 11. aggagctcatggcggattgagacacc<br>12. agggatccctctttatatctaagcttggc<br><br>13. acctgcagttcgccatgtgcttggcg<br>14. acggatccaaccgatatcaaattgtgtgg | *espF* allelic exchange |
| 15. SACB primer<br>16. SACB primer | 15. gcaactcaagcgtttgcgaaag<br>16. ggcttgtatgggccagttaaag | to check *espF* allelic exchange |
| 17. espF 600 primerf<br>18. espF 600 primerf | 17. tcaacaaatgggtgaagtag<br>18. acaggaaactacgtgggcag | to check *espF* allelic exchange |
| 19. espF o157 xbaI site<br>20. espF o157 xhoI site | 19.agtctagagccaagcttagatataaagagg<br>20. acctcgagccctttcttcgattgctcatag | O157 *espF* cloning in pET21d for Purification of 6xHis-tagged EspF |
| 21. espF O 127 xbaI site<br>22. O127 espF xhoI site | 21. agtctagattagtggttgggtacgag<br>22. agtctagattagtggttgggtacgag | EPEC *espF* cloning in pET21d Purification of 6xHis-tagged EspF |
| 23. O26 espF xbaI site<br>24. O26 espF xhoI site | 23. agtctaga agctgtacgaaagtttctatg<br>24. acctcgagtgccttttcgacagttc | O26 *espF* cloning in pET21d Purification of 6xHis-tagged EspF |
| 25. O26 *espF* gat fore<br>26. O26 *espF* gat rev | 25. GGGG -ACA-AGT-TTG -TAC-AAA-AAA-GCA-GGC-T(CC-GCC) atgcttaatggaattagtcaagc<br>26. GGGG -AC -CAC- TTT- GTA- CAA-GAA-AGC-TGG ctacacaaaccgcatag | Ptimer for gateway cloning of EHEC O26 *espF* |
| 27. *espF*O157 and *espF*O127 gat fore<br>28. *espF*O157 and *espF*O127 gat rev | 27. GGGG -ACA-AGT-TTG -TAC-AAA-AAA-GCA-GGC-T(CC-GCC) atgcttaatggaattagtaacgc<br>28. GGGG -AC -CAC- TTT- GTA- CAA-GAA-AGC-TGG ctacccttttcttcgattgctcatag | Gateway cloning of $espF_{O157}$ and $espF_{O127}$ |

**Table 2.4 Primers used for RT-PCR and promoter cloning in the study**

| Primer name | Sequence | Primer use |
|---|---|---|
| 1. Primer forward for RANK | gcagtgcatttgggctcctgc | amplification of RANK from bovine terminal rectal epithelial primary cells |
| 2. Primer reverse for RANK | acgcctcctcacacagggtca | amplification of RANK from bovine terminal rectal epithelial primary cells |
| 3. Primer forward for RANKL | gccatggtggaaggttcgtggt | amplification of RANKL from bovine terminal rectal epithelial primary cells |
| 4. Primer reverse for RANKL | ggcccaacctcggtcatggt | amplification of RANKL from bovine terminal rectal epithelial primary cells |
| 5. Primer forward for E-cadherin | caaggcgtctgccctggaagg | amplification of Ecaherin from bovine terminal rectal epithelial primary cells |
| 6. Primer reverse for E-cadherin | tgttgtgcggcagcatggga | amplification of Ecaherin from bovine terminal rectal epithelial primary cells |
| 7. Primer forward for slug | ccgcgctccttcctggtcaa | amplification of slug from bovine terminal rectal epithelial primary cells |
| 8. Primer reverse for slug | gtggtccacacggcgatggg | amplification of slug from bovine terminal rectal epithelial primary cells |
| 9. Primer forward for vimentin | cgcgacaacctggccgaaga | amplification vimentin from bovine terminal rectal epithelial primary cells |
| 10. Primer reverse for vimentin | cgctccaggtcaagacgtgcc | amplification of vimentin from bovine terminal rectal epithelial primary cells |
| 11. Primer forward for relb | tgggggtggcctcctgtccc . | amplification of *relB* from bovine terminal rectal epithelial primary cells |
| 12. Primer reverse for relb | ggtgcccctgcttgtggggg | amplification of *relB* from bovine terminal rectal epithelial primary cells |
| 13. Primer forward for GAPDH | gatgctggtgctgagtatgtagtg | amplification of GAPDH from bovine terminal rectal epithelial primary cells |
| 14. Primer reverse for GAPDH | atccacaacagacacgttgggag | amplification of GAPDH from bovine terminal rectal epithelial primary cells |
| 15. bt_VIMprom_Nhe_for | AAAAGCTAGC TGACTCAGCGACCCCACCCTTC | amplification of RANKL promoter |
| 16. bt_VIMprom_Xho_rev | TTTTCTCGAG GTAGCACGGACTGGCTCCGGAA | amplification of RANKL promoter |
| 17. bt_SNAI2prom_Nhe_for | AAAAGCTAGC GGTGACTTTATACTGAAAAAGG CAC | amplification of Slug promoter |
| 18. bt_SNAI2prom_Xho_rev | TTTTCTCGAG AGCATCTCTGCCCCGCAGGTG | amplification of Slug promoter |
| 19. hs_RANKLprom_NheI_for | AAAAGCTAGC CACTCTTCCTCAACATTTACTGA GG | amplification of vimeintin promoter |
| 20. hs_RANKLprom_XhoI_rev | TTTTCTCGAG CTCCCTCCCCTTCTTGTCTG | amplification of vimeintin promoter |

### 2.2.2d Agarose gels and electrophoresis

DNA agarose gel electrophoresis in 1 x TAE or 1x TBE were used to separate DNA fragments. According to the fragment size range, appropriate agarose concentrations were used e.g. a 1% gel was used for fragments more than 2 Kbp, 1.5% gel for fragments between 500 bp and 2 Kbp and a 2% gel for fragments less than 500 bp.

Agarose gels consisted of the appropriate amount of agarose (Melford) added to 100 ml of Tris-BORATE-EDTA (TBE). The solution was heated in microwave for 1-5 min until the agarose dissolved; following cooling, safe view (NBS Biologicals) or Gel red (Biotium, Inc.) dyes were mixed with the solution in a 10 µl/100 ml agarose. DNA was mixed with 10x DNA loading buffer (Invitrogen) in a ratio of 5ul of DNA to 1 µl of buffer, and then the DNA was loaded into the agarose gel wells. A 100 bp or 1 Kb standard ladder (Invitrogen) was also loaded in an additional well. The gel was run at 80-120 volts according to the expected DNA fragment size for 30-50 min. The gels were imaged under ultraviolet light using a Flowgen Multi-Image cabinet and stored using Image capture software.

### 2.2.2e Purification of PCR product

PCR products were purified either directly or by separation of the DNA fragment by 1% TAE agarose gel electrophoresis. The DNA band of the required size was cut out from the gel. Both the direct purification and the gel purification were performed using the Promega Gel and PCR clean up system according to the manufacturer's instructions.

### 2.2.2f Determination of DNA concentration

The concentration and purity of the DNA was determined by measuring the UV absorbance at 260 nm and 280 nm. The DNA concentration was calculated with the $OD_{260}$nm (1 $OD_{260}$nm = 50 µg/ml dsDNA or 33 µg/ml ssDNA). The purity was estimated with the $OD_{260}$nm/$OD_{280}$nm ratio, with a ratio of approximately 1.8 indicating a low degree of protein contamination.

## 2.3 Cloning of different genes used in this study

### 2.3.1 Materials used in cloning techniques

### 2.3.1a TFB1 solution (1 litre)

| | |
|---|---|
| 30mM KoAC | 2.94 g |
| 10mM $CaCl_2.2H_2O$ | 1.47 g |
| 100mM KCl | 7.45 g |

15% Glycerol 150 ml

Dissolved in 900 ml distilled water and autoclaved, then 100ml of 500 mM autoclaved $MnCl_2$ (50 mM final concentration) were added

### 2.3.1b TFB2 (1 litre)

75mM $CaCl_2.2H_2O$ 11.03 g

10mM KCl 0.745 g

15% Glycerol 150 ml

Dissolved in 900 ml of distilled water and autoclaved. After that 100 ml of autoclaved 100 mM Na-MOPs PH7 (2.09 g MOPs with 10 mM NaOH) were added.

**Table 2.5. Plasmids used in this study**

| Plasmid | Description | source |
|---|---|---|
| 1. pMB102 | low copy cloning vector with inducible *lac* promoter | Gift from Dr Tammari Schneiders, University of Belfast |
| 2. pAT1 | pMB102 containing $espF_{O127}$ | This study |
| 3. pAT2 | pMB102 containing $espF_{O157}$ | This study |
| 4. pAT3 | pMB102 containing $espF_{O26}$ | This study |
| 5. pUC-gfp | pUC18 derivative containing gfp | Laboratory stock |
| 6. pAJR146 | rpsM-GFP+ transcriptional fusion in pACYC184 | Laboratory stock |
| 8. pDONR-207 | Gateway entry cloning vector | Invitrogen |
| 9. pDG28 | Vector containing *sackan* cassette. Used to obtain *sackan* cassette | Laboratory stock |
| 10. PIB307 | vector for allelic exchange used for creation the next two plasmids | Laboratory stock |
| 11. pAT4 | pIB307 containing *espF* flanking regions and *sackan* cassette. Used for *espF* allelic exchange in EHEC O157:H7 strain TUV93-0 | This study |
| 12. pAT5 | PIB307 containing espF flanking regions. Used for deletion of *sackan* cassette from chromosome after *espF* allelic exchange in EHEC O157:H7 strain TUV93-0 | This study |
| 13. pKD4 | as a template for amplification of kanamycin cassette | Laboratory stock |
| 14. pKM201 | Thermal sensitive red-gam expressing plasmid used for Preparation of hyper-recombinant EHEC O157:H7 strain TUV93-0 for *espF* deletion | Laboratory stock |
| 15. pTREX-DEST30-prA | vector to generate amino terminus protein A fusions for LUMIER binding assays | Laboratory stocks |
| 16. pRenilla | Vector to generate amino terminus luciferase fusions for Lumier binding assays | Laboratory stocks |
| 17. pAT6-7 | *espF*O157, *espF*O26 and *espF*O127 cloned into pTREX-DEST30-prA respectively | This study |
| 18. pAT8-10 | *espF*O157, *espF*O26 and *espF*O127 cloned into pRenilla respectively | This study |
| 19. pAT11-13 | *espF*O157, *espF*O26 and *espF*O127 cloned into peGFP-DEST respectively | This study |
| 20. pHJ1 | SNX9in pDONR223 | Gift from Prof. Juergen Haas, University of |

| | | |
|---|---|---|
| | | Edinburgh, UK. |
| 21. pAT14-15 | pTREX-DEST30-SNX9 pRenilla-SNX vectors expressing SNX9-protein A or -luciferase fusion proteins | This study |
| 22. pHJ2 | N-WASP in pDONR223 | Gift from Prof. Juergen Haas, University of Edinburgh, UK |
| 23. pAT16-17 | pTREX-DEST30-N-WASP pRenilla-N-WASP vectors expressing N-WASP-protein A or -luciferase fusion proteins | This study |
| 24. pHJ3 | MAD2L2in pDONR223 | Gift from Prof. Juergen Haas, University of Edinburgh, UK |
| 25. pAT18-20 | pTREX-DEST30- MAD2L2, pRenilla-MAD2L2vectors expressing N- MAD2L2-protein A or -luciferase fusion proteins and MAD2L2 in DSred fusion plasmid pDSRED2-DEST | This study |
| 26. pHJ4 | NFICin pDONR2234 | Gift from Prof. Juergen Haas, University of Edinburgh, UK |
| 27. pAT21-23 | pTREX-DEST30- NFIC, pRenilla- NFICvectors expressing NFIC -protein A or -luciferase fusion proteins and MAD2L2 in DSred fusion plasmid pDSRED2-DEST | This study |
| 28. pFOP and PTOP- flash | pFOP & pTOP -Flash to study transcriptional activity of β - catinin | Invitrogen |
| 29. pGM1 | pGL3 containing RANKL promoter | Gift from Dr Georg Malterer, University of Muenchen Germany |
| 30. pGM2 | pGL3 containing Slug promoter | Gift from Dr Georg Malterer University of Muenchen Germany |
| 31. pGM3 | pGL3 containing vimeintin promoter | Gift from Dr Georg Malterer University of Muenchen Germany |
| 32. pCR3 | Mammalian , CMV IE | Laboratory stock |
| 33. pRC1&2 | *sopB* wildtype & $sopB^{c462s}$ cloned into pTrc99-FF4 | Gift from Dr Vassilis Koronakis, University of Cambridge ,UK |
| 33. pGM4 | *sopB* cloned into pCR3 plasmid | Gift from Dr Georg Malterer University of Muenchen Germany |

### 2.3.2 Cloning techniques of *espF* alleles

### 2.3.2a Restriction endonuclease digestion

Restriction endonuclease reactions were performed according to the manufacturer's recommendations. In general, 1.5 μg DNA was digested for 2 h at the appropriate temperature with 10-20 U enzyme. Efficiency of the cleavage reaction was determined by 1% TAE agarose gel electrophoresis stained with ethidium bromide.

### 2.3.2b Preparation of chemically-competent bacteria

An overnight colony of bacteria was cultured in 5 ml LB at the appropriate temperature with or without antibiotic at 200 rpm. 1 ml of this culture was inoculated to 100 ml of LB and was incubated until $OD_{600}$ = 0.4–0.6, The bacteria were chilled

on ice for 15 min, divided into two Falcon tubes and centrifuged at 4000 rpm, 4°C for 8 m. The supernatants were discarded and the pellets were suspended in 0.4x TFB1 (40 ml/100 ml bacterial culture), and placed on ice for 15 min and then centrifuged at 4000 rpm, 4°C. The bacterial pellets were suspended in 0.04 x TFB2 (4 ml/100 ml of bacterial culture) and kept on ice for 15 min and then stored as 200 µl aliquots at -70°C.

### 2.3.2c Preparation of electro-competent cells

An overnight colony was grown on 5ml LB at appropriate temperature and 200 rpm agitation with or without antibiotic according to the culture. Then 1 ml of this culture was inoculated into 100 ml of LB and incubated in the previous conditions until $OD_{600}$= 0.3-0.5. The culture then was divided into two 50 ml Falcon tubes and chilled on ice for 15 min then centrifuged at 4000 rpm, 4°C for 10 min. The pellets were washed twice with 25 ml of 10% cold glycerol. The pellets were washed again with 12.5 ml of ice-chilled glycerol and centrifuged as before. The washed pellets were suspended on 0.2 ml of 10% ice-chilled glycerol and 50 µl aliquots stored at -70°C.

*espF* alleles were amplified from the different strains using the primers defined in Table 2 and the products cloned into pTS1 (Table 3). *Bam*HI and *Hind*III sites were used to clone the alleles from EHEC strains O157:H7 and O26:H11. For EPEC O127 *espF*, as this contains a natural *Hind*III site, an alternative cloning strategy was used: pTS1 was digested with *Hind*III followed by Klenow polymerase (Roche) treatment to create a blunt end, and then restricted with *Bam*HI. The EPEC O127:H6 *espF* PCR product was digested with *Bam*HI and *Eco*RV and then cloned into the restricted pTS1. Restriction digests were set up as per manufacturer's instructions (New England Biolabs) with the recommended enzyme buffers. All clones were checked by sequencing and matched published database sequences for the alleles (where available).

### 2.3.2d Ligation

Before ligation, the amount of plasmid and insert DNA was determined by running both of them in an agarose gel and comparing the bands with known standards. Ligations were performed using T4 ligase enzyme overnight at room temperature.

| | |
|---|---|
| Plasmid | 1 µl |
| Insert | 7 µl |
| Ligase buffer | 2 µl |
| Ligase enzyme | 1 µl |

### 2.3.2e Electrotransformation of circular DNA

Before transformation the 2 mm gap cuvettes were chilled on ice for at least 5 min. 1 µl of ligated plasmid, 0.5 µl of miniprep plasmid or midiprep plasmid were mixed with the competent cells and left for 5 m on ice before being transferred to the 2mm gap cuvettes. The cuvettes were dried and the cells shocked at 2500 volts for 0.005 sec in an electroporator. 500 µl of SOC or LB broth were added to the cells immediately after shocking and the cells were incubated at the appropriate temperature for 1-2 h. Then 100 µl of the culture were plated onto LB agar plates containing the appropriate antibiotic.

### 2.3.2f Chemical transformation

Plasmids or ligation reactions were added to the chemical competent cells and chilled on ice for 30 min. The mixture was heat shocked in a water bath at 42°C for 1 min. Then 500 ml of SOC were added and the cells incubated at the appropriate temperature for 1-2 h to allow recovery. After that, the culture was plated onto LB agar plates containing the appropriate antibiotic.

### 2.3.2g Purification of plasmid DNA

The plasmids used in this study were isolated by midiprep alkaline lysis according to manufacturer's instructions (PureYield TM Plasmid Midiprep System kit, Promega, UK). The mini prep from the plasmid was used for diagnostic digest and was purified using Plasmid DNA Purification Gene Jet Kit: Purchased from Fermentas life

Science or QIAprep Spin Miniprep Kit: Purchased from Qiagen according to manufacturer's instructions.

### 2.3.2h Gateway cloning for different *espF* for Protein-protein interactions

Gateway cloning of the *espF* alleles from the different *E coli* strains was carried out to simplify construction of vectors for yeast-2-hybrid analysis of EspF interactions with eukaryotic proteins. *espF* alleles were amplified using a proofreading Taq polymerase and the primers defined in Table 2 and cloned into the Gateway™ vector pDONR-207 (Table 3) using BP clonase (Invitrogen) to form entry clones. The obtained clones were checked for the correct insert by DNA plasmid extraction and restriction digestion using *Ban*II. Once in the Gateway™ system, clones were easily transferred to the required destination vectors including pTREX-DEST30 (protein A fusions) and p*Renilla* (luciferase fusions).

The recombination reactions (BP reactions) were performed as the description below:

| | |
|---|---|
| pDONR201 Vector | 150µg |
| Purified PCR product | 2-7 µl |
| BP Clonase enzyme mix | 1 µl |

The reaction was incubated overnight at room temperature. One µl of the reaction was transformed to DH5α competent cells, recovered on S.O.C medium for 1-2 h at 37°C and plated on LB plates containing 15 µg/ml gentamycin and incubated overnight at 37°C. The obtained clones were checked for the insert by plasmid extraction and digestion using *Ban*II.

#### 2.3.2h.1 Creating an expression clone (LR Reaction)

Expression clones can be created by excision, integration and recombination of entry clone containing the PCR product, recombination sites using the LR clonase complex. The destination vectors contain the *ccdB* gene between the "left" and "right" recombination sites. The advantage of this arrangement is that there is

virtually no background from vector that has not recombined with the PCR products, because the presence of the *ccdB* gene will inhibit the growth of all standard *E. coli* strains. CcdB protein product interferes with *E. coli* DNA gyrase thereby inhibiting the growth of most *E. coli* strains used in the lab, such as DH5α™, TOP10 or XL-1 Blue (Bernard & Couturier, 1992).

### 2.3.2h.2 LR Reaction

| | |
|---|---|
| Destination vector (300ug/µl) | 1 µl |
| Entry Clone (150ng/µl) | 1 µl |
| $dH_2O$ | 1 µl |
| LR Clonase enzyme mix (Int, IHF, and Xis.) | 1 µl |

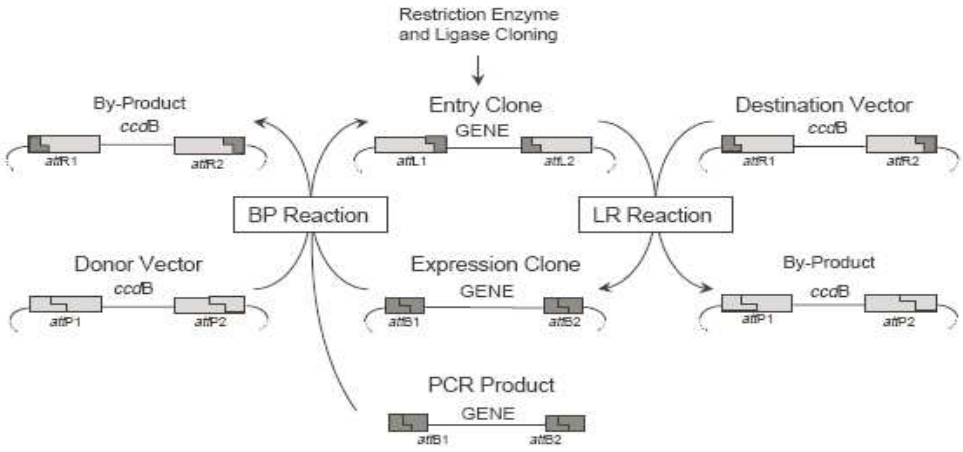


**Fig. 2.1 Gateway cloning technology for cloning and sub-cloning genes.** Once a DNA segment is cloned into the Gateway system, it can be easily transferred from an entry clone into a destination vector (via the LR reaction) to generate an expression clone. A DNA segment in an expression clone can be easily transferred into an attP pDONR vector (via the BP reaction) to generate an entry clone and then into different destination vectors, all without the need for restriction enzymes and ligase. This diagram was directly taken from Invitrogen gateway cloning kit.

The reaction was incubated for 3-4 h at room temperature. 2 µl of the reaction was transformed into DH5α chemical competent cells, recovered on S.O.C medium for 1-2 h at 37°C and plated on LB plates containing appropriate antibiotic and incubated overnight at 37°C. The resulting clones were checked for the insert by plasmid extraction and digestion using appropriate restriction enzymes (*Eco*RI and *Bam*HI for Bait and EGFP. *Xba*I and *Hind*III for His and GST fusion construct plasmids).

## 2.4 Allelic exchange for deletion of *espF* in *E. coli* O157:H7

In order to construct plasmids that would facilitate chromosomal exchange, flanking regions of *espF* were PCR amplified (primers 11-18, Table 3) and cloned into a temperature sensitive pIB307. A *sackan* cassette from pDG28 was excised with *Bam*HI, cleaned and cloned into the *Bam*HI site of pIB307 containing the *espF* flanking regions. Individual plasmids containing *espF* flanking regions and *sackan* cassette were then exchanged into the chromosome of TUV93-0, to replace *espF* with the *sackan* cassette. To achieve this, the plasmid was electroporated into the target strain and transformants were selected on LB-CAM plates at 30°C (permissive). Transformants were then inoculateCAM broth and cultured overnight at 30°C. 10 fold dilutions were spread onto LBC agar plates down to $10^{-7}$. These were cultured overnight at 42°C (a restrictive temperature that selects for chromosome integration). Six colonies were then selected and the process repeated twice. At the end, a colony was taken from each plate (about 6 in total). These were inoculated into one LB broth overnight at 30°C (to lose the plasmid) and then diluted to allow overnight growth at 30°C in LB broth. This was repeated 3 times. After that 10 fold dilutions were made and spread onto LB Kan agar plates at 30°C. Individual colonies were replica plated onto LB Kan agar and LB Cam agar at 30°C to identify those that had undergone successful allelic exchange. Putative strains containing the cassette in place of *espF* were checked by PCR.

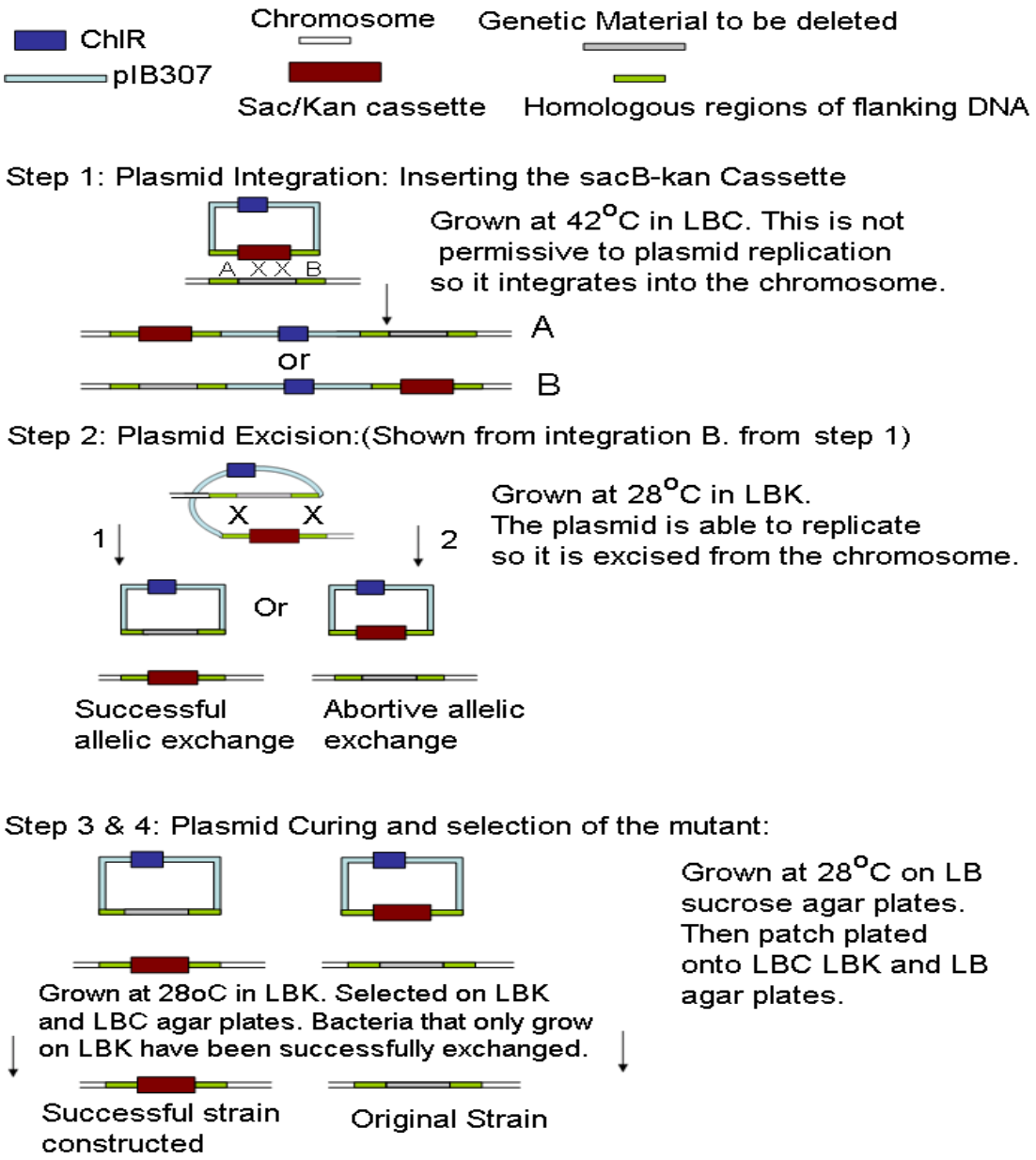


**Fig.2.2 Allelic exchange to substitute *espF* of *E. coli* O157 with *sackan* cassette; taken from with minor modification (Emmerson *et al.*, 2006).** The insertion of the *sacB-kan* cassette into the chromosome can be done in 4 steps 1. Plasmid insertion into the wild-type strain chromosome at the non tolerant temperature for plasmid replication (42°C). 2. and 3: this new strain was grown at 28°C in the presence of kanamycin to allow the bacteria that got excised and cured the plasmid, leaving the cassette on the chromosome. 4. The bacteria were straked on to LBC or LBK, the strain that can only survive on LBK are successful constructs of the intermediate strain.

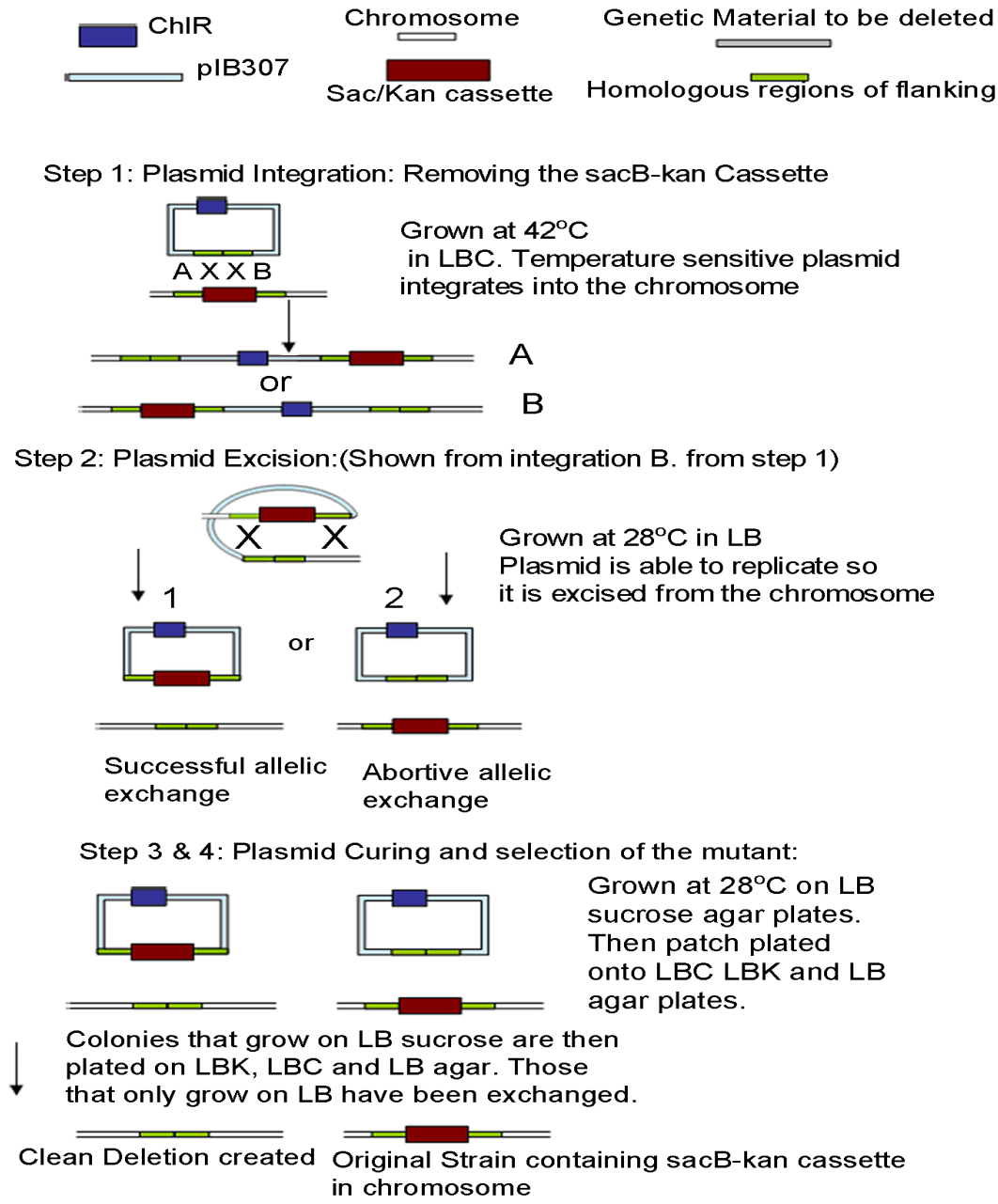


**Fig. 2.3 Allelic exchange to remove *sackan* cassette in *E. coli* O157 (taken from Emmerson *et al.*, 2006).** 1. To insert the plasmid into the intermediate strain chromosome it was selected at the non permissive temperature for plasmid replication (42°C). 2 and 3, these bacteria were grown at 28°C in the absence of antibiotics to enhance the bacteria that have excised (Step 2) and later cured (Step3) the inserted plasmid. Step 4: the bacteria were allowed to grow on media containing sucrose, LBK or normal LB to select for successful strain constructs.

### 2.4.1 Removal of *sackan* cassette from the chromosome

To achieve this, the plasmid pIB307 containing both *espF* flanking regions (but no cassette) was electroporated into TUV93-0 containing the *sackan* cassette integrated into its chromosome and transformants were selected on LB-CAM plates at 30°C. Ten transformants were then inoculated into LB CAM broth at 42°C and sub-cultured four times over a 48h period. Serial dilutions of these cultures were plated onto LB-CAM plates at 42°C. 10 single colonies were then inoculated into LB broth at 30°C for 48 h of logarithmic growth, and serial dilutions were plated onto LB-sucrose agar (6%, wt/vol; no NaCl). Individual colonies were replica plated onto LB, LB-CAM, and LB-KAN at 30°C to identify those that had undergone successful allelic exchange. Those that did not grow on both LB-CAM and LB-KAN but grew in LB agar were checked by PCR for loss of the *sackan* cassette and deletion of *espF*.

## 2.5 Analysis of type III secretion profiles and EspF secretion

To analyse type III secretion profiles and EspF secretion from the EPEC O127:H6 Δ*espF* and complemented strains; were cultured in M9 media. EspD was detected as previously described (Naylor *et al.*, 2005b; Roe *et al.*, 2007), using mononclonal antibody kindly supplied by Prof. Chakraborty (Giessen). EspF was detected using polyclonal antibodies supplied from Prof. Sasakawa (Tokyo) (Nagai et al., 2005) and Prof. Hecht (University of Illinois at Chicago) (Viswanathan *et al.*, 2004a). Briefly EPEC O127:H6 Δ*espF* and complemented strains was grown with shaking overnight at 37°C in Luria-Bertani broth supplemented with ampicillin (100 μg/ml). The cultures were then diluted 1:100 in M9 minimal salts X5 (Sigma) supplemented with 0.4% (w/v) glucose, 0.05% (w/v) Casamino Acids, and 2mM $MgSO_4$ and were grown standing at 37°C and 5% $CO_2$ to an $OD_{600}$ of 0.4 at this time IPTG was added to the culture to 1mM. The cultures continued to grow to $OD_{600}$ = 1 and were centrifuged (5000 g) for 30 min to pellet the bacteria. The supernatant containing the secreted proteins was precipitated using 10% (w/v) trichloroacetic acid (TCA) overnight at 4°C. TCA precipitated supernatants were centrifuged (5000g) for 30 min at 4°C. The supernatants were discarded and the pellets were left to dry and resuspended in 200 μl of 1.5M tris-HCl pH8.8. EspD and EspF were detected by SDS page and Western blotting.

## 2.6 Protein techniques

### 2.6.1 Materials and supplements used in protein techniques

### Table 2.6 Antibodies used in this study.

| Antibody | Source | use |
|---|---|---|
| Anti-vimentin high affinity, mouse monoclonal antibody | Roche Diagnostcs | It was used at 1/1000 for immunoblotting and 1/100 for immunofluorescence. |
| Anti-slug, rabbit monoclonal antibody | SantaCruz Biotechnology | It was used at, 1/1000 for immunoblotting and 1/2000 for immunofluorescence. |
| Anti-β-actin, mouse monoclonal antibody | Sigma | It was used at 1/1000 for immunoblotting |
| Anti-β-Catenin rabbit polyclonal | Sigma | It was used at 1/1000 for immunoblotting and 1/100 for immunofluorescence. |
| ATP synthase subunit alpha mouse monoclonal antibody | Invitrogen | It was used at 1/1000 for immunoblotting |
| E-Cadherin rabbit polyclonal | Cell Signaling | It was used at 1/1000 for immunoblotting. |
| Phospho GSK3 beta (Ser9) rabbit polyclonal IGg | Thermo Scientific | It was used for immunoblotting at 1/1000 |
| GSK3 beta rabbit monoclonal antibody | Thermo Scientific | It was used for immunoblotting at 1/250 |
| Goat Anti LEF1 | Sigma | It was used for immunoblotting 1/250 |
| LGR5 (C_16) mouse polyclonal | SantaCruz biotechnology, INC | It was used for immunofluorescence at 1/100 |
| RANK (H-300) rabbit polyclonal | SantaCruz biotechnology, INC | It was used at 1/1000 for immunoblotting |
| RANKL (L300) rabbit polyclonal | Cell Signaling | It was used at 1/1000 for immunoblotting |
| RelB rabbit polyclonal | Santa Cruz biotechnology, INC | It was used at 1/200 for immunoblotting and 1/50 for immunofluorescence |
| Anti Nanog | | It was used at 1/100 for immunofluorescence |
| Pancytokeratin rabbit polyclonal | Cell Signaling | It was 1/100 for immunofluorescence |
| Pan-Cadherin | | it was used at 1/1000 for immunoblotting |
| Goat anti-mouse HRP coupled | Jackson, Hamburg,Germany. | It was used at 1/1000 for immunoblotting |
| Goat anti-rabbit HRP coupled | Jackson, Hamburg, Germany | It was used at 1/1000 for immunoblotting |
| Rabbit anti- goat HRP coupled | Jackson, Hamburg, Germany | It was used at 1/1000 for immunoblotting |
| Alexa fluor®488 goat anti-mouse IgG | Invitrogen | It was used at 1/500 for immunofluorescent. |
| Alexa fluor®594 goat anti-rat IgG | Invitrogen | It was used at 1/4000 for immunofluorescent |
| histone core antibody, sheep polyclonal | gene tex | It was used at 1/200 for immunoblotting |
| Rabbit polyclonal Phospho-β- catenin (Ser33/3/37/thr41) | Cell Signaling | It was used at 1/1000 for immunoblotting |
| AKT Rabbit polyclonal | Cell Signaling | It was used at 1/1000 for immunoblotting |
| Phospho-AKT (Ser473) Rabbit polyclonal | Cell Signaling | It was used at 1/1000 for immunoblotting |
| mouse monoclonal PCNA (PC10) | Santa Cruz Biotechnology | It was used at 1/50 for immunofluorescent |
| Rabbit anti goat HRP coupled | Sigma | It was used at 1/1000 for immunoblotting |
| Goat anti mouse HRP coupled antibody | Invitrogen | It was used at 1/1000 for immunoblotting |
| Goat anti mouse antibody TRITC | ABD Serotec | It was used at 1/100 for immunofluorescent |
| Goat anti- rabit antibody TRITC | Invitrogen | It was used at 1/100 for immunofluorescent |
| Alexafluor 488-conjugated goat anti-rat IgG | Invitrogen | It was used at 4 µg/ml for immunofluorescent |
| Goat anti-rabbit immunoglobulins/Biotinylated | Dako | It was used 1/500 for immuno-histochemistry staining |

| | | |
|---|---|---|
| Mouse monoclonal anti-his | Qiagen | It was used at 1/5000 for immunoblotting |
| Anti mouse -GP2/glycoprotein rat mono clonal antibody | MBL (Cell Biology) | It was used at 5 µg/ml for immunofluorescent |

**Table 2.7 Cell signal inhibitors used in this study**

| Chemical | Source | Final concentration |
|---|---|---|
| MG132: Proteasomal-inhibitor drug | Calbiochm. Diluted in DMSO | 10µM 5h before the challenge |
| AKT inhibitor | Calbiochem | used at 20µM 5h before the challenge |
| SN50 NF-KB inhibitor | Calbiochem | used at 50µM 5h before the challenge |
| GSK-3 β inhibitor SB 415286 | Sigma | used at 50µM for 5h before the challenge |
| PI3 kinase inhbitor LY 294002 | Calbiochem | used at 50µM 5h before the challenge |
| OPG | Sigma | used at 50ng/ml an hour before the challenge with bacteria |
| β-catenin/Tcf inhibitor, FH535 | Calbiochem | used at 10 µM 5h before the challenge |
| Cycloheximide: Protein synthesis blocker | Sigma Aldrich diluted in DMSO | used at 1µg/ml overnight before the challenge |
| Recombinant human soluble RANK Ligand (RANKL) | Prospec | used at 50ng/ml 24h before the challenge or analysis |
| Bovine RelB siRNA (set 1) | Invitrogen | GGACCACGGAUGAACUGGAGAUCAUAUGAUCUCC AGUUCAUCCGUGGUCC |
| Bovine RelB siRNA (set 2) | Invitrogen | ACGACAUAACUGAGGGUAAGGACUUAAGUCCUU ACCCUCAGUUAUGUCGU |

### 2.6.1a Protease inhibitors:

Complete, EDTA free, protease inhibitor cocktail tablets, purchased from Roche Diagnostics. One tablet was used for 50 ml of lysis buffer.

### 2.6.1b BCA protein assay reagent:

Protein quantification kit, purchased from Thermo Scientific. It was used according to manufacturer's instructions.

### 2.6.1c Protein Ladder:

Pre-Stained Standard, purchased from Biorad, supplied ready to use. 5µl were loaded for small gels. (2) His-tagged protein marker purchased from Qiagen.

### 2.6.1d Cell staining reagents:

1. DAPI, purchased from Invitrogen. Used 1/5000 in PBS

2. Alexa Fluor 647 phalloidin purchased from Molecular Probes and it was used 1/40 in PBS

3. Phalloidin TRITC purchased from Sigma. Used 1/100 in PBS

4. Phalloidin FITC purchased from Sigma. Used 1/100 in PBS

5. DAKO Autostainer Plus System (DAKO, Capinteria, CA) was used for immuno-histochemistry staining

6. Vectastain Elite ABC kit (Vector Laboratories) and NovaRED Peroxidase Substrate kit (Vector Laboratories) were used for immuno-histochemistry staining

### 2.6.1e Cell lysis buffers

1. NP40 1 litre consists of

| | |
|---|---|
| 1 M Tris PH 7.5 | 20 ml |
| 5 M NaCL | 30 ml |
| 1 M MgCL2 | 5 ml |
| NP40 | 10 ml |
| DH2O | up to1 litre |

2. protein Lysis buffer

| | |
|---|---|
| Tris-HCl pH 7.4 | 20 mM |
| NaCl | 150 mM |
| Triton x-100 | 3% |
| EDTA | 1 mM |

Protease inhibitors (Sigma) according to manufacturing instruction**.**

**2.6.1f The Proteo-Extract R Subcellular Proteome Extraction Kit** purchased from Calbiochem® & Novagen®. Used for extraction of proteins from different cell components (cytoplasmic, membranous and nuclear)

### 2.6.1g 12% SDS gel consists of

| | |
|---|---|
| Acrylamide (40% solution) | 3.75 ml |
| 1.5M Tris PH (8.8) | 3.75 ml |
| SDS 10% (w/v) | 0.1 ml |
| $DH_2O$ | 3.6 ml |
| APS 10% (w/V) | 0.1 ml |
| TEMED (Sigma) | 0.01 ml |

Stacking gel (4%)

| | |
|---|---|
| Acrylamide (40% solution) | 1 ml |
| 0.5 M Tris PH (6.8) | 2.5 ml |
| 10% (w/v) SDS | 0.1 ml |
| DH2O | 6.4 ml |
| 10% (w/V) APS | 0.1 ml |
| TEMED | 0.01 ml |

### 2.6.1h SDS Running buffer- per litre

| | |
|---|---|
| Trizma base | 15 g |
| Glycin | 72 g |
| SDS | 5 g |
| D $H_2O$ | Up to 1 litre |

### 2.6.1i Transfer buffer- per litre

2.42 g Tris (hydroxymethyl) Methylamin

11.54 g glycine

200 ml Methanol

Distilled $H_2O$ to one litre

### 2.6.1j ECL reagents

### 2.6.1j.1 ECL purchased from thermo scientific

### 2.6.1j.2 ECL prepared in the lab:

A) Luminol (Fluka 09253)

250 mM in DMSO (0.886 g / 20 ml) aliquots in 1ml and kept in dark at -20°C.

B) p-Coumaric acid (Sigma C9008)

90 mM in DMSO (0.296 g/20 ml) aliquots in 0.44 ml and kept in dark at -20°C.

ECL solution1:

| | |
|---|---|
| Stock Luminol | 1ml |
| P-Coumaric acid | 0.4 ml |
| 1.0 M TRis | 10 ml |
| $DH_2O$ upto | 100 ml |

ECL solution 2:

| | |
|---|---|
| $H_2O_2$ (30%) | 64 µl |
| 1.0 M Tris (pH 8.5) | 10 ml |
| $DH_2O$ | Up to 100 ml |

Solution 1 and 2 kept at 4°C.

### 2.6.1k Denature/purification buffer (100ml):

| | |
|---|---|
| 0.1 M $NaH_2PO$ | 1.56 g NaH2PO4·H2O (MW 137.99 g/mol) |
| 0.01 M Tris | 0.12 g Tris (MW g/mol) |
| 8 M Urea | 48.048 g Urea (MW g/mol) |
| $H_2O$ | 100 ml |

Adjust pH to using conc. By NaOH and HCL.

### 2.6.1l Buffers for purification under native conditions

#### 2.6.1l.1 Lysis buffer:

| | |
|---|---|
| 10 mM imidazole in PBS | pH to 8.0 |

#### 2.6.1l.2 Wash buffer:

| | |
|---|---|
| 20 mM imidazole in PBS | pH to 8.0 |

#### 2.6.1l.3 Elution buffer:

| | |
|---|---|
| 250 mM imidazole in PBS | pH to 8.0 |

### 2.6.2 Extraction of cell proteins

#### 2.6.2a Total cell lysate

Bovine terminal rectal epithelial cells were released from tissue culture plates using a tissue scraper, and 600 µl of lysis buffer that consisted of 20 mM Tris-HCl pH7.4 150 mM NaCl, 3% Triton X-100, 1 mM EDTA, and protease inhibitors (Sigma) according to manufacturing instruction**.**

#### 2.6.2b Extraction of proteins from cell fractions

The ProteoExtractR Subcellular Proteome Extraction Kit (Calbiochem® & Novagen®) is designed for reproducible extraction of subcellular proteomes from mammalian cells. Based on different solubilities of certain subcellular compartments, the S-PEK utilizes proprietary chemistries to yield four sub-proteome fractions which are enriched in cytosolic, membrane/ organelle, nuclear, and cytoskeletal proteins. The kit was used according to the manufacturer's instructions. In brief the kit was used as follows: The cells were washed twice by adding one ml ice-cold wash buffer at 4°C 5 m each. 400 µl S-PEK Extraction Buffer I was added to each well and incubate at 4°C under gentle agitation. The supernatant was collected and kept as cytosolic fraction. S-PEK Extraction Buffer II was added at 400µl/well and incubated for 30min at 4°C under gentle agitation. The supernatant was collected and kept as membranous fraction S-PEK Extraction Buffer III 200 µl/well and incubate at 4°C under gentle agitation for 10 m. The supernatant was collected and kept as nuclear fraction. Protease inhibitor was added to all S-PEK Extraction Buffer was added at 2 µl/well and benzonase was added to S-PEK Extraction Buffer III at 0.5 µl/well.

#### 2.6.2c Protein procedures

##### 2.6.2c.1 SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE) electrophoresis was performed using the Biorard system. The resolving gels (12%) were prepared with 3.3 ml $H_2O$, 4.0ml 30% Acrylamide mix, 2.5 ml 1.5 M Tris (pH 8.8) and 0.1ml 10% SDS. Prior to pouring the gel, 0.1 ml of 10% Ammonium persulfate (APS) and 0.01 ml of TEMED (Sigma) were added. The solution for generating the resolving gel

was mixed and, after pouring, the gel was overlaid with propanol. After polymerisation, the isopropanol was removed and the stacking gels were prepared with 6.1 ml $H_2O$, 1.3 ml 30% Acrylamide mix, 2.5 ml of 0.5M Tris (pH 6.8) and 0.1 ml 10% SDS. Prior to pouring the gel 0.1 ml 10% Ammonium persulfate and 0.01 ml TEMED (Sigma) were added. The stacking gel solution was poured on top of the separation gel and a comb was fixed. After polymerisation, the glass plates containing the gel were assembled in the gel electrophoresis apparatus. Samples were loaded on the gel together with a protein ladder. The cell lysate was added to an equal volume of 2x sample extraction buffer (Sigma). The mixture was boiled for 5min, loaded onto a 12% acrylamide denaturing gel and run at 150 V for 60 to 80 min.

### 2.6.2c.2 Western blot

Proteins were transferred to a nitrocellulose membrane (Amersham Biosciences, UK) using a Trans-Blot semi-dry Transfer Cell (Bio-Rad, UK). Blotting was performed at 15 V for 30m. The nitrocellulose membrane was blocked with fresh blocking buffer (5% (w/v) non-fat dried milk in 0.1% (v/v) Tween-20 in PBS) and incubated for an hour at RT or overnight at 4°C. The next day, it was washed three times in fresh buffer (0.1% (v/v) Tween-20 in PBS). Primary antibody was diluted to appropriate concentration in 5% milk in the previous buffer and left on the membrane for 1h on a gyro rocker at RT or overnight at 4°C. The membrane was washed 3 times for 15 min each as above. The secondary antibody was added to the nitrocellulose membrane at a dilution of 1:1000 or according to manufacturer instructions in 5% (w/v) non fat dried milk in 0.1% (v/v) Tween-20 in PBS and left for 1 h as above. Following washing, the blotted proteins were detected using the ECL Western blotting detection system (GE health care, UK), according to the manufacturer's instructions. The membranes were exposed to Fuji Medical X-Ray film (Fujifilm Europe GmbH) for different time periods and films were developed using OPTIMAX X-Ray Film Processor.

### 2.6.3 Purification of 6xHis-tagged EspF

EHEC O157:H7 *espF* was cloned into pET21d using primers (19&20) table (2.3). This plasmid was electrotransformed into *E. coli* strain BL21 (DE3). From this clone an overnight culture was grown in 25 ml of LB with Ampicilin 100 µg/ml at 37°C and 200 rpm agitation. These amount of culture were then inoculated in a litre of LB with antibiotic under the previous conditions until $OD_{600}$= 0.5. The culture then was induced for protein expression using IPTG 1mM final concentration for 4 h. The culture was divided into two 500 ml bottles and centrifuged at 4000g for 20 min at 4°C, the pellet then was either used or frozen at -20°C until use.

### 2.6.3a Preparation of cleared *E. coli* lysates under native conditions

The cell pellet was thawed for 15 min on ice and was suspended in lysis buffer at 5 ml per 3 g wet weight. One mg/ml of lysozyme and protease inhibitor cocktail specific to be used with histidine-tagged protein purification (Sigma) was added to the suspended pellet. The pellet was then incubated on ice for 30 min. The mixture was Sonicated on ice using a sonicator equipped with a micro-tip. Using four 20 sec bursts using Soniprep150 (SANYO MSE) at power 6 with a 30 seconds cooling period between each burst. The lysate was centrifuged at 14,000 xg for 30 min at 4°C to pellet the cellular debris and supernatant was saved. 1 ml of 50% Ni-NTA slurry (resin) (Sigma) was added to 5 ml cleared lysate and mix gently by shaking at gyroracker for 30-60 min. The lysate–Ni-NTA mixture was loaded into a disposable chromatography column with the bottom outlet capped. The bottom cap was removed and the column flow-through was collected. The flow-through saved for SDS-PAGE analysis.

The column then was washed twice with 4 ml wash buffer; wash fractions were collected for SDS-PAGE analysis. The protein was eluted 4 times with one ml elution buffer. The eluate was collected in four tubes and analyze by SDS-PAGE.

### 2.6.3b Preparation of cleared *E. coli* lysates under denaturing conditions

The cell pellet was thawed for 15 min on ice and suspended in denature purification buffer (pH8) at 5ml per 3 g wet weight. Cells were stirred for 30 min on gyroracker

at room temperature. Lysis was complete when the solution becomes translucent. The lysate was centrifuged at 14,000 x *g* for 30 min at room temperature to pellet the cellular debris, and the supernatant was saved (cleared lysate). 5µl of 2x SDS-PAGE sample buffer was added to 5 µl supernatant and stored at –20°C for SDS-PAGE analysis. 1ml of the 50% Ni-NTA slurry was added to 5 ml lysate and mix gently by shaking at gyroraker for 30–60 min at room temperature. The lysate–resin mixture was loaded carefully into a disposable chromatography column with the bottom cap still attached. The bottom cap was removed and the flow-through was collected. Flow-through sample was collected for SDS-PAGE analysis. The column then washed twice with 4ml of denature purification buffer (PH6.3). Wash fractions kept for SDS-PAGE analysis. The recombinant protein was eluted 4 times with one ml denature purification buffer (PH4.5). Samples were collected for analysis by SDS-PAGE.

### 2.6.3c Dialysis

The elute was poured into dialysis tube with pour diameter 14 KD and was soaked into sterile solution of PBS in a ratio 1 ml of elute in a litre of PBS for overnight. The PBS was changed for another overnight and the buffer free protein was collected and saved at -20°C**.**

## 2.7 Yeast techniques

### 2.7.1 Solutions for Yeast production

### 2.7.1a YPD Medium for growing up yeast

For 1 litre

20g Peptone

10g yeast extract

Make up in 900 ml, and autoclave.

Add 100 ml of 20% sterile-filtered glucose once autoclaved.

If making YPD plates, add 30 g Agar per Litre.

### 2.7.1b SD Medium (for transformed yeast) – For 1 Litre

26.7 g minimal SD base

0.64 g –Leu –Trp drop-out supplement

20 g Agar

Make upto 900 ml

Autoclave, when cool, 100 ml amino acid supplements : Trp for AH109 (minus Leu); Leu was added for Y187 (minus Trp).

Stock Amino Acid solutions – 10 x stocks

Tryptophan – 200 mg in 1 Litre

Leucine – 1 g in 1 Litre

### 2.7.1c SBEG – 500 ml

| | |
|---|---|
| Sorbitol, 1 M final conc | 91.1 g |
| 1 M Bicine, pH 8.35 with NaOH, sterile | 5 ml |
| PEG | 15 ml |
| $DH_2o$ | up to 500 ml |
| Filter sterilised. Bicine | final concentration : 10 mM |

### 2.7.1d PEG/Bicine Solution – for 100 ml

| | |
|---|---|
| PEG (powder) | 40 g |
| 1 M Bicine, pH 8.35 with NaOH, sterile | 20 ml |
| $DH_2O$ | up to 100 ml |

Filter sterilised. Final Bicine concentration 200 mM

### 2.7.1e NB Buffer – for 100 ml

| | |
|---|---|
| Na Cl | 0.88 g |
| 1M Bicine | 1 ml |
| $DH_2O$ | up to 100 ml |

Final concentration of Bicine is 10 mM.

Final concentration of NaCl is 0.15 M

### 2.7.1f Mating medium

| | |
|---|---|
| SD-LW | 472.5 ml |
| 5% YPDA | 25 ml |

| | |
|---|---|
| Pen/Strep | 2.5 ml |

### 2.7.1g Diploid Selection Medium 500ml

| | |
|---|---|
| SD-LW | 497.5 ml |
| Pen/Strep | 2.5 ml |

### 2.7.1h Detection medium

| | |
|---|---|
| SD-LWH (SD no L, W, H) | 497.5 ml |
| Pen/Strep | 2.5 ml |

50µM 4MuX

3-AT (standard range: 0-20 mM 3-AT)

4-MuX: 4-Methylumbelliferyl-alpha-D-galactoside (FW:338.3, Sigma M-7633)
Dissolve in dimethylformamide – 100 mg in 5.917 ml to make 50 µM (1000 x)
3-AT: 3-amino-triazol. 4.2 g in 50 ml to make 1 M stock (100 xs for 10 mM 3-AT)
Both are toxic and should be handled under a fume hood.

### 2.7.2 Yeast -2-hybrid (Y2H) system for detecting protein-protein interactions

The Y2H was first described by Fields & Song in 1989 and at present used to identify protein-protein interactions in eukaryotic cells. Y2H is based on the theory that a transcription factor consists of a DNA binding domain (BD) that binds to the promoter and an activation domain (AD) that recruits the transcription complex. These domains are independently not functional but when they are brought together the activity of the transcription factor is restored (Fields & Song, 1989). In the Y2H system a protein of interest (X) is fused to the DNA-binding domain (DNA-BD) of yeast transcription factor (Bait), whereas a Protein Y is fused to the transcriptional activation domain (TA) (Prey).

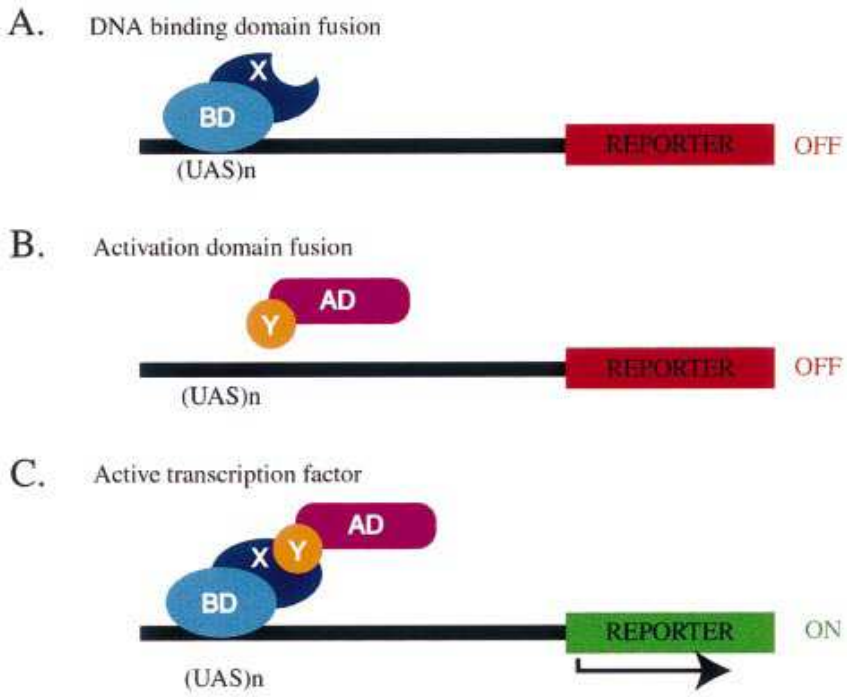


**Fig. 2.4 The mechanism yeast-two-hybrid system**

This system was used to identify the protein-protein interaction. The protein to be screened (X) is fused to the DNA-binding domain (DNA-BD) of yeast transcription factor (Bait) while the other protein from the library to be identified (Y) is fused to the transcriptional activation domain (TA) (Prey). If these two proteins interact together the transcription factor activity is reconstituted and auxotrophic genes such as *hiS3* that give growth to the cells in which an interaction has occurred are then expressed. This figure was taken directly from (Stephens & Banting, 2000).

Both fusions are targeted to the nucleus of the yeast and if protein X and Y interact, the transcription factor activity is reconstituted and auxotrophic genes such as *hiS3* that give growth to the cells in which an interaction has occurred are then expressed (Fig. 2.4**)**.

### 2.7.2a Production and transformation of competent yeast

Yeasts are eukaryotic micro-organisms typically measuring 3–4 µm in diameter, however some yeasts can reach over 40 µm. they are classified in the kingdom Fungi. They are unicellular organism however some of them can be multicellular through the formation of a string of connected budding (Walker *et al.*, 2002).

Two yeast strains were used in this study, AH109 and Y187 provided by (Ragnhild Eskeland, MRC). Bait plasmid (pGBKT7) contains Trp gene that enable AH109 to grow on Leu+ve, Trp-ve plates. Prey plasmid (pGADT7) contains Leu gene that help Y187 grow on Trp +ve, Leu -ve plates. For different EspFs screen: Yeast strain Y187 for Prey and Yeast strain AH109 for Bait

### 2.7.2b Production of competent yeast

An overnight culture of each yeast strain was produced by inoculating one fresh growing colony using a metal inoculating loop into 50 ml YPD media in a 250 ml flask using sterile techniques. The cultures were incubated at 30°C and rotated at 260 rpm overnight. The overnight culture was inoculated into 300 ml YPD and was grown under the previous conditions until $OD_{600}$ = 0.5. The culture was divided into 6 sterile tubes previously child on ice. The cells were pelleted by centrifugation at 930 xg for 10 min at 4°C. The supernatant was discarded carefully as the pellet does not stick as well as bacteria and the cells were re-suspended in 25 ml ice child SBEG solution. The cells were pelleted by centrifuging at 930 x *g* for 10 min at 4°C and the supernatant was discarded. The palette was suspended into 840 µl cold SBEG solution. All suspended yeast cells were collected together to be use as competent yeast.

### 2.7.2c Transformation competent yeast

The transformation must be done at the same day at room temperature. 1 µg of plasmid was added to an Eppendorf. 100 µl of the appropriate competent yeast strain added to each Eppendorf, and mixed well with the DNA by pipetting several times. 750 µl PEG/Bicine solution was added and mixed by pipetting up and down. The complex was incubated at 30°C and incubated at 900 rpm for an hour. The cells were heat shocked by incubation at 45°C in a water bath for 5 min. the cells were pelleted by centrifuging for 2 min at 2700 x *g*. The supernatant was discarded and the pellet suspended in 1ml NB buffer. The cells were centrifuged under the previous conditions and the cells were suspended in 200µl NB buffer. The whole 200 µl of cells were plated onto SD PLUS TRP (minus Leu for Prey) or SD PLUS LEU (minus Trp for Bait) under sterile conditions (preferably in a hood, or on a bench next to a flame) and left to dry in the hood, and then incubated at 30°C. Colonies started to appear after 2-3 days.

### 2.7.2d Production of glycerol stocks for yeast cultures

A yeast colony was picked and added to 3-5ml SD drop-out media in a 15 ml centrifuge tube (Plus TRP minus LEU for Prey; Plus LEU minus TRP for Bait). The culture was grown for 2-3 days at 30°C and shaking at 260 rpm or until visible yeast pellet was observed at the bottom of the tube. The culture was stored at -80°C in a final sterile glycerol concentration of 25%.

### 2.7.2e Direct mating for Y2H screens

This protocol is designed for mating bait against multiple defined preys. The other orientation (prey against multiple baits) works analogously. Before starting this assay, please ensure the prey and bait constructs are transformed in different but compatible yeast strains (Y187 and AH109). All the growth media contains pen-strep, so contaminations should not occur.

Yeast bait and prey clones streaked from glycerol stocks on selective agar plates and incubated for 1-3 days at 30°C. The haploid yeast was inoculated in 5 ml liquid medium for pre-culture should be at the day. The 5 ml freshly grown yeast was inoculated in 25 ml of a single-selective liquid medium - SD minus Trp for Bait and

SD minus Leu for Prey. The cultures were incubated overnight at 30°C and shaking at 220 rpm in an appropriate vessel. So that cells don't grow too densely overnight as high density of the culture impairs mating efficiency. The original protocol suggests an $OD_{600}$ of 1.0 to 1.2 as a benchmark to achieve high mating efficiency. 100 µl mating media (double-selection –LW SD/5% YPDA; minus Leu and minus Trp) was add to the required number of wells of a U-bottom micro-titre plate. This was the mating plate. A U-bottom plate must be used at this stage to enable close proximity of yeast cells. The bait cultures suspended and 25 µl transferred to each appropriate well of the mating plate. The prey cultures suspended and 25 µl transferred from each culture to the mating plate. The plate was centrifuged at 2000 rpm for 30 sec at room temperature to bring cells in close proximity to each other at the bottom of each well. This was to facilitate mating. The plate(s) were incubated at 30°C overnight without shaking to allow the cells to mate. It is important to set up the mating in the morning when the cells haven't grown to saturation. The wells of a flat-bottom microtitre plate sated up with 150 µl of fresh double-selective media (SD –L-W/pen-strep no YPDA).This to be the diploid selection plate. The yeast pellets suspended in the mating plate and 10 µl transferred from each culture to the diploid selection plate. The plate(s) incubated at 30°C without shaking for 2 days. The diploid cells checked visually for growing (to saturation or close to saturation). The detection media was prepared as described overleaf. This was a triple-knock-out SD media (no Trp, Leu or His) with a fluorescent detection compound 4-MuX and 3-AT, which inhibits auto-activation of baits. Usually a range of 3-AT concentrations is tested from 0, 0.5, 1, 2.5, 5 and 10, sometimes up to 20mM 3-AT. Duplicate assay plates prepared by adding 150 µl detection media (detection plate) or SD-LW (control diploid selection plate) to a sufficient number of wells of flat-bottom microtitre plates. The SD-LW plates will confirm that diploid yeast cells are in fact growing during the detection assay. The diploid cultures suspended in the diploid selection plate and 10µl transferred from each culture to each detection and control plate, covered with gas-permeable lids and placed in a plastic bag. The cells incubated at 30°C for 3-7 days, no shaking. 3 days is usually sufficient to detect an interaction. The cells suspended in the control plate and absorbance measured at $OD_{600}$ to confirm yeast cell growth and that the mating has in fact worked. This should be blanked with double knock-

out selective media. The fluorescence readout was measured using a fluorescence reader: excitation at 365 nm; emission at 448nm. Any measurement of fluorescence will indicate that a protein interaction has occurred. According to the original protocol by Manfred Koegl, cells have to be passaged once more (10 µl culture into 150 µl detection medium) to see a difference between positive and negative wells in some cases, since high fluorescence signals from false-positive interactions are not maintained in the second passage.

## 2.8 LUMIER assays

For LUMIER assays, proteins were transiently expressed in HEK293 cells as hybrid proteins with the *Staphylococcus aureus* protein A tag or *Renilla reniformis* luciferase fused to their amino termini. 20 ng of each expression construct was transfected into HEK293 cells using 0.05 µl of lipofectamine 2000 (Invitrogen) in 96 well plates. After 40 h, the medium was removed and cells were lysed on ice in 10 µl of ice-cold lysis buffer (20 mM Tris pH 7.5, 250 mM NaCl, 1% TritonX-100, 10mM EDTA, 10mM DTT, Protease Inhibitor Cocktail, Phosphatase Inhibitor Cocktail (both Roche) and 25 units/µl Benzonase (Novagen) final concentration). Sheep-anti-rabbit IgG-coated magnetic beads were also added (Invitrogen, Dynabeads M280, 2mg /ml final concentration) and incubated on ice for 15 min. 100 µl of washing buffer (PBS, 1mM DTT) was added per well and 10% of the diluted lysate removed to determine the luciferase activity present in each sample before washing. The rest of the sample was washed 6 times in washing buffer. Luciferase activity was measured in the lysate as well as in washed beads. Negative controls were wells transfected with the plasmid expressing the luciferase fusion protein and a vector expressing a dimer of protein A. For each sample, four values were measured: the luciferase present in 10% of the sample before washing (“input”), the luciferase activity present on the beads after washing (“bound”), and the same values for the negative controls (“input nc”, and “”bound nc”). Normalised signal to noise ratios were calculated as follows (bound/input)/(bound nc/input nc) (Braun *et al.*, 2008).

## 2.9 Cell Culture

### 2.9.1 Tissue culture media and supplements

### 2.9.1a Basic Media

DMEM: purchased from Sigma, supplied in 500 ml sterile bottles, supplemented with 4.5 g/L glucose and stored at 4°C.

RPMI 1640 medium purchased from Gibco-BRL, supplied in 500 ml sterile bottles and stored at 4°C.

### 2.9.1b Other sterile solutions and supplements

Foetal Calf Serum (FCS): Purchased from Gibco, liquid supplied sterilised in 500 ml bottles. FCS was aliquoted into 50 ml sterile bottles and stored at -20°C.

Glutamine: Purchased from Lonza, L-glutamine solution was available in 100 ml sterile bottles supplied at 29.2 mg/ml in 0.85% NaCl, pH 4.7-6.0. The stock solution was stored at -20°C in 5 ml aliquots. 5 ml of L-glutamine was added to 500 ml of media. Non-essential Amino Acid: Purchased from Lonza, liquid supplied in 100 ml sterile bottles. The 100 x solution was diluted with media to 1x solution and stored at 4°C. Penicillin/streptomycin: Purchased from Lonza, Penicillin/streptomycin solution was formulated to contain 20000 U/ml penicillin and 20000 μg/ml streptomycin. It was aliquoted into 5 ml aliquots and stored at -20°C. It was used at 5 ml per 500 ml media. Phosphate-Buffered Saline (PBS): Purchased from Lonza, liquid supplied in 500 ml sterile bottles. PBS was formulated to contain 0.0067 M PO4.and stored at 4°C. Trypsin EDTA: Purchased from Lonza, Liquid supplied sterilised in 100 ml sterile bottles. Trypsin EDTA solution contains 200 mg/L EDTA and 170.000 U/L trypsin.

**Table 2.8 Macrophage and Caco2 growth medium**

| Reagent | Supplied conc. | Required final conc. | Volume added |
|---|---|---|---|
| **DMEM** | 4.5 g glucose/litre | As supplied | 440 ml |
| **FBS** | 100% | 10% | 50 ml |
| **L-glutamine** | 200 mM (100X) | 1X | 5 ml |
| **Pen-Strep** | 100% | 1% | 5 ml |
| | | Total volume | 500 ml |

**Table 2.9 HeLa cells growth medium**

| Reagent | Supplied conc. | Required final conc. | Volume added |
|---|---|---|---|
| **MEM** | As supplied | As supplied | 440 ml |
| **FBS** | 100% | 10% | 50 ml |
| **L-glutamine** | 200 mM (100X) | 1X | 5 ml |
| **Pen-Strep** | 100% | 1% | 5 ml |
| | | Total volume | 500 ml |

### 2.9.1c Reagents used for isolation of bovine primery cells:

DMEM Sigma D5796

MEM D-Valine Lonza BE02-020F

Hanks BSS Sigma H9269

FBS Sigma F0643

Gentamicin (50mg/ml) Sigma G1397

Pen/Strep (10,000 Units/ml) Invitrogen 15140122

Fungizone (250µg/ml) Invitrogen 15290026

L-glutamine (200mM) Invitrogen 25030024

Collagenase Sigma C2674: Add 1.67 ml DMEM and store in 250 µl aliquots at -20°C

Dispase I Roche 04942086001: Add 1ml DMEM. Store at $4^{o}$C.

Insulin Sigma I2643: Add 1450 µl DMEM. Store in 125 µl aliquots at -20°C

Epidermal Growth Factor Sigma E4127 (Reconstitute in 1ml of 1%BSA in PBS. Store in 10µl aliquots at -20°C)

Vitrogen Collagen Nutacon 5409

### 2.9.1d Media for washing tissue after flushing of luminal contents

HBSS 500 ml

Gentamicin (25µg/ml) 250 µl

Fungizone (5µg/ml) 5 ml

Pen/strep (100 Units/ml) 5 ml

### 2.9.1e Digestion Medium

| | |
|---|---|
| DMEM | 100 ml |
| FBS (1%) | 1 ml |
| Gentamicin | 250 µl |
| Pen/Strep | 1 ml |

### 2.9.1f Enzymes: added once digestion medium has been added to cells

| | |
|---|---|
| Collagenase | 250 µl (75 U/ml) |
| Dispase I | 250 µl (20 µg/ml) |

### 2.9.1g Differential Centrifugation Solution

| | |
|---|---|
| DMEM | 500 ml |
| D-Sorbitol (2%) | 10 g |

Dissolve sorbitol in small quantity of DMEM and then filter sterilize it before adding to the DMEM bottle

### 2.9.1h Collagen Solution

| | |
|---|---|
| Sterile Distilled Water | 45 ml |
| Acetic Acid | 150 µl |
| Vitrogen Collagen | 6 ml |

Filter sterilise water and acetic acid before adding to collagen.

### 2.9.1i Primary culture Medium

| | |
|---|---|
| DMEM | 480 ml |
| 2% FBS | 10 ml |
| L-glutamine (2 mM) | 5 ml |
| Gentamicin (25 µg/ml) | 250 µl |
| Pen/Strep (100 Units/ml) | 5 ml |
| Epidermal Growth Factor (10ng/ml) | 5 µl |
| Insulin (0.25U) | 125 µl |

Filter sterilise all additives before adding to DMEM

### 2.9.1j Feeding Medium

| | |
|---|---|
| MEM-D Valine | 465 ml |
| 5% FBS | 25 ml |
| L-glutamine (2 mM) | 5 ml |
| Gentamicin (25μg/ml) | 250 μl |
| Pen/Strep (100 Units/ml) | 5 ml |
| Epidermal Growth Factor (10 ng/ml) | 5 μl |
| Insulin (0.25 U) | 125 μl |

Filter sterilise all additives before adding to MEM-D Valine

**Table 2.10 Monoclonal antibodies used in immuno cytochemical screening of bovine rectal primary epithelial cell cultures**

| Monoclonal Antibody | Specificity | Cellular Expression | Source | Reference |
|---|---|---|---|---|
| CC21 | CD21 | Follicular Dendritic Cells, Mature B Cells | IAH | (Naessens & Howard, 1991) |
| CC20 | Bovine CD1b | Dendritic Cells | IAH | (Howard *et al.*, 1993) |
| CC15 | Bovine WC1 | γδ T Cell | IAH | (Howard *et al.*, 1989) |
| ILA-12 | CD4 | T helper Cells | ILRAD | (Teale *et al.*, 1987) |
| ILA-51 | CD8 | Cytotoxic T Cells | ILRAD | (Teale *et al.*, 1987) |
| ILA-156 | CD40 | B cells, Antigen Presenting Cells | ILRAD | (Norimatsu *et al.*, 2003) |
| ILA-111 | CD25 | Activated T and B Cells, Macrophages | ILRAD | (Choy *et al.*, 1990) |
| ILA-43 | CD2 | αβT cells, Natural Killer cells | ILRAD | (Teale *et al.*, 1987) |
| Anti-hPH | β-subunit of hPH | Fibroblasts | Acris GmbH | (BAI *et al.*, 1986) |

ILRAD - International Laboratory for Research on Animal Diseases, Nairobi, Kenya
IAH- Institute for Animal Health, Compton

### 2.9.2 Techniques used in cell cultures

### 2.9.2a Maintenance of cell lines

All cell lines were maintained in incubators at 37°C or 38.5°C supplied with 5% $CO_2$. Cells were grown on a variety of tissue culture plasticware depending on the nature of the experiment. Cultures were grown to 90-95% confluency, washed with

PBS and trypsinised from the flasks with trypsin solution (Lonza, Belgium). Cells were incubated at 37°C until all cells were dissociated from the flask. Cells were recovered by adding growth media containing 5 or 10% serum. Cells were centrifuged at 1000 rpm for 10 min in a benchtop centrifuge. The cells pellets were re-suspended in growth medium and counted by use of a haemocytometer. Cells were seeded at a density required by the experimental design.

### 2.9.2b Splitting of cells

1X solution of Trypsin-EDTA (Invitrogen (Gibco) 15400-054) [10X] was prepared in a balanced salt solution (PBS)]. Cell supernatant was poured and 10 ml of Trypsin-EDTA was added swirl, to ensure the entire growing surface is covered and the flask was incubated for 5-10 min. The flask was then agitated to loosen the cells (ensure cells lifting by looking down microscope). 5 ml media was add to quench Trypsin-EDTA and the contents of the flask transferred into Falcon tube and centrifuged for 5min and the cell pellet were plated with fresh media.

### 2.9.2c Freezing of cell lines

Cells were resuspended in 1 ml of pre-cooled freezing medium. The freezing medium consists of equal amount of FCS and 20% DMSO in FCS (Fluka, Germany). Cells were aliquoted in sterile freezing ampoules (Nunc, UK) at a density 2 x $10^6$ cells/vial. Cells were transferred to a cryopreservation box (Nalgene) and frozen overnight at -80°C. The next day, the cells were transferred to the liquid nitrogen storage tank.

### 2.9.2d Thawing of frozen cell lines

Frozen ampoules were transferred from the liquid nitrogen in dry ice and thawed rapidly by warming in a 37°C water bath. Thawed cells were transferred into 20 ml universal tubes and recovered by adding equal volumes of growth media slowly and then adjusted to 5 ml. Cell suspension were centrifuged for 1200 rpm for 5 min and the supernatant were removed and the pelt was resuspended in fresh medium and cultured into 25 ml flask.

### 2.9.2e Phagocytosis assays.

The murine macrophage cell line (RAW 264.7) was cultured in DMEM (Sigma) supplemented with 10% heat inactivated foetal bovine serum (FBS, Sigma), 1 unit of penicillin, 1µg/ml of streptomycin and 2mM L-glutamine (final concentrations). Cells were grown at 37°C in 5% $CO_2$ and moisture. The bacteria, transformed with pUC-gfp (Table 3) were inoculated from LB broth overnight cultures into DMEM to $OD_{600}$ = 0.3. For wildtypes strains it was diluted 1/10 and 100 µl was added to each well (MOI of 20 bacteria per cell). When analysed, the *espF* alleles were induced with IPTG (1mM). At $OD_{600}$ = 0.7 the bacterial cultures were then diluted 1:5 into pre-warmed DMEM and 300 µl added to the macrophages (initially seeded at 1x $10^5$ cells/well the day before). The cells were then incubated at 37°C in 5% $CO_2$ in a moist box for the desired incubation time. The cells were washed three times with sterile phosphate buffered saline (PBS) and fixed using 2.5% paraformaldehyde , PH 7.4. After three washes with PBS, the samples were incubated for 90 min with the relevant anti-LPS antibody (MAST Group Ltd) at 1:100 and following washes incubated with Alexa Fluor 594-conjugated goat anti-rabbit immunoglobulins antibody (1/2000, Molecular Probes) for an hour. The slides were then washed three times with PBS and mounted with Hydromount (National Diagnostic) and cover slips applied. Slides were examined by fluorescence microscopy using a Leica Q fluorescence microscope and appropriate filter sets. All bacteria, internal and external, express GFP whereas only the bacteria external to the macrophages stain with the anti-O157 LPS antibody. This differential staining allows the proportion of internalized bacteria to be quantified.

### 2.9.2f Bacterial binding assays on caco-2 cells

Caco2 cells were infected with Δ *espF* EPEC containing the following plasmids

1. Δ*espF* EPEC + (pAJR145) GFP chloramphenicol(cam
2. Δ*espF* EPEC + pTS1 plasmid containing EPEC *espF*(Amp) + GFP(Cam)
3. Δ*espF* EPEC + pTS1 plasmid containing *espF* EHEC O157:H7 (AMP) + GFP (Cam)
4. Δ*espF* EPEC + pTS1 plasmid containing *espF* EHEC O26:H11 (Amp) + GFP (Cam).

For the assays, the bacteria were cultured as for the macrophage assays to an $OD_{600}$ = 0.7. The bacteria were then diluted 1:100 in DMEM prior to infection. A multiplicity of infection of 10:1 (bacteria: Caco2 cell) was used and the infection allowed to progress for 2 h at 37°C in a 5% $CO_2$. Cells were washed gently twice with PBS and fixed in 4% PFA and then kept in a fridge overnight. PFA was removed and the bacteria stained and analyzed as described for the macrophage assay.

### 2.9.2g Transfection of adherent HeLa and Caco2 cells using Effectene (QIAGEN) transfection reagents

Effectene reagent (Qiagen, UK) is a lipid based reagent that is suitable for the transfection of plasmid DNA into cultured eukaryotic cells. It is used with a special condensed DNA enhancer. The enhancer is used first to condense the DNA molecules, whilst the effectene reagent subsequently coats the condensed DNA molecules with cationic lipids.

Cells were plated in 48 well plates at a density of $5x10^4$ in 400 µl DMEM with FCS and antibiotics 48h before transfection to reach optimal confluence for transfection (40-70%, approx. $2x10^5$ cells per well). 150 ng of total DNA was diluted EC buffer to a final volume of 50 µl then 1.2 µl of enhancer was added to the above solution and mixed together by vortex and incubated for 5 min at room temperature to allow formation of condensed DNA. Then 4 µl of effectene was added to the above solution and incubated for 10 min at room temperature to allow formation of the DNA transfection complex. At the same time the medium was removed from the plates and the cells were washed with PBS and 200 µl of fresh medium added (DMEM containing serum and antibiotic). After the lipid-DNA complex formation, 200 µl of fresh medium (DMEM containing serum and antibiotic) was then added to the DNA mixture. This complex was poured onto adherent cells drop by drop, and swirling carried out to ensure an even distribution. Protein expression was determined 48-72 h post transfection and stable cells were selected by removing the media and adding 200 µg/ml of G418 (Invitrogen) in fresh medium. The transfection efficiency was checked after 24 h using eGFP expression as a positive readout.

### 2.9.2h Addition of Mitochondrial marker

For use of this marker, the cells slides were fixed and permeablized using 200 µl of 2% (v/v) formalin and 0.2 Triton X-100 in PBS [4ml of 10% formalin, 16 ml PBS and 40µl of Triton] for 20min at room temperature, and then washed twice with PBS. Mito Tracker (Molecular Probes) was added at different concentrations: 125, 250 or 500 nM in PBS for 30 min and then washed two times with PBS before mounting and analysis as described for the macrophage assay.

### 2.9.2i Bovine primary rectal epithelial cell cultures

Terminal rectal tissue from adult cattle was obtained from a local abattoir. The specimens were placed in Hank's Balanced Salt Solution (HBSS) containing gentamicin (25 µg/ml) on ice and transported to the laboratory. The specimens were longitudinally opened and luminal contents flushed out with HBSS. Two pieces of mucosal epithelium 3 cm and 25 cm proximal to recto-anal junction were excised and washed vigorously several times in HBSS with gentamicin (25 µg/ml), penicillin (100 U/ml), streptomycin (30 µg/ml) and amphotericin B (200µg/ml) in a sterilized tissue collecting pot to remove mucus and any adhering contaminants/ materials. The procedure for primary cell culture was essentially as described by (Booth *et al.*, 1995; Hoey *et al.*, 2003) with a few modifications. 24-well tissue-culture plates (Coring incorporated, USA) and chamber slides (Nagle Nunc international USA) were coated with bovine collagen solution (Nutacon 5409) 500 µl/well and allowed to dry overnight into the class II cabinet. Collagen coated plates and chamber slides were stored at -20°C until required. Collagen-coated plates were removed from -20°C and rehydrated with 500 µl PBS or HBSS (Sigma H9269). Pots containing tissues were sprayed outside with 70% ethanol before taking into the class II cabinet. The rectal pieces were removed and placed on paper towel in the class II hood. The mucosal epithelium was scraped with a sterile glass slide and the scrapings collected into a 50ml centrifuge tube.

In order to wash the scrapings the tube was filled up to 50ml with HBSS, mixed vigorously, centrifuged at 1200 rpm for 2 min and the supernatant was discarded. This step was repeated until the supernatant became quite clear (about 5 times).

Sometimes a lot of mucus attached to the crypts, to remove this; the mucus above the pellet was sucked up by pipetting. The crypts pellet is more compact and has a slightly greyer colour. Floating material was removed after the 4th wash. 40 ml of supernatant was discarded after the last wash and the pellet was transferred to fresh a 50 ml tube. Digestion medium was added to the pellet (no more than 10ml) to make the final volume 25 ml. 250 µl of Collagenase (75 U/ml) (Sigma) and Dispase I (20 µg/ml) (Roche) were added. Digestion was performed with the tube taped horizontally to the shaker at 37°C for 80 min. After digestion, the digestion mix was pipetted up and down gently to loosen cells. One drop was taken onto a Petri dish to check for the integrity of the crypts. Crypts were seen under the microscope as small cylindrical structures of variable sizes and shapes with lots of other digested tissue debris around.

Digested material was put into another tube and allowed to settle for 2min. The tube was filled up to 50 ml with HBSS and pipetted gently to disrupt crypts clumped together. The digestion mix then centrifuged for 2 min at 1200 rpm. One drop of the supernatant was examined under the microscope for the presence of crypts by taking a drop from 20 ml mark on the tube. If there were still crypts in the supernatant, the mix should be split into 2 tubes and diluted with HBSS, mixed by gentle pipetting and centrifuged at 1200 rpm for 2 min. This step was repeated again for crypts in supernatant from the 25 ml mark. (If clear, 25 ml of the supernatant was removed and the tube was filled up with differential centrifugation medium and mixed gently. The mix was centrifuged at 700 rpm for 2 min). The supernatant was checked for crypts at the 25 ml mark and removed if clear. The tube was filled up with differential centrifugation medium (optional), pipetted up and down gently to mix and centrifuged at 1200 rpm for 2 min. A drop of supernatant was checked for presence of crypts after each spin from the 20 ml mark on tube. If there were still crypts there, check at 30ml mark and so on. Remove clear supernatant and add more differential centrifugation medium. Each time the amount of medium was reduced by 10 ml. This process was repeated until supernatant became clear of crypts. Supernatant was kept when there were a few crypts just in case there were none in the pellet. The pellet was washed with HBSS and resuspended in an appropriate

amount of Primary culture medium depending on size of pellet. The required final concentration should be 5000 crypts per ml; this can be adjusted by count of the number of crypts in 10 µl on a Petri dish. The appropriate amount of culture medium was added.

HBSS was removed from collagen coated plates and 800 µl of primary culture medium was added. 3 or 4 drops of the crypts were added (from a 10 ml pipette) to each well to add 500-700 crypts to each well. The crypts were cultured in primary culture medium for 24 h. At this stage most of the crypts had attached to the surface and the non-adherent tissue debris was removed and media was replaced with MEM D-Val selective medium supplemented 1% or 2.5% batch tested FCS, 0.25 U/ml insulin, 10 ng/ml EGF and 30 µg/ml gentamicin. This medium inhibits any fibroblast contaminants in the culture (Frauli & Ludwig, 1987; Lazzaro *et al.*, 1992; Hoey *et al.*, 2003). 50% of the medium was replaced every two days. The epithelial cells proliferated from attached crypts and formed a confluent monolayer within 6 to 8 days of culture. The cultures were incubated at 37°C in an atmosphere of 5% $CO_2$, 95% air with 90% relative humidity.

Bovine rectal tissues with intact anal canal about 30 cm proximal to recto-anal junction were brought from the abattoir in a polystyrene box with ice to transport the tissue.

### 2.9.2j Characterisation of bovine primary rectal cultured cells.

The epithelial origin of the cells was confirmed by immuno-staining for cytokeratin intermediate filaments. The cells at five days of culture were fixed with 2% paraformaldehyde, permeabilized with cold acetone for 5 min, washed with phosphate-buffered saline (PBS) and stained with a pan-cytokeratin monoclonal antibody (Sigma 1:300) for 3 h at 25°C. The cells were washed with PBS and the monoclonal was detected using FITC-labelled goat anti-mouse mAb (1:80) (Sigma). The cell nuclei were stained with TO-PRO Iodide (Molecular Probes). The stained cells were mounted in fluorescence mounting medium Fluoromount (DAKO) and examined using a Leica DMLB epifluoresence microscope. To ascertain if lymphoid cells were present in primary cell cultures from the crypts isolated from lymphoid

rich mucosal tissue, immuno-staining was carried out with a panel of seven monoclonal antibodies (Table 2.10) specific for different immune cell types of cattle. The cells were fixed either in paraformaldehyde (3.5%) or non-formaldehyde zinc acetate based fixative. Briefly, cells at 72 h of culture were washed twice in PBS and fixed in freshly prepared zinc salt fixative solution (0.1M Tris base buffer with Ca acetate 0.5% pH 7-7.4, containing Zn acetate 0.5% and Zn chloride 0.5%). After initial blocking of endogenous peroxidase activity with 0.3% hydrogen peroxide for 5 min and the non-specific binding with 25% normal goat serum for 30 min the cells were immuno-labelled overnight at 4°C (Table 2.4). The primary antibody binding was detected using goat anti-mouse peroxidase labelled secondary antibody (Horizontal En Vision Plus HRP system, Dako, Ely, UK) for 30 min at RT. The slides were developed in a citrate buffer containing 3,3'diaminobenzidine hydrochloride for 7-8 min. Cell monolayers were counterstained with haematoxylin, rinsed, dehydrated and mounted. Tris-buffered saline (TBS, 0.05M Tris HCl, 0.15M NaCl, pH 7.6) was used to wash the slides between each stage of the labelling procedure and to dilute normal goat serum and antibodies. As a negative control primary antibody was replaced with TBS in the staining protocol.

Uptake of inert micro-particles has been used as a functional assay for M-cells with in vitro cultures (Kerneis *et al.*, 1997). FITC-conjugated latex beads of 0.5 μm size (Polysciences Inc., Germany) were diluted (1:1000) in DMEM containing 2% fetal bovine serum. Aliquots (100 μl) of diluted beads were pipetted evenly on to 6-day old cultures and incubated at 37 °C for 45 min. The cells were washed three times in phosphate-buffered saline, fixed and permeabilized with 2% (w/v) PFA/ 0.25% (v/v) Triton X-100 at room temperature for 20 min. Staining of F-actin was done with Phalloidin-FITC/ TRITC (diluted 1:20 in PBS; Molecular Probes) for 45 min at room temperature in the dark. For co-localization studies with vimentin, the cells were incubated with mouse anti-vimentin monoclonal antibody (1:100) (Sigma) and incubated overnight at 4°C. This primary antibody was detected with Alexa 594-tagged rabbit anti-mouse or goat anti-mouse monoclonal antibodies as per the manufacturer's instructions. The cell nuclei were stained with TO-PRO Iodide (Molecular Probes). The mounted slides were examined by confocal microscopy. To

examine the position of the beads, 0.4 μm optical sections were acquired and processed via Imaris Surpuss Module (Bitplane) computer software programme. For co-localisation studies with micro-particles the infected cells were washed three times with pre-warmed (37°C) MEM/HEPES and further incubated with FITC-conjugated latex beads of 0.5 μm size (Polysciences Inc., Germany) for 45 min and processed for immuno-fluorescence as described above.

### 2.9.2k Staining primary Bovine Epithelial cells for FACS

The percentage of cells expressing vimentin in the bovine terminal rectal primary epithelial cell cultures were detected using flow cytometry (FACS). Briefly, the cells were removed from plates by trypsinisation. The cells were transferred to sterile universal and centrifuged at 200 x g for 5 min. The supernatant removed and the pellet washed once with PBS. The cells were fixed and permeabilised in 500 μl Cell Permeabilisation solution (Becton Dickenson) for 10min at room temperature. The cells washed in PBS with 0.5% Bovine Serum Albumin and 0.1% Sodium Azide. The cells divided between an appropriate numbers of tubes. One tube left without primary antibody as control for each vimentin antibody and spin down at 2500 rpm for 3 min. Primary antibody diluted in FACS medium (DMEM, 1% FBS, 0.1% Sodium Azide) was added to the cell pellet and vortexed. The cells were incubated at $4^{o}$C for 30 min, spined down as above and washed twice in FACS medium. The secondary antibody added to each tub including a tube with no primary staining (negative control). The cells then incubate at $4^{o}$C for 30 min, centrifuged as before, and washed twice in FACS medium. The cell pelts were re-suspended in 200 μl FACS medium and measure fluorescence on the FACS Calibur.

The work presented in this figurer 3.2.8a A, B, and C. and 6.2.1 was done by Dr Arvind Mahajan and Ms Edith Paxton while figures 5.2.4, 5.2.6 and 6.2.1 were done by Ms Edith Paxton.

### 2.9.2l Trancytosis assays

Caco-2 and primary bovine terminal rectal epithelial cells were allowed to establish monolayers on 0.4 μm pore size polycarbonate filters of 35 mm Transwell chambers (Corning incorporated, USA) by growing them for 2-3 weeks until confluences.

Before seeding primary bovine terminal rectal epithelial cells, the Trans well plates were coated with collagen (Nutagen). Integrity of cell monolayer, polarization, and formation of tight junctions were tested by measuring the TER using epithelial Voltohmmeter (WPI, USA). Only filters of cell monolayers that displayed the required TER were used for bacterial transcytosis assay: for Caco-2, 200–300 Ω/cm2; bovine terminal rectal primary epithelial cells, 100 Ω/cm2. To establish M-cell co-culture system RajiB cells were seeded in the lateral chamber of the Trans well developed Caco-2 monolayers for 6 days to signal differentiation of human colon-derived Caco-2 cells and convert some of them to M like cells.

*E. coli* were prepared as for the phagocytosis assay while *S.* Typhimurium were grown overnight in LB and diluted 1 in 100 in LB and left to grow in the conditions as for *E. coli*. Before the challenge with bacteria the cells were washed twice with MEM-Hepes without antibiotic an hour before the bacterial culture reach $OD_{600}$ = 0.4 and 300 µl of that media was added to each well. 3 wells of confluent cells were infected using 100 µl of bacterial suspension for each well. The infected cells were incubated at 37°C, 5% $CO_2$ and moisture for 1h for *E. coli* or 30 min in case of *S.* Typhimurium. The medium was removed from the lateral well. The collected bacteria were 10 fold serial diluted in PBS. 100 µl of each dilution was triplicate plated on LB plate or LB plate with ampicillin. The plates were incubated at 37°C for overnight and counted next day. To use inhibitor and RANKL with Salmonella, The inhibitors were added to the cell one overnight before challenging with bacteria.

### 2.9.2m Kanamycin protection assay to detect intracellular *E. coli* in bovine terminal rectum epithelium

Bacteria were prepared as for the phagocytosis assay. The bovine primary epithelial cells were washed twice with MEM-Hepes without antibiotic one hour before the bacterial culture reached $OD_{600}$ = 0.4 and wells of confluent epithelial monolayer were infected using 100 µl of bacterial suspension for each well. The infected cells were incubated at 37°C, 5% $CO_2$ and moisture for 1.5 h. The bacterial suspension was removed and the wells were washed 4 times with PBS to remove the non attached bacteria. 500 µl of MEM-Hepes containing 750 µg/ml of kanamycin was

added to each well of the infected cells. The infected cells were then incubated for 3hr and then washed 3 times with PBS. 300 µl of 0.1% triton (in PBS) was added to each well to lyse the cells. The bacteria were collected after scraping of the cells, serially diluted in PBS, and triplicate plated onto LB plates with appropriate antibiotics. The plates were incubated at 37°C for overnight and colonies counted the next day.

### 2.9.2n Using cell signalling inhibitors to check the pathway *Salmonella* Typhimurium using to convert epithelial cell to antigen sampling M cell

The bovine rectal epithelial cells were treated for 5 h with final concentrations 50 µM for SN50 NF-KB inhibitor, PI3 kinase inhbitor LY 294002, GSK-3 β inhibitor, SB 415286. AKT inhibitor was used at 20 µM. Proteasomal-inhibitor MG132 and β-catenin/Tcf inhibitor, FH535 10 µM. DMSO was used alone as control. Thereafter, the cells were infected with *Salmonella* Typhimurium for 2 h or 100 ng / ml of RANKL was added for overnight. The cells then were harvested. The protein concentrations of the cellular lysates were measured and 10 µg of protein from each sample was subjected to SDS-PAGE and immunoblotting. The same inhibitors were added to the cells on the transwell plates and the cells were challenged with *S.* Typhimurium for transcytosis assay.

### 2.9.2o Establishment of an *in vitro* M cell co-culture model (Martinez-Argudo *et al.*, 2007).

Co-culture of Caco-2 cells with Raji B cells and measurement of bacterial translocation. Caco-2 cells were grown for 14 days (in DMEM (Sigma) supplemented with 10% Fetal bovine serum, 1% L glutamine and 1% penicillin/streptomycin (Sigma) on transwell polycarbonate inserts– 3 µm pore) to allow differentiation and development of microvilli. These cells were then co-cultured with Raji B cells $0.5x10^6$ in the basal compartment for 6 days. Bacteria were prepared as describe for the phagocytosis assays and 100µl of bacterial suspension added to each upper chamber. Bacterial counts from the lower chamber were determined at 60 min. relative to the inoculum.

- Raji B cells were grown in RPMI 1640 medium (Gibco-BRL) supplemented with 10% fetal calf serum, 1% Glutamine (Gibco), 0.1 mg $ml^{-1}$ streptomycin and 100 unit $ml^{-1}$ penicillin (Sigma) at 37°C in 5% $CO_2$. (Martinez-Argudo *et al.*, 2007).
- Caco-2 cells were grown in DMEM (Sigma) supplemented with 20% foetal calf serum, 1% nonessential amino acids, 1% Glutamine and 1% of pen-strep (Sigma) at 37°C in 5% $CO_2$.

### 2.9.2p Measurement of bacterial translocation through an *in vitro* M-like cell co-culture

Bacteria were prepared as for the phagocytosis assay. The transwells were washed three times with DMEM without antibiotic an hour before the bacterial challenge. 100 µl of bacterial suspension were added to each upper chamber. Samples from the basolateral chamber were plated at 60 min after being 10 fold diluted. Serial dilutions from the inoculum were plated to calculate the number of bacteria inoculated. The number of translocated bacteria was determined and related to the number of bacteria added.

### 2.9.2q Immunofluorescence

To isolate the primary terminal rectal epithelium, fresh rectal tissues were obtained from a local abattoir. The primary bovine rectal epithelial cells were isolated from the bovine terminal rectal mucosa using Collagenase and Dispase I enzymes to separate the intestinal crypts from the adherent tissues. These crypts were grown in collagen-coated glass coverslips until confluent as described previously (Mahajan *et al.*, 2005). The isolated cells were grown on glass coverslips or in 8-well culture glass slides (BD Falcon, USA). The glass slides and coverslips were coated with bovine collagen solution (Nutacon 5409) and allowed to dry overnight into the class II cabinet. Collagen-coated plates were removed from -20°C and re-hydrated with 500µl PBS or HBSS (Sigma H9269). The cells were incubated at 37°C and 5% $CO_2$. After 24 - 48 h of incubation (depending on concentration of crypts), half the media was removed and replaced with Feeding media (this gets removed any remaining fibroblasts in the culture). Cells were fed every other day until being confluent. Once the cells become confluent, they were challenged with *S.* Typhimurium for 2 h. Cells

were fixed and permeablised with a solution consisted of 2% (v/v) formalin and 0.2% (v/v) triton X-100 in PBS. The cells were washed gently with PBS three times. the cells were blocked by 3% BSA diluted in PBS buffer for 1hr, then cells were stained for with 1/100 primary antibodies diluted in PBS, overnight at 4°C. The cells were washed with PBS three times. The monoclonal antibody was detected with TRITC or FITC-labelled, secondary antibody (1:100), 1 h in darkness at RT. The cells were washed three times with PBS and stained with phalloidin 647 or TRITC. Cells were washed 3 times with PBS and then stained with DAPI 1/5000 (Invitrogen, UK) for 15 min and then washed 2 times with PBS. The glass coverslips were fixed onto a glass slide with fluorescence mounting medium (Vectashield). The slides were stored at 4°C in darkness. The slides were examined using Zeiss axiovert confocal microscope, objective x10.

Uptake of inert micro-particles and *S.* Typhimurium (SL1344) has been used as in vitro functional assay for M-cells in cultures (Clark *et al.*, 1994; Kerneis *et al.*, 1997). FITC-conjugated latex beads of 0.2 μm size (Polysciences Inc., Germany) were diluted (1:1000) in DMEM containing 2% foetal bovine serum. Aliquots (100 μl) of diluted beads were pipetted evenly on to 6-day old cultures and incubated at 37°C for 45 min; then the cells were washed three times in phosphate-buffered saline and *Salmonella* Typhimurium (SL1344) (MOI 1:100) were added to the cell for 10 min. The cells were washed three times in phosphate-buffered saline, fixed and permeabilized with 2% (w/v) PFA/ 0.25% (v/v) Triton X-100 at room temperature for 20 min. Staining of F-actin was done with Phalloidin-647 (diluted 1:40 in PBS; Molecular Probes) for 45 min at room temperature in the dark. For co-localization studies with vimentin, the cells were incubated with mouse anti-vimentin monoclonal antibody (1:100) (Sigma) and incubated overnight at 4°C. This primary antibody was detected with Alexa 594-tagged rabbit anti-mouse or goat anti-mouse polyclonal antibodies as per the manufacturer's instructions (Invitrogen). The cell nuclei were stained with either TO-PRO Iodide (Molecular Probes) or DAPI (Merck). The mounted slides were examined by confocal microscopy. To examine the position of the beads, 0.4 μm optical sections were acquired and processed via Imaris Surpuss Module (Bitplane) computer software programme.

### 2.9.2r Confocal microscopy

Confocal data were acquired using a 1024 x 1024 pixel image size, a Zeiss Plan Apochromat 1.4 NA x63 oil immersion lens and a multi-track (sequential scan) experimental set up on a Zeiss LSM510. Image data, acquired at Nyquist sampling rates, were deconvolved using Huygens software (Scientific Volume Imaging, Netherlands), and the resulting three-dimensional models were analyzed and orthogonal views were created using NIH ImageJ software, final figures were assembled in Adobe Photoshop.

### 2.9.2s Scanning electron microscopy

Samples were fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer; pH 7.3, for 24 h then washed in three 10 min changes of 0.1M sodium cacodylate. Specimens were then post-fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate for 45 min, and then washed in three 10 min changes of 0.1M Sodium Cacodylate buffer. These sections were then dehydrated in 50%, 70%, 90% and 100% normal grade acetones for 10 min each, then for a further two 10-min changes in analar acetone. Dehydrated samples were then critical point dried in a Polaron E 3000 series II drying apparatus, mounted on aluminium stubs , coated in an Emscope SC 500 sputter coater with a 10nm thick layer of gold palladium, and viewed in a Hitachi S-4700 Scanning electron microscope.

### 2.9.2t Transmission electron microscopy

For TEM, samples were fixed in 3% glutaraldehyde in 0.1 M Sodium Cacodylate buffer, pH 7.3, for 2 h then washed in three 10min changes of 0.1M Sodium Cacodylate. Specimens were then post-fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate for 45 min, and then washed in three 10 min changes of 0.1 M sodium cacodylate buffer. These sections were then dehydrated in 50%, 70%, 90% and 100% normal grade acetones for 10 min each, then for a further two 10-min changes in analar acetone. Samples were then embedded in Araldite resin. Ultrathin sections, 60 nm thick were cut, stained in uranyl acetate and lead citrate then viewed in a Phillips CM 120 transmission electron microscope.

## 2.10 Dual luciferase reporter assay

The dual luciferase reporter assay system (Promega, UK) provides an efficient method to measure the response of a reporter promoter. It relies on the use of two luciferase plasmids one of which is the firefly reporter (Photinus pyralis) and the second is the Renilla plasmid (Renilla reniformis). Firstly the firefly luciferase reporter is measured by adding Luciferase Assay Reagent II (LAR II) to generate a "glow-type" luminescent signal. After quantifying the firefly luminescence, this reaction is quenched, and the *Renilla* luciferase reaction is initiated by simultaneously adding Stop & Glo® Reagent to the same tube. The Stop & Glo® Reagent also produces a "glow-type" signal from the *Renilla* luciferase, which decays slowly over the course of the measurement.

Dual luciferase reporter assays were performed as the following: Caco-2 cells were seeded out onto 24 well plates at a density of $8x10^4$ cells per well and incubated overnight at 37°C and 5% $CO_2$. The next day cells were transfected using effectin (Quiagene) according to the manufacturing instructions. The transfection mix was added to the cell containing 20 ng Renilla, 180 ng of *sopB* cloned into pCR3 expression vector/pCR3 expression vector used as control and 100 ng of luciferase reporter plasmid (pTOP flash, pFOP flash, Slug, RANKL and Vimentin promoters cloned in luciferase reporter vector pGL3).

### 2.10.1 Assays for Transcriptional Response to a β-Catenin/Tcf Transcriptional Promoter Element.

Activated Wnt signaling pathway leads to the dephosphorylation, stabilization, and nuclear translocation of β-catenin. The stabilized β-catenin complexes with the TCF/LEF transcription factors, leading to the activation of Wnt-responsive genes *slug, vimentin, c-myc, siamois*, and *cyclin* D1. The effect of SopB on β-catenin/Tcf transcriptional activity was studied by transient co-transfection of Caco-2 cells with sopB/PCR3and the pTOP-Flash reporter plasmid in which the expression of luciferase is promoted by three tandem copies of the optimal Tcf-response element (CCTTGATC) adjacent to the minimal c-fos promoter. Control cultures were transiently transfected with sopB/PCR3 and the pFOP-Flash reporter plasmid

containing all of the regulatory elements of the pTOP-Flash plasmid, except that the Tcf response motifs contain mutations (CCTTGGCC) that prevent its functional activation by Tcf. 48h after transfection cells were washed once with PBS and the complete growth media was exchanged. LiCl 10 mM final concentration was added as positive control for pTOP flash, pFOP in PCR3 transfected cells.

### 2.10.2 Effect of SopB on transcriptional activity of Slug, RANKL

Slug-induced repression of E-cadherin at transcriptional level and triggers a complete epithelial to mesenchymal transition.

Receptor activator of NFκB ligand (RANKL) expression is known to be associated with transformation of epithelium to mesenchymal cell EMT that may be through induction of transcription factor Slug.

To study the effect of SopB on Slug promoter activity Caco-2 cells transiently transfected with SopB and Slug promoter cloned from bovine genome. SN50 (NFκB inhibitors) was added to the cells transfected with *sopB* to examine if SopB induce slug transcription through activating the NFκB pathway

To study the effect of SopB on RANKL promoter activity Caco-2 cells transiently transfected with SopB and RANKL promoter cloned from bovine genome. SN50 NF-KB inhibitor β- Catenin/TCF inhibitor, FH535 SB 415286 AKT inhibitor lY 294002 were added to the cells 48 h after transfection to study the path way that SopB use to induce RANKL promoter activity.

### 2.10.3 Luciferase assay to measure effect of RANKL on transcription of Slug and Vimentin

To investigate effect of RANKL on transcriptional activity and the down stream signaling pathways Slug and Vimentin specific luciferase based assays were conducted in presence of pharmacological inhibitors specific to NFκB or Wnt pathway: 100 ng / ml of RANKL was added 48 after Caco-2 cell was transfected with 100 ng/ well from Vimentin or Slug promoter and 20 ng Renilla plasmid. In the main time of adding RANKL, the pharmacological inhibitors was added to the cells as following: AKT inhibitor 20 μM; 50 μM final concentration from LY 294002 (PI3

kinase inhbitor), SN50 (NFκB inhibitor), or 10 μM from FH535 (β -catenin/Tcf inhibitor). Positive control SB 415286 (GSK-3 β inhibitor) was used in 50 μM final concentration. These agents were added to the cells 48 h after transfection. In some of the assays LiCl (10 mM final concentration) was added as positive control.

15 h later the media was aspirated and drained completely followed by addition of 50 μl of 1 x Passive lysis buffer (PLB; Promega, UK). Then, plates were left for 1 h on a shaker to allow for efficient lysis of the cells. The total cell lysate were transferred into 96 well black plate (BD Falcon,USA). Analysis was performed by addition of 30 μl of LARII reagent (Promega, UK) to each well. The reading was performed on a polar star plate reader (Polar star optima, BMG Biotech) on the luminescence setting. After the plate was read, 30 μl of 'Stop and Glo' (Promega, UK) was added to each well to quench the firefly luciferase activity and read again on the same settings to measure Renilla luciferase activity. Relative luciferase activity was calculated by normalizing the firefly luciferase value to that of the Renilla luciferase. To standardise the luciferase activity of samples within the same experiment set, the relative luciferase values were divided by the control samples mean.

### 2.11 Small interfering RNA (siRNA)

The abbreviation, also known as short interfering RNA or silencing RNA, stands for a class of double-stranded RNA molecules, 20-25 nucleotides in length that play a variety of roles in biology. The most important usage for siRNA is the RNA interference (RNAi) pathway, where it interferes with the expression of a specific gene.

Bovine primary terminal rectal epithelial cells were grown until 40-60% confluence. For RelB knockdown a final concentration of 40nM bovine RelB siRNA was used. The siRNAs were transfected into cells by using lipofectamine transfection reagent (Invitrogen) as described by the manufacturer. After 48 h, the transfected cells were treated with RANKL (100 ng/ml) overnight then infected with wildtype *Salmonella* Typhimurium for 2 h or left untreated and harvested for Western blotting or RT-PCR analysis. All siRNA duplex oligonucleotides were synthesized by Invitrogen). Sequences are shown in Table 2.7.

## 2.12 *In vivo* mice studies to investigate role of SopB in epithelial cells transformation:

*S. Typhimurium* SL1334 and its *sopB* mutant were transformed with the pAJR146 plasmid (Table 2.5) were grown overnight in LB-CAM at 37°C with minimum aeration. Following centrifugation the bacterial pellets were re-suspended in LB and the $OD_{600}$ measured. The bacteria were diluted to give a concentration of $10^8$/100 µl in LB and 100 µl were injected to each C57/BL6 mouse two loops were ligated. One loop for bacterial inoculums and the other were receiving media alone to serve as a control. 5 mice were used for each bacterial strain. The infection period was between 90min. After infection the Peyer's patches were isolated from the rest of the intestine – they were used for confocal studies – washed, fixed, stained for GP-2 (green) and actin (far red 647 nm (blue)), and mounted for microscopy. The remaining intestine from each loop was washed and divided up for RNA extraction – RNA protect was added (2 ml/sample). Protein extraction – Protein lysate buffer (with protease inhibitor) was added (2 ml/sample), Histo-pathology – were fixed with 10% formalin (2 ml/sample) and Cryo- sectioning – OTC were added (2 ml/sample). This experiment was carried ou with help of Dr David Donaldson at the Roslin Institute.

### 2.12.1 GP-2 receptors staining in mouse Ligated intestinal loop assay

For the ligated intestinal loop assay, mice were anaesthetized with avertin and kept warm on a 37°C warming pad during the assay. pAJR146 containg *S.* Typhimurium and *sopB* mutant ($10^8$ C.F.U.) were injected into the ligated intestinal loop. After incubation for 90min, the mice were killed and Peyer's patches were excised from the intestine.

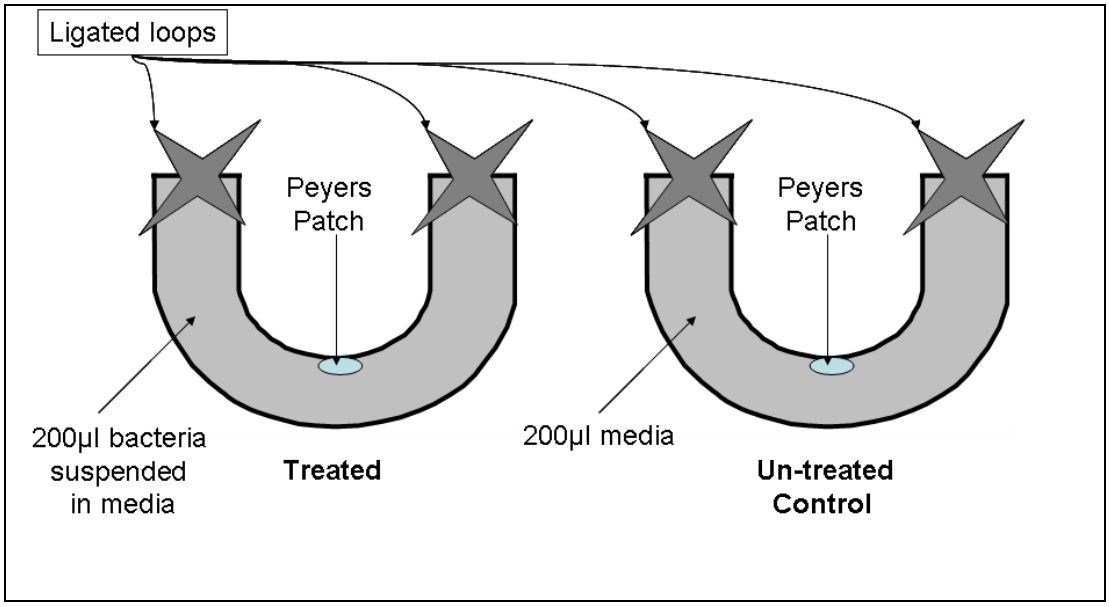


**Fig. 2.5 Mouse ligated loops to study the interaction of *S.* Typhimurium with FAE (~2-3 cm)**

### 2.12.2 Whole-mount immuno-staining of the follicle associated epithelium (FAE) of Peyer's patches (PP)

In order to detect M cells in the FAE of PP, sections of intestinal tissue containing Peyer's patches were whole mount stained for GP2 as previously described (Hase *et al.*, 2009). Briefly, PP were excised and washed with cold HBSS. The PP were then fixed with cytofix/cytoperm (BD biosciences) for 1hr on ice followed by washing with PBS. Non-specific staining was blocked by incubating the tissues in PBS containing 0.5% BSA and 0.1 saponin (w/v). M cells were visualised by an incubation with anti-mouse GP2 (clone 2F11-C3, 5 µg/ml) (MBL international) followed by Alexafluor 488-conjugated goat anti-rat IgG (4 µg/ml) (Invitrogen) and the tissues were counter-stained with Alexafluor 647-conjugated Phalloidin (7.5 U/ml) (Invitrogen). Stained PP were mounted in cavity slides in PBS/30% Glycerol (v/v)/0.05% Sodium Azide (w/v) and stored at 4$^{o}$C. Z-stack images of the FAE of individual PP follicles were obtained using a Zeiss LSM5 confocal microscope (Zeiss).

### 2.12.3 Immuno-histochemistry staining

The cryo specimens were cut into 3-mm-thick sections. The frozen sections were taken out for 30 min to get to RT. Immuno-histochemical staining was performed using DAKO Autostainer Plus System (DAKO, Capinteria, CA). It was used according to manufacturer instructions. Briefly, the slides were fixed with acetone for 10 min at RT, air dried for 15 min. The sections were treated with Methanol/H2O2 (1%) for 10 min at RT to block the endogenous peroxidases. The sections were washed in PBS/BSA (0.2%) for 5 min and blocked with 100 µl of normal goat serum diluted 1/20 (Jackson immunoresearch) (normal serum according to the secondary) at RT for 15 min. The sections were incubated for 1 h with primary, polyclonal antibody against Slug (1:400) at RT or 4 $^{o}$C overnight. Negative controls were performed by omission of the primary antibody. The sections were washed as above and incubated with the secondary antibody 1/500 in PBS/BSA (0.2%). (for slug goat anti-rabbit immunoglobulins/Biotinylated (Dako cat no, Eo432) Washed mentioned above. The staining signal was detected using Vectastain Elite ABC kit (Vector Laboratories) and NovaRED Peroxidase Substrate kit (Vector

Laboratories). The sections were counterstained with Mayer's haematoxylin and then mounted. The images were acquired using a laser-scan Nikon E800 Microscope.

## 2.13 Statistical design and analyses

All statistical analyses were carried out in R (v 2.10.1 © The R Foundation for Statistical Computing) and Minitab software. Overall differences between strains were first assessed, and if there were statistically significant differences then post-hoc Tukey pair-wise comparisons were carried out.

Two types of statistical models were used, when considering differences between strains in the percentage of intracellular bacteria at either set time intervals as determined by fluorescence microscopy, or as a measure of phagocytosis were examined by General Linear Models with binomial errors to account for the percentage nature of the data. To ensure that any differences between experiments was accounted for in the time interval analysis, which of 3 experiments the data came from was also entered as a covariate.

For all other statistical analyses, analysis of co-variance of log10 transformed number of bacteria post-translocation were carried out to assess differences between strains. To adjust for differences in pre-translocation levels and experiments the number of bacteria pre-translocation and which of the 3 experiments the translocation carried out were entered as covariates. Log transformation was undertaken to normalize the residuals. Statistical significance was taken when $P<0.05$. Statistical analyses in chapter 3 and chapter six excpt figure (6.2.6) were carried out and blotted by Dr Darren Shaw.

# *Chapter 3*

## *Comparative Analysis of EspF Variants in the Inhibition of E. coli Phagocytosis by Macrophages and the Inhibition of E. coli Translocation Through Human- and Bovine-Derived M-cells*

# 3. Comparative Analysis of EspF Variants in the Inhibition of *E. coli* Phagocytosis by Macrophages and the Inhibition of *E. coli* Translocation Through Human- and Bovine-Derived M-cells

## 3.1 INTRODUCTION

Enterohaemorrhagic *E. coli* EHEC are emerging zoonotic pathogens, particularly in industrialized countries (Beutin, 2006). HEC strains cause sporadic outbreaks of severe disease in humans, the most important being hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS), the latter results in kidney damage and may lead to death (Griffin & Tauxe, 1991; Boyce *et al.*, 1995). Shiga toxins (Stx) produced by EHEC strains are the main factors responsible for these serious outcomes in humans. By contrast, Enteropathogenic *E. coli* (EPEC) are another pathogenic type of *E. coli* that can also cause severe intestinal disease in humans but there is no clear evidence these strains are zoonotic although EPEC strains do circulate and cause diseases in animals (Moon *et al.*, 1983). Human EPEC infections are not usually associated with HC and HUS as the strains do not produce Shiga toxins.

Our understanding of EHEC pathogenesis is primarily based around studies on the EHEC O157 and EHEC O26 serogroups that are associated with most human EHEC infections in Europe, North America, and Japan (Karmali, 1989; Karmali, 2004; Karch *et al.*, 2005). Both serogroups are considered to be present in ruminants, in particular cattle as the primary reservoir (Karmali, 1989; Bettelheim, 2000; Naylor *et al.*, 2003; Naylor *et al.*, 2005a). While there are many EPEC serotypes, extensive research has been carried out on the sequenced human EPEC O127 strain E2348/69. EHEC and EPEC strains express a type III secretion system (T3SS) that is important for colonisation of the human or animal host (Moon *et al.*, 1983; Jarvis *et al.*, 1995; Hueck, 1998; Shaw *et al.*, 2001). The T3SS injects effector proteins into host cells that manipulate cellular processes to promote the colonisation and persistence of the bacterium in the gastrointestinal tract ( Nougayrede *et al.*, 2001; Elliott *et al.*, 2002; Dean *et al.*, 2006; Iizumi *et al.*, 2007; Echtenkamp *et al.*, 2008; Marchès *et al.*, 2008). The primary phenotype associated with T3S is intimate attachment between

the bacterial outer-membrane protein intimin and the T3SS translocated intimin receptor (Tir) (Kenny *et al.*, 1997). In both EHEC and EPEC, the genes encoding this protein secretion system are expressed from the locus of enterocyte effacement (LEE) pathogenicity island ( Jarvis *et al.*, 1995; Hueck, 1998). While several effector proteins are also expressed from the LEE, a number of additional secreted effector proteins have been identified that are expressed primarily from integrated phage elements scattered throughout the O157 chromosome (Tobe *et al.*, 2006). EHEC and EPEC strains have different combinations of effector proteins, potentially reflecting host adaptation and differences in pathogenesis.

EspF is a LEE-encoded effector protein that requires the CesF chaperone to be translocated by the T3SS into host cells (Elliott *et al.*, 2002). EspF has multiple proline-rich domains which act by binding to SH3 domains or Enabled/VASP homology 1 (EVH1) domains of host cell signalling proteins (Crane *et al.*, 2001). For example $EspF_{EPECO127}$ binds to Sorting Nexin 9 (SNX9) via its SH3 amino terminal region (Marchès *et al.*, 2006; Alto *et al.*, 2007). EspF is involved in disruption of tight junctions and increases monolayer permeability in part through the redistribution of occludins ((McNamara *et al.*, 2001; Nougayrède & Donnenberg, 2004; Viswanathan *et al.*, 2004a; Nougayrède *et al.*, 2007). EspF sequences differ between EPEC and EHEC strains and the EHEC O157 variant has a more modest impact on transepithelial electrical resistance (TER) (Viswanathan *et al.*, 2004a). EspF in combination with other effectors inhibits the water transporter SGLT-1(Dean *et al.*, 2006). $EspF_{EPECO127}$ is targeted to mitochondria with the N-terminal region of EspF functioning as an import signal. $EspF_{EPECO127}$ causes an increase in mitochondrial membrane permeabilization in addition to the release of cytochrome C from mitochondria into the cytoplasm and subsequent caspase-9 and caspase-3 cleavage leading to cell death (Crane *et al.*, 2001; Nougayrède & Donnenberg, 2004; Nagai *et al.*, 2005; Nougayrède Nougayrède *et al.*, 2007). More recent work has demonstrated that EspF can lead to loss of nucleolin from the nucleolus, an activity driven by EspF's activity on mitochondria (Dean *et al.*, 2010). $EspF_{EPECO127}$ also plays an important role in inhibition of bacterial uptake by macrophages (Quitard *et al.*, 2006), preventing macrophage phagocytosis via inhibition of phosphatidyl

inositol-3 (PI3) kinase dependent pathway of bacterial uptake (Nougayrede *et al.*, 2001; Quitard *et al.*, 2006).

Intestinal epithelium is composed of multiple cell types including absorptive enterocytes, enteroendocrine, goblet, and Paneth cells. These cells derive through asymmetrical division migration and differentiation from pluripotent stem cells. An additional specialised epithelial cell type, termed M-cells ("membranous" or "microfold" cells), are associated particularly with epithelium overlying gut-associated lymphoid tissue. This is referred to as follicle-associated epithelium (FAE) and is a site of active immunological function. In contrast to villous epithelium, FAE contains no or fewer goblet cells (Owen, 1999), defensin- and lysozyme-producing Paneth cells (Giannasca *et al.*, 1994; Giannasca *et al.*, 1999) and expresses low amounts of membrane-associated hydrolases (Owen & Bhalla, 1983). The M-cells generally lack the distinct microvilli and thick filamentous brush border glycocalyx (Frey *et al.*, 1996) and instead have variable microfolds. Together, these features of M-cells promote contact of antigens with gut epithelium and result in sampling of antigens from the intestinal lumen and transfer to antigen presenting cells (APCs) within an intra-epithelial pocket (Neutra *et al.*, 1996) on its basolateral side (Ermak *et al.*, 1994; Farstad *et al.*, 1994; Iwasaki & Kelsall, 2000). $EspF_{EPECO127}$ has also been shown to inhibit EPEC translocation across antigen-transporting epithelial M-cells in an *in vitro* model (Martinez-Argudo *et al.*, 2007) based on a published co-culture system (Kerneis *et al.*, 1997).

In cattle, the terminal rectum is rich in lymphoid follicle-associated epithelium (FAE) containing M-cells (Mahajan *et al.*, 2005). As this is the predominant site colonized by EHEC O157 in cattle (Naylor *et al.*, 2003), we hypothesized that EspF may have an important role in cattle colonisation by inhibiting translocation of the organism by M-cells and that the sequence differences of EspF in EHEC O157:H7 may reflect selection for its function in the bovine host. To test this, we have compared the capacity of different *espF* alleles to inhibit phagocytosis and limit bacterial translocation through M-cells derived in a co-culture system with their capacity to inhibit bacterial uptake into and translocation through cells cultured from the bovine terminal rectum.

## 3.2 RESULTS

### 3.2.1 Strain specific susceptibility to phagocytosis by cultured macrophages.

Previous research has demonstrated that EspF is able to inhibit bacterial uptake into macrophages, although the majority of previous research examining this inhibition has been focused on the $EspF_{O127}$ variant from EPEC O127 E2348/69. As an initial experiment EHEC O157 & O26 and EPEC O127 were compared for their capacity to inhibit their non-opsonised phagocytosis into cultured macrophages. EPEC O127 bound in significantly higher numbers to the macrophages (Fig. 3.2.1A, $P<0.001$) and the majority remained external (Fig. 3.2.1B and C). EHEC O26 exhibited adherence phenotype similar to EHEC O157 but significantly less than EPEC O127 (Fig 3.2.1A, $P<0.001$), however, resisted uptake by macrophages (Fig. 3.2.1B and C). By contrast EHEC O157 adhered at lower levels and a significantly higher proportion was phagocytosed (Fig. 3.2.1B-C). For example at 90 min post addition 63% (+/-3%) of EHEC O157 bacteria were internalised compared to only 33% (+/-5%) of EPEC O127 or 31% (+/-.3%) for O26 (Fig. 3.2.1B).

### 3.2.2 PCR amplification of *espF* of different EHEC serotypes

To functionally analyse the differences in function of EspF from different EHEC and EPEC strains, the different *espF* genes were amplified by polymerase chain reaction (PCR). DNA templates used for the amplification of *espF* were extracted from *E. coli* O26:H11, *E. coli* O5:H-, *E. coli* O26:H2, *E. coli* O111:H12, *E. coli* O103:H2, *E. coli* O118:H16, *E. coli* O103:H-, *E. coli* O157:H7, and *E. coli* O127:H6. The primers were designed based on alignments of the different published *espF* sequences. The primers are defined in Table 2.3 (Materials & Methods) and the amplification conditions were the same for all the ORFs (Materials & Methods). Variable sizes of PCR products (0.7-0.8 kb) were obtained on amplification of the *esp*F alleles from EPEC and the different EHEC serotypes (Fig. 3.2.2a).

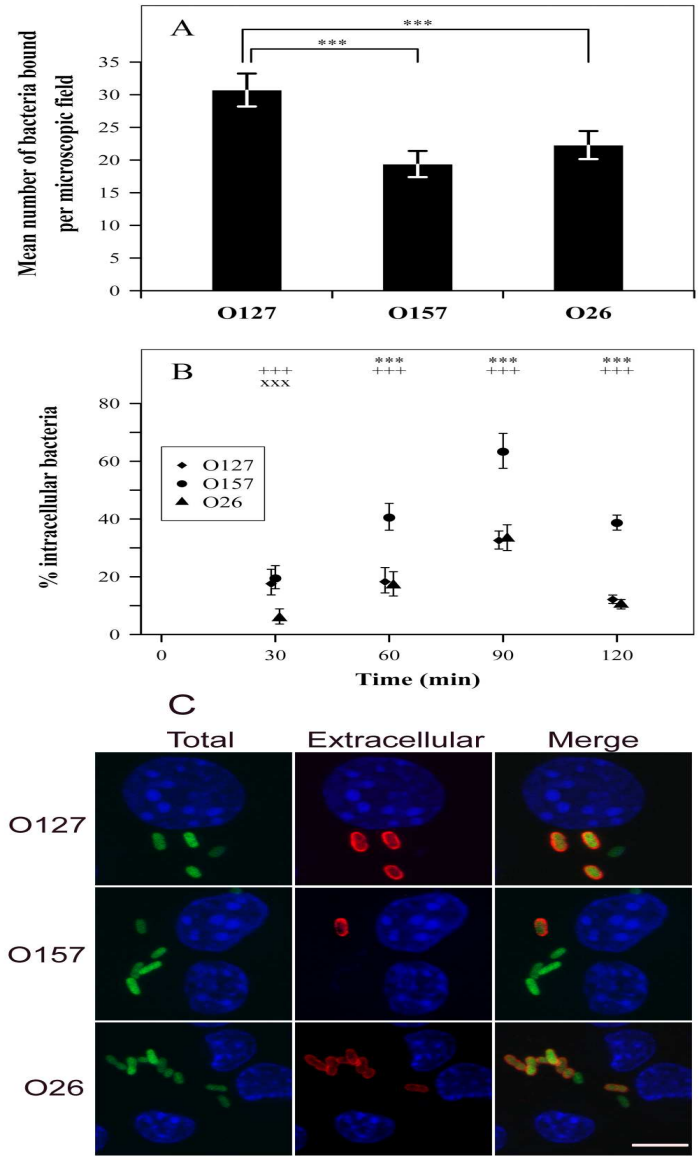


**Fig. 3.2.1 A comparison of EHEC O157, O26 and EPEC O127 interactions with RAW 264.7 macrophages.** Confluent monolayers of the mouse macrophages were infected with EPEC O127:H6 (E2348/69), EHEC O26:H11 (ZAP 1139) EHEC O157:H7 (ZAP1163) strains and the number of bacteria inside (green) or outside (red) of the macrophages determined by fluorescence microscopy as detailed in the Materials and Methods. (A) Mean (± 95% confidence intervals) number of adherent bacteria per macrophage at 30 min following addition of the bacteria at an MOI of 100. (B) Percentage (±95% confidence intervals) of intracellular bacteria at the time points shown as determined by fluorescence microscopy for EHEC O157 (●), EPEC O127 (◆) and EPEC O26 (▲). *** denotes a significant difference of <0.001 for O157 vs EPEC;+++ for O157 vs O26, and xxx for EPEC vs O26. (C) Confocal images were acquired using a Zeiss Plan Apochromat 1.4 NA x63 oil immersion lens and a multi-track (sequential scan) experimental set up on a Zeiss LSM510. Scale bar 5μm.

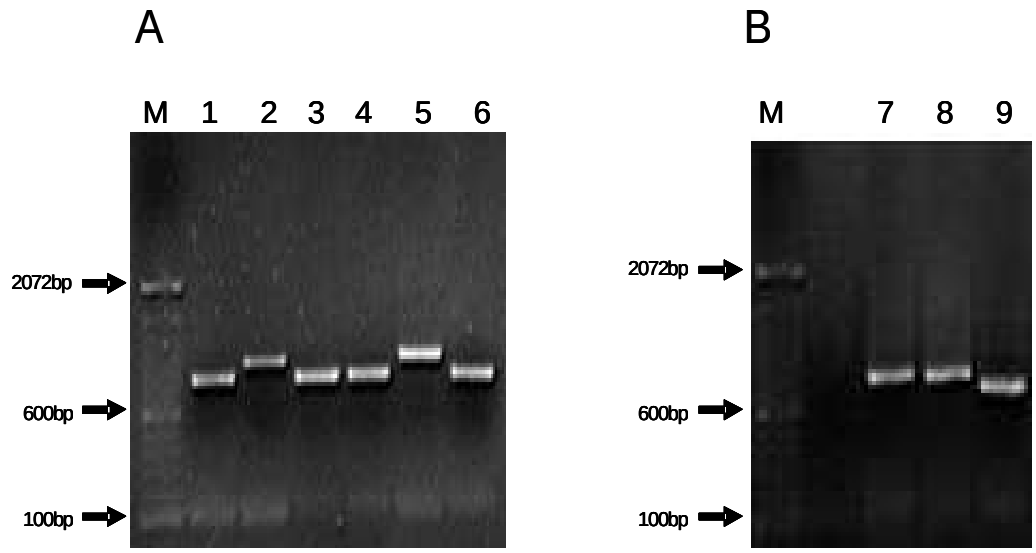


**Fig. 3.2.2a PCR amplification of *esp*F from different EHEC serotypes**. Samples were run on 2% agarose gels as described in materials and methods. Lane M, 100bp DNA ladder. Lanes 1-9 (A,B) *esp*F of different EHEC serotypes (A) O26:H11 (Lane 1), O5:H- (Lane 2), O26:H2 (Lane 3) O111:H12 (Lane 4), O103:H2 (Lane 5), O118:H16 (Lane 6), O103:H- (B)(Lane 7), O157:H7 (Lane 8), and EPEC O127:H6 (Lane9).

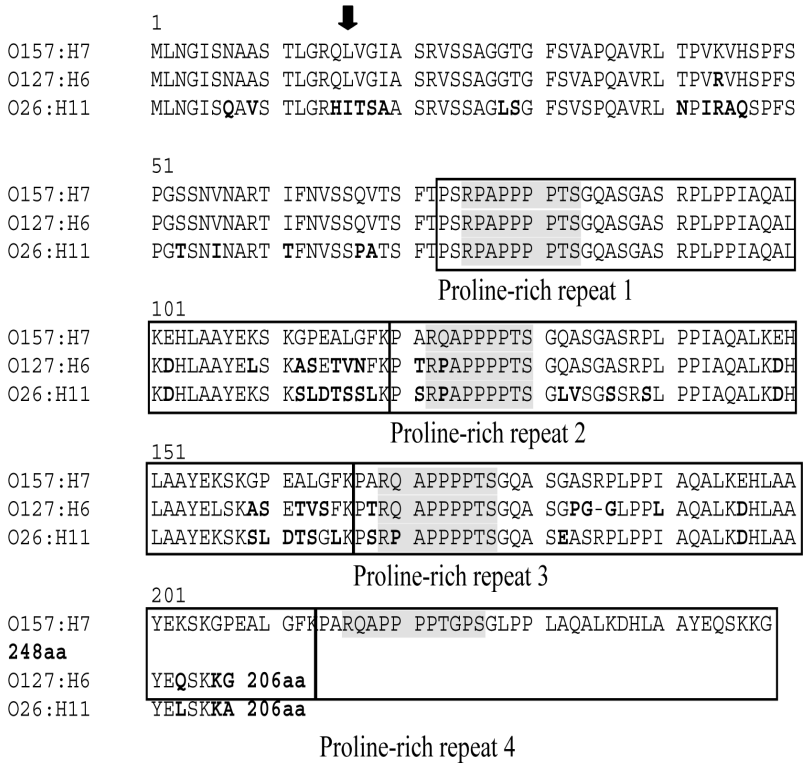


**Fig. 3.2.2b Alignment of EspF amino acid sequences from EHEC O157:H7 (EDL933); EPEC O127:H6 (E2348/69) and EHEC O26:H11 (ZAP1139).** The proline-rich repeats (PRR) are boxed with EHEC O157 containing an additional 4th repeat. Amino acid differences from the EHEC O157 sequence are bolded. A binding site for Sorting Nexin 9 (SNX9) is highlighted in grey within the PRRs (Alto *et al.*, 2007). The putative N-WASP binding region is within the middle of the PRRs (Alto *et al.*, 2007), although the most significant sequence diversity between these variants is at the ends of each PRR. The leucine at position 16 (arrow) has been shown to be essential for EspF translocation into mitochondria and this is changed to the similar aliphatic amino acid isoleucine in EHEC O26 (Nagai *et al.*, 2005). Analysis of NCBI *E. coli* O157:H7 sequences showed no significant variation in the predicted EspF amino acid sequence. The predicted EspF from an *E. coli* O26:H2 strain was identical to that shown for *E. coli* O26:H1. The other O26 strains *espF* sequence were similar to O26:H11.

Analysis of NCBI *E. coli* O157:H7 sequences showed no significant variation in the predicted EspF amino acid sequence. The predicted EspF from an *E. coli* O26:H2 strain was identical to that shown for *E. coli* O26:H11.

EHEC and EPEC serotypes express different variants of EspF (Fig. 3.2.2b). EHEC O157 contains four polyproline repeat regions compared to three in the EPEC O127 and EHEC O26 strains. In addition, the three sequences differ within the polyproline repeat regions and in the amino terminus of the protein shown to be important for organelle targeting (Alto *et al.*, 2007; Dean *et al.*, 2010).

### 3.2.3 Cloning of *espF* of EPEC O127:H6 and EHEC strains O157:H7 and O26:H11

To determine the relative contribution of the different *espF* alleles in inhibiting phagocytosis, these were amplified from *E. coli* O157, O127, and O26 and cloned into pMB102 (Table 2.2) under control of a pTAC inducible promoter. *espF* of *E. coli* O26:H11 and O157:H7 and EPEC O127:H6 were amplified by PCR using primers as described in (Table 2.3 Materials & Methods). PCR products as well as pTS1 were digested with restriction endonucleases (Table 2.3) and ligated together using T4 ligase enzyme before being transformed into DH5α competent cells. To confirm that the cloned products were the correct size and that the cloning procedures were successful pTS1 was extracted from the transformed colonies for *espF* from EHEC O157 and O26 were digested using restriction endonucleases (Fig. 3.2.3a). The colonies for EPEC *espF* were screened by PCR (Fig. 3.2.3b) as the *Hind*III site was lost from the plasmid by making it blunt as EPEC *espF* containing *HindIII* site in its sequence. All these clones were confirmed by sequencing.

### 3.2.4 Strain specific susceptibility to phagocytosis is associated with *espF* allele expression.

EHEC and EPEC serotypes express different variants of EspF (Fig. 3.2.2b). EHEC O157 contains four polyproline repeat regions compared to three in the EPEC O127 and EHEC O26. In addition the three sequences differ within the polyproline repeat regions and in the amino terminus of the protein shown to be important for organelle targeting (Alto *et al.*, 2007; Dean *et al.*, 2010).

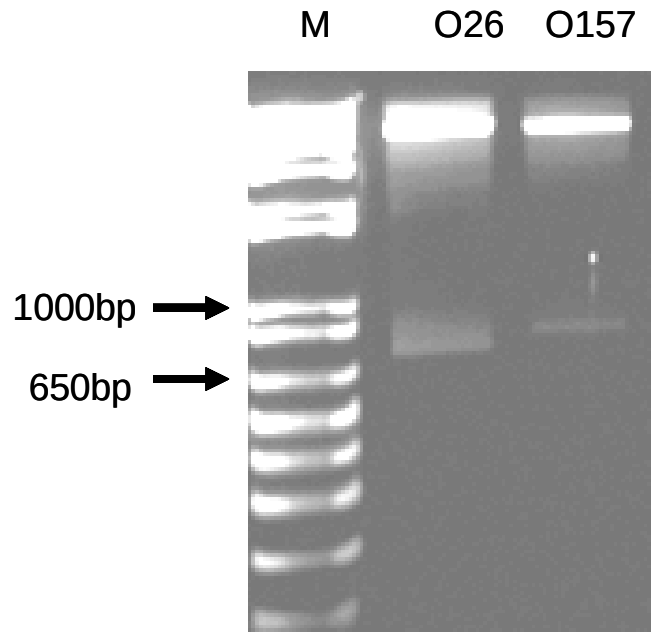


**Fig. 3.2.3a Diagnostic digests of pTS1. Clones of O26:H11 espF and O157:H7 *espF*** obtained by digestion with *Bam*HI and *Hind*III enzymes as described in materials and methods. Electrophoresis was carried out through a 0.8% agarose gel. Lane M, 1Kb DNA ladder. Successful clones were at expected size bands appear at 700bp and 800bp respectively for the O26 and O157 clones.

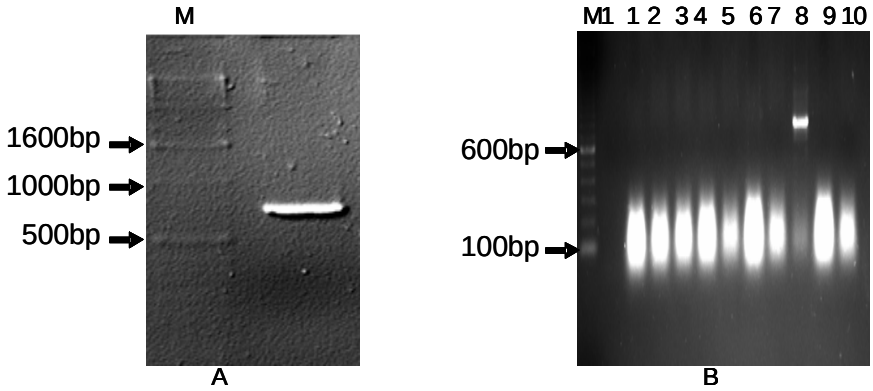


**Fig. 3.2.3b PCR amplification of *espF* of EPEC O127:H6 for cloning into pTS1(A). Diagnostic PCR of *espF* clones into the pTS1 expression plasmid (B).** A DNA segment of expected size (700bp) was seen in lane 8. Lane M, 1Kb DNA ladder and M1 is 100bp ladder. Samples were electrophoresed in 2% agarose gel as described in Materials and Methods.

To determine the relative contribution of the different *espF* alleles in inhibiting phagocytosis, these were amplified from *E. coli* O157, O127 and O26 and cloned into pMB102 (Table 2.5 Materials & Methods) under control of a pTAC promoter that is inducible with IPTG. The sequences were confirmed as identical to those published on the NCBI database for these serotypes resulting in predicted amino acid sequences shown in Fig. 3.2.2b. The clones were transformed into EPEC E2349/69Δ*espF* (Table 2.1 Materials & Methods) and secretion profiles examined Fig. 3.2.4a. All strains secreted comparable levels of the translocon protein EspD; EspF secretion was detected from all three complemented strains by Western blotting (Fig. 3.2.4a), but with potentially lower levels detectable for the O26 variant. (Fig.3.2.4a). The complemented strains were then compared in the macrophage phagocytosis assay at a 90 min post-infection time point (Fig.3.2.4b). All three alleles were able to complement the *espF* knockout (Fig. 3, $P<0.001$), although the $espF_{O157}$ allele was significantly less effective at inhibiting uptake in comparison to the $espF_{O127}$ and $espF_{O26}$ alleles (Fig.3.2.4b, $P<0.001$). These results confirm that the capacity of EPEC O127 and EHEC O157 strains to inhibit phagocytosis correlates with the activity of the respective *espF* allele.

### 3.2.5 The role of the EspF in adherence to epithelial cells

It has been reported that EPEC EspF has no role in A/E lesion formation, altering signal transduction or invasion of host cells (McNamera & Donnenberg 1998; McNamera *et al.,* 2001). In this study *espF* clones from EHEC O157:H7 and O26:H11 were compared with the EPEC O127 *espF* clone to investigate if the EHEC variants play any role in bacterial attachment or invasion of eukaryotic cells.

To study the role of the EspF varaints in adherence to epithelial cells, Caco2 cells were infected with the Δ*espF* EPEC strain transformed with the different *espF* alleles.and incubated for 1h at 37°C, 5% $CO_2$. The infected cells were fixed in 4% PFA. Bacteria were imaged and quantified following fluorescence microscopy, either directly if expressing a GFP plasmid or following O-antigen specific antibody staining followed by detection with an Alexafluor 594 conjugated secondary antibody. There were no significant differences between different *espF* alleles and adherence to the Caco2 cells (Fig. 3.2.5).

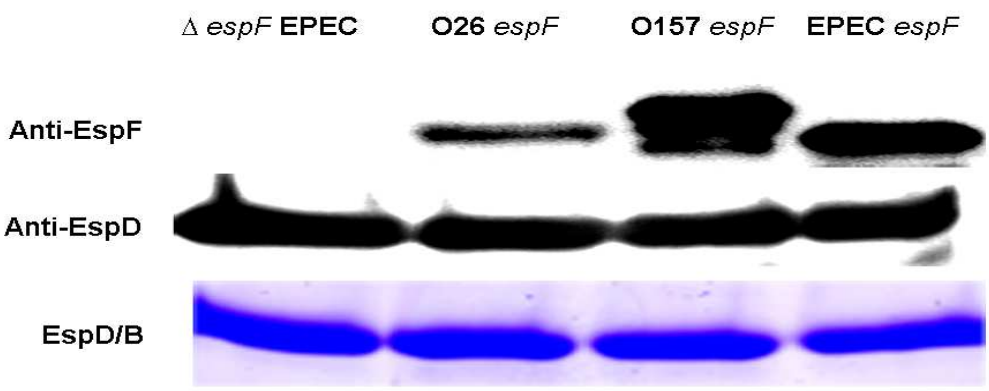


**Fig. 3.2.4a Levels of EspD and EspF secretion by EPEC O127 Δ*espF* and complemented with the three *espF* alleles.** Supernatants were prepared and separated in a standard SDS denaturing gel which was then stained with Colloidal Coomassie blue. The bottom panel shows the staining for the main EspD/B band. The middle panel shows the same samples with detection for EspD. The top panel shows detection of EspF. The supernatants were prepared from equal volumes of bacteria (50 ml) cultured to an optical density at 600 nm of 1.0. Experimental details are given in the Materials and Methods section.

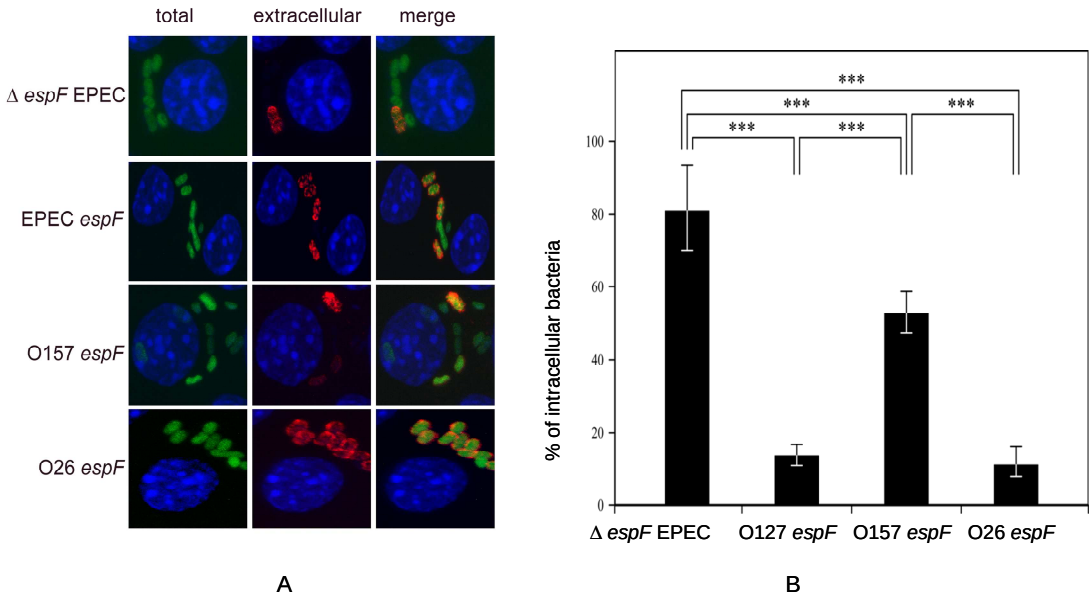


**Fig. 3.2.4b Comparative analysis of *espF* of EPEC O127:H6 and EHEC strains O157:H7 and O26:H11 interaction with macrophages.** (A) confluent monolayer of RAW264.7 macrophages was infected with a panel of GFP−labelled Δ*espF* EPEC strains transformed with *espF* cloned from EPEC O127:H6, EHEC O157:H7 and EHEC O26:H11. The proportions of extra−cellular bacteria were determined at 90 min. The infected cells were fixed in 4% PFA. Bacteria were stained with O−antigen specific antibody detected with Alexafluor 594 conjugated secondary antibody. The intra cellular (green) versus total bacteria (green + red/orange) were imaged and quantified as described in Materials and Methods. Majority of EPEC O127:H6 and EHEC O26 were seen outside of macrophages while EHEC O157 were internalize. **(B)** Inhibition of phagocytosis (mean ± 95% confidence intervals) by different *espF* alleles. *** denotes a statistical significance of P<0.001**.**

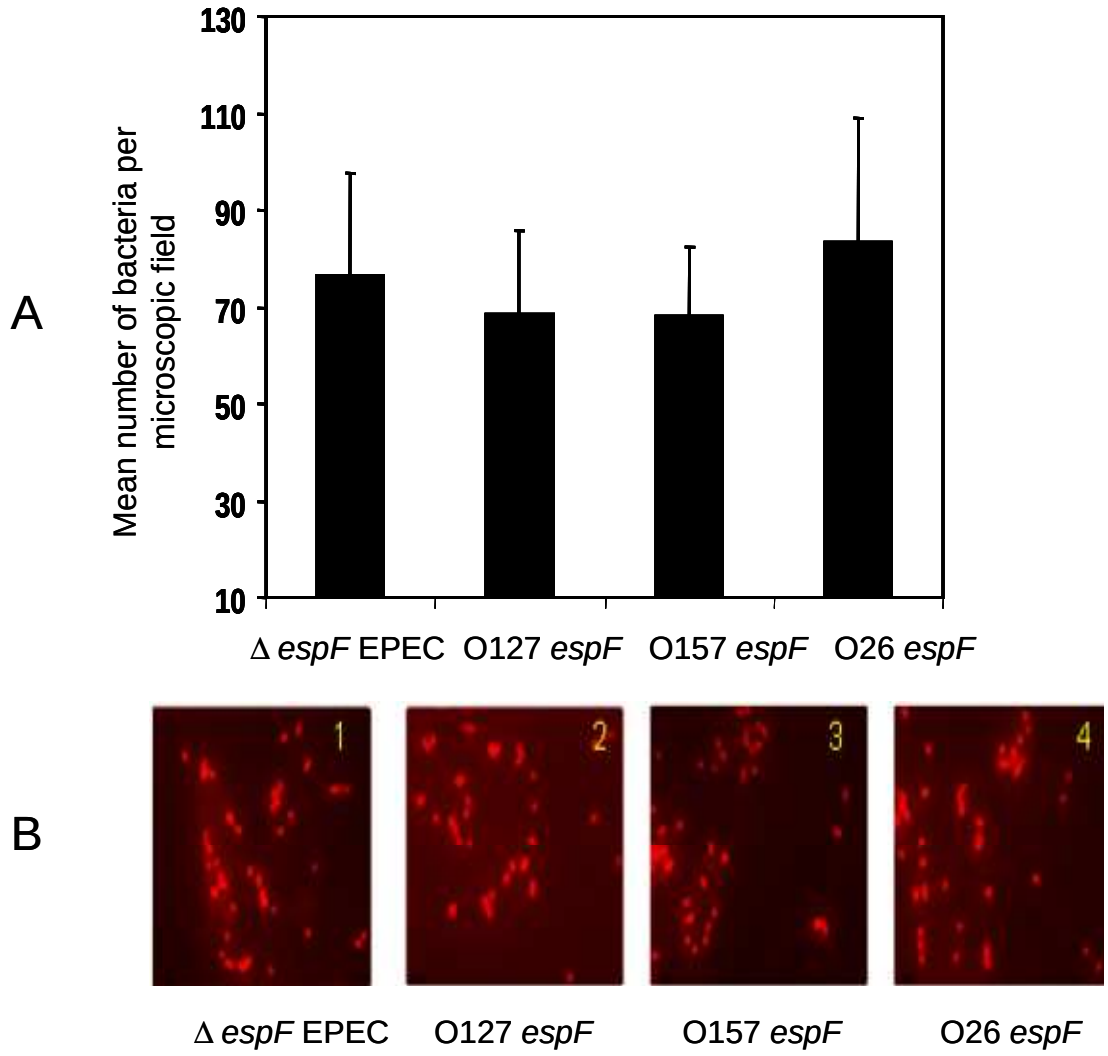


**Fig. 3.2.5 Adherence to Caco-2 of EPEC O127 Δ*espF* and the strain complemented with the three *espF* alleles.** Adherence was determined at 60 min post addition to the Caco-2 and detected by counting bacteria per microscope field. The experiments were repeated in triplicate with at least 10 fields enumerated for each strain in each repetition. Details of the assay are provided in Materials and Methods. There were no significant differences between the adherence levels of the strains.

### 3.2.6 Deletion of *espF* alleles from *E. coli* O157 strain TUV93-0 to study its role on the interaction of *E. coli* with primary cultures of bovine rectal epithelial cells

Previous work has established that EHEC O157:H7 colonises the terminal rectum of cattle, the main reservoir host (Naylor *et al.*, 2005a).

To investigate the role of EspF in EHEC O157:H7 colonization of bovine rectal epithelium I aimed to construct an *espF* deletion in the EHEC O157:H7 strain TUV93-0. To facilitate exchange into the wildtype locus on the chromosome, the flanking regions of *espF* gene were PCR amplified (Fig. 3.2.6a) and cloned into pIB307 (Table 2.5 Materials & Methods), a temperature sensitive plasmid (Fig. 3.2.6b). The construction of a clean deletion is a two step process, in the first a counter-selectable marker is introduced (sacB) adjacent to a selectable marker (kan), in the second step the clean deletion is introduced by removal of this two gene cassette. The *sackan* cassette from pDG28 (Table 2.5 Materials & Methods) was excised with *Bam*HI (Fig. 3.2.6c), cleaned up and cloned into the *Bam*HI site of pIB307 (Fig. 3.2.6d). The plasmid (pAT4) containing the two *espF* flanking regions and the *sackan* cassette was used for recombination of the *sackan* cassette into the chromosome of strain TUV93-0 to replace *espF* with the cassette (Fig. 3.2.6e and Material & Methods). Once this strain was confirmed, allelic exchange was then carried out using the pIB307 plasmid containing the two *espF* flanking regions (pAT5) to obtain clean deletion *espF* O157:H7 strain TUV93-0 (Fig. 3.2.6f and Material & Methods).

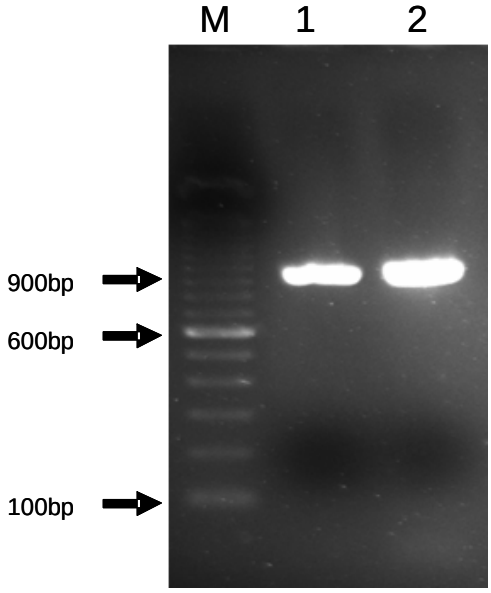


**Fig. 3.2.6a PCR amplification of *espF* flanking region from *E. coli* O157:H7.** About 1Kbp of the upstream (Lane1) and downstream (Lane2) *espF* flanking of *E. coli* O157:H7 were amplified using primers as described in Materials and Methods. Samples were run on a 1.5% agarose gel. Lane M, 100bp DNA ladder.

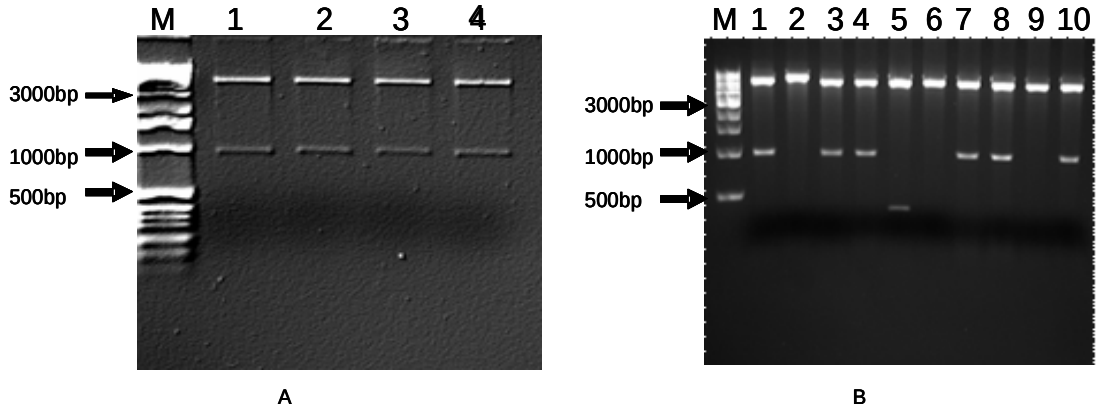


**Fig. 3.2.6b Diagnostic digests of pIB307 clones containing with two *espF* flanking regions.** The up stream (A) and down stream (B) flanking region of *espF* were digested with *Sac*I & *Bam*HI; and *pst*I & *Bam*HI enzymes, respectively Lane M, 1Kb DNA ladder. Lanes 1-4 (A) and Lanes 1,3,4,7,8,10 (B) show successful clones with the expected 1000bp DNA inserts. The digested products were separated on a 0.8% agarose gel.

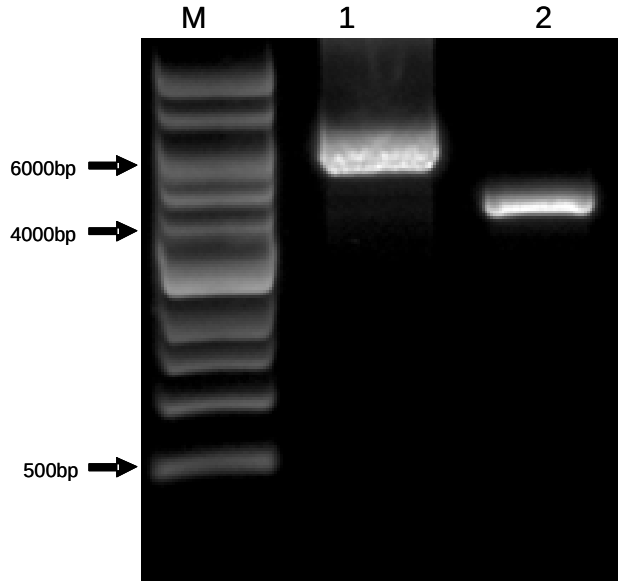


**Fig. 3.2.6c Electrophoresis of pIB307 cloned with 2 *espF* flanking regions and *sackan* cassette.** pIB307 cloned with 2 *espF* flanking regions were digested with *Bam*HI (lane1) and *sackan* cassette after being digested with *Bam*HI from pDG28 and gel purified (lane2). Lane M, 1Kb DNA ladder.Agarose electrophoresis was run on a 0.8% agarose as decribed in materials and methods.

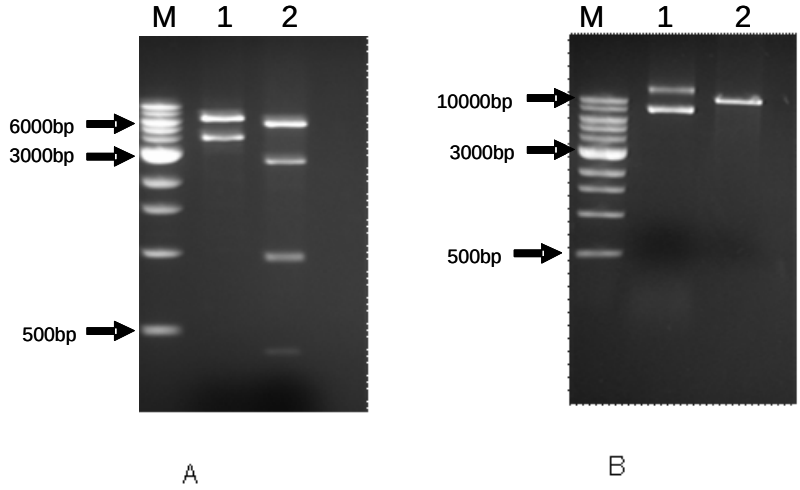


**Fig. 3.2.6d Diagnostic digest for pIB307 cloned with the two *espF* flanking regions and the *sackan* cassette.** Digestion with *Bam*HI enzyme released the cassette at 4000bp (Lane1). *Bam*HI and *Pst*I digestion gave four expected bands as *Pst1* cut twice within the cassette (lane2 (A). *Sac*I digestion gave an expected segment 10000bp demonstrating successful cloning of the two *espF* flanking regions and the *sackan* cassette (Lane 2); pDG28 plasmid without the *Sac*I sites was used as a control (Lane1) (B). Lane M, 1Kb DNA ladder. The digested products were electrophoresd on a 0.8% agarose gel as described in materials and methods.

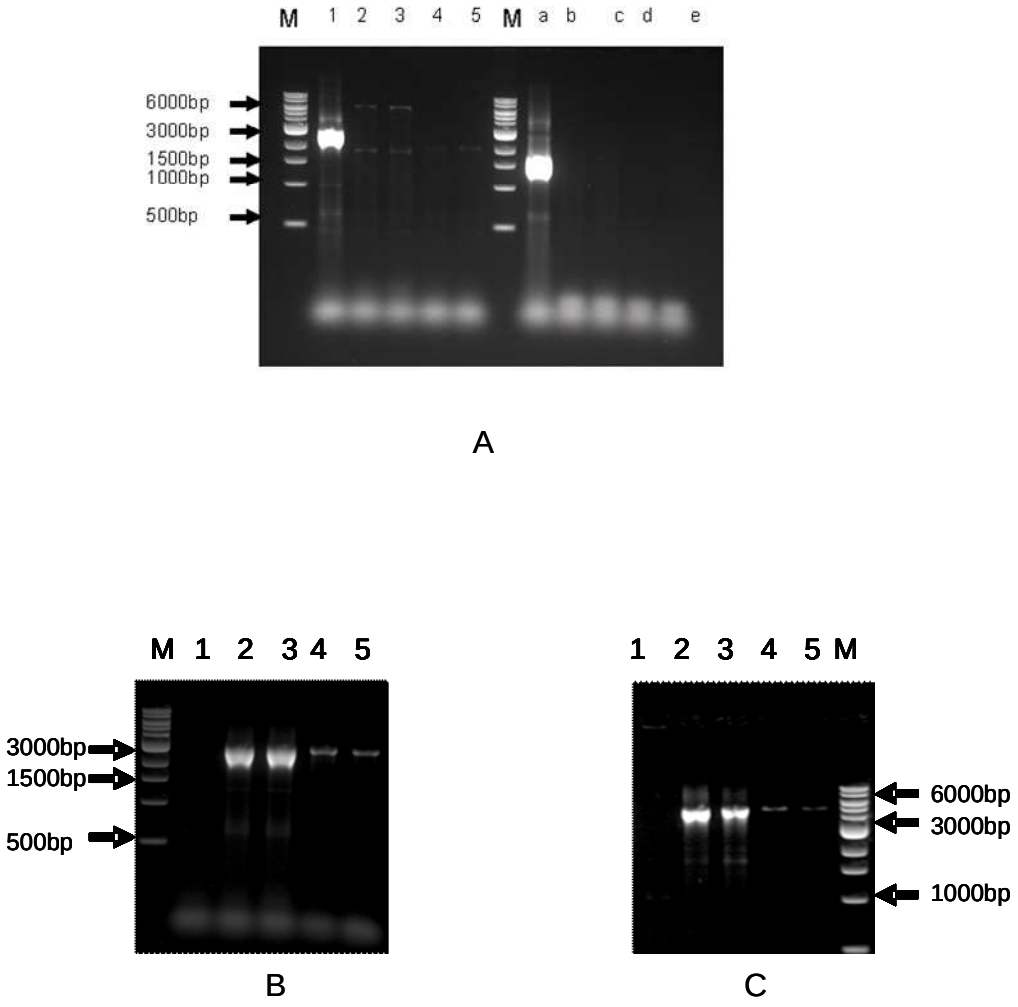


**Fig. 3.2.6e Diagnostic PCR for *espF* O157:H7 knock-out.** The obtained strains and control TUV93-0 were amplified using 1kp primers external to *espF* lane1-5 (A). Lane1 is control wild type TUV93-0 , lane 2-5 are the tested knockout strain which should have expected band at 6000bp while the control strain at 2800bp. In addition primers start from *espF* ATG and 1kp away from *espF* reverse primer were used in lane (a - e) (A) while a is the control wiled type that release expected band at 1800bp and (b – e) are the tested knockout strain that should give no bands with that primers. 1kp primer external to downstream of *espF* and 3 primer of *sacB (B) and* 1Kb primer external upstream to *espF* and 5 primer of *sacB* (C). Lane 1 is control wild type strain TUV93-0 that should give no bands with *sacB* primers, lane 2-5 are the tested knockout strain which should have expected band at 2800bp (B) and at 5500bp (C) Samples were run on 1% agarose gel as described in materials and methods. Lane M, 1Kb DNA ladder.

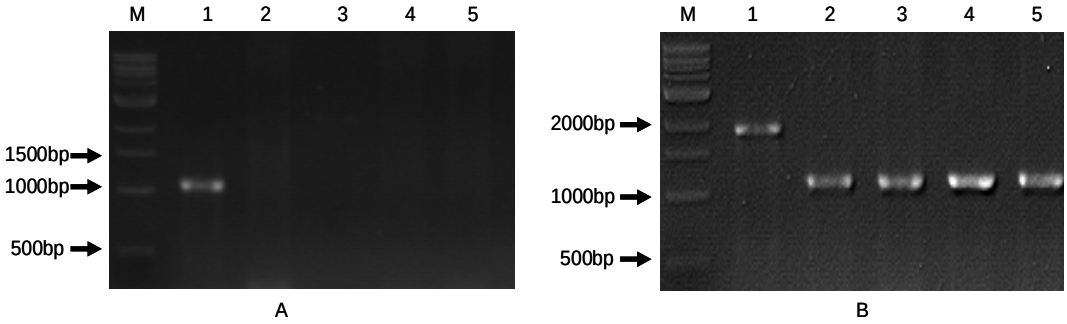


**Fig. 3.2.6f Diagnostic PCR for sackan cassette deletion to obtain clean espF mutant in *E. coli* O157:H7 strain TUV93-0.** PCR amplification from the obtained strains and control TUV93-0 containing *sackan* cassette at the site of *espF* were done using *sacB* primers for detection of *sackan* cassette (A). Lane 1 is control Δ *espF* O157:H7 strain TUV93-0 containing *sackan* cassette tha should give band at 1.2Kb, lane 2-5 are the tested knockout strain which should have no bands, so all were successful. Primer pairs 600bp up and down streams to *espF* were also used *(B).* Lane 1 is control wild type O157:H7 strain TUV93-0 that should give band at 1.8Kb. Lane 2-5 are the tested knockout strain which should have expected bands at 1.2Kb, so all were successful. Lane M, 1Kb DNA ladder. Samples were run on 1.5% agarose gel as described in Materials and Methods.

### 3.2.7 Strain specific variation in M-cell translocation is associated with EspF variation.

Co-culturing of human Caco-2 cells and lympho-epithelial B cells *in vitro* leads to the differentiation of a subset of epithelial cells into antigen-transporting cells (M-cells) (Kerneis *et al.*, 1997; Lo *et al.*, 2004). This "M cell" culture system can then be used to analyse bacterial translocation and the capacity of bacteria to inhibit this trafficking (Martinez-Argudo *et al.*, 2007). Evidence for M-cell translocation in the assay is provided by comparison of translocation through a standard Caco-2 monolayer which should occur at significantly lower levels (Fig. 3.2.7 A-C). To determine the contribution of *espF* to the translocation of EHEC O157 through this M-cell culture system, a defined deletion of *espF* was constructed in *E. coli* O157:H7 TUV93-0 (Table 2.1). Translocation of the EHEC O157 Δ*espF* strain was significantly higher than the wild type strain across both the Caco-2/rajiB co-culture and the Caco-2 monolayer. The levels of translocation were restored to those of the wild type by complementation with $espF_{0157}$ *in trans* (Fig. 3.2.7A).

The EHEC O157, O26 and EPEC O127 strains were then compared in the same co-culture translocation assay. EHEC O157 demonstrated significantly higher levels of translocation compared with EPEC O127 and EHEC O26 (Fig. 3.2.6b B, $P<0.001$). To examine whether the *espF* alleles have an effect on the level of translocation inhibition, EPEC E2349/69Δ*espF* transformed with the three amplified and cloned *espF* alleles was analysed in the same assay. All three complemented the *espF* mutation in the EPECΔ*espF* strain to a significant level (Fig. 3.2.7C, $P<0.001$). As with the phagocytosis assay, the $espF_{O157}$ allele was the least effective of the three being significantly less effective than the $espF_{O127}$ allele at inhibiting translocation. This was the case for translocation across both the Caco-2/rajiB co-culture and the Caco-2 monolayers, although translocation through the co-culture system always occurred at significantly higher levels ($P<0.001$). Given the correlation with strain origin, these differences again indicate variation in EspF activity between the different *espF* alleles

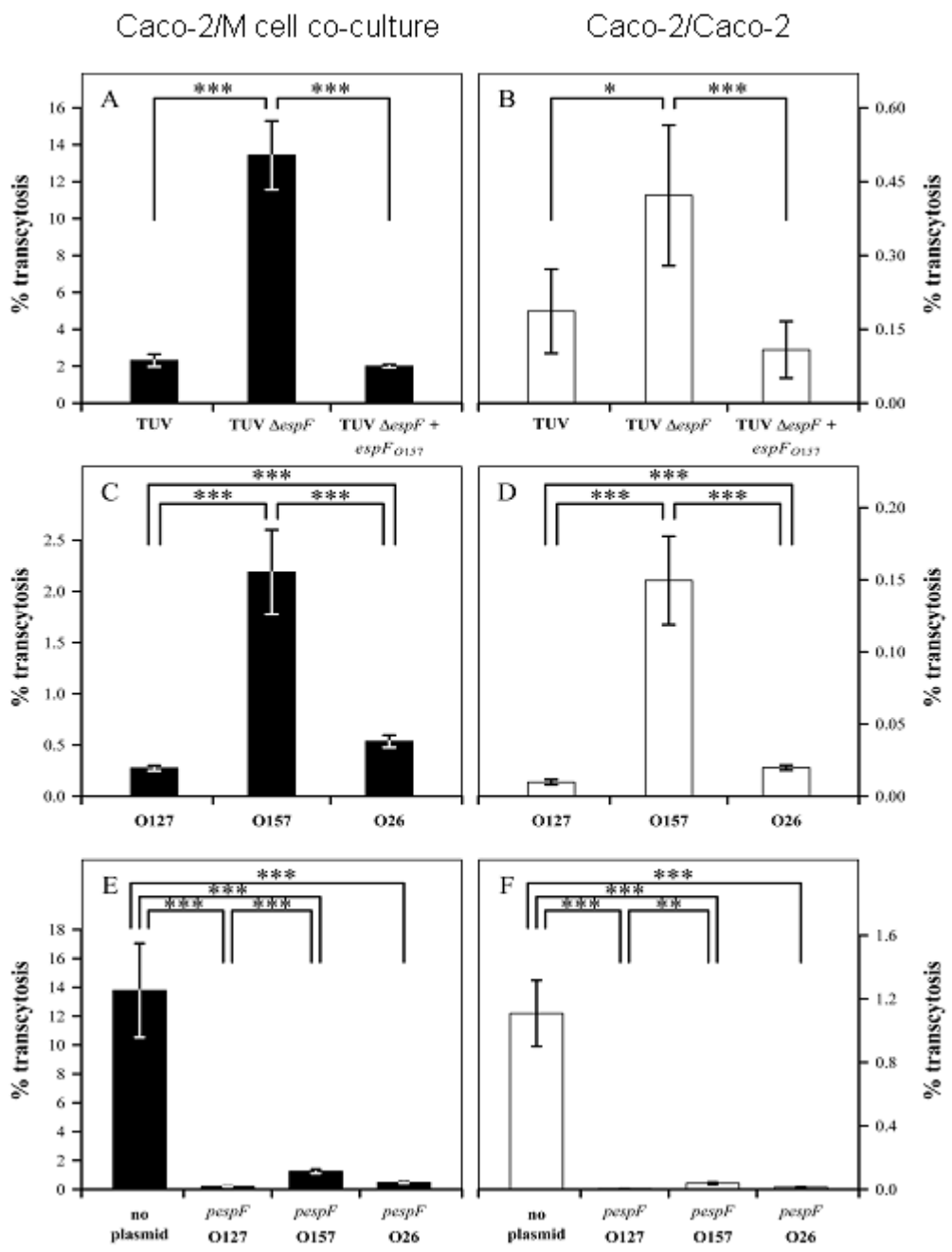


**Fig. 3.2.7 Translocation of EPEC and EHEC strains across a Caco−2 and lympho−epithelial M−cell co−culture system** (black bars (A,C,E)) and Caco−2 cells only (white bars (B,D,F)). (A,B) *espF* is required to inhibit EHEC O157 TUV93−0 translocation through M−cells. Translocation of EHEC O157 TUV93−0 compared with an isogenic *espF* deletion mutant and complemented with *espFO157* (pAT1, Table 2.5). (C,D) Comparative translocation of EPEC O127:H6 strain E2348/69, EHEC O157:H7 ZAP 198 EHEC O26:H11 across the co−culture system. (E,F) Comparative translocation of an EPEC O127Δ*espF* mutant complemented with the three defined *espF* alleles. ** & *** statistical significance of P<0.05, P<0.01 & P<0.001, respectively.

### 3.2.8 The significance of *espF* alleles on the interaction of *E. coli* with primary cultures of bovine rectal epithelial cells.

The terminal rectum of cattle contains a high number of lymphoid follicles and M-like cells that are present in the follicle-associated epithelium (FAE) (Mahajan *et al.*, 2005). Primary cells were cultured from a mixed population of crypts isolated from this bovine terminal rectum (Materials & Methods). The cells in the monolayers expressed cytokeratins (Fig. 3.2.8a A) indicative of epithelial cells. Screening with a panel of antibodies (Table 2.10 Materials & Methods) provided no indication of contaminating cells, such as fibroblasts and other mesenchymal cells (This work was done by Dr Arvind Mahajan). It was apparent that a small proportion of cells expressed Vimentin, an intermediate filament protein indicative of M-cells (Fig. 3.2.8a A). A subset of Vimentin-expressing cells endocytosed latex fluorescent microparticles (Fig. 3.2.8a C&C1). *Salmonella enterica* serovar Typhimurium preferentially targets M cells in the gut (Clark *et al.*, 1994) and therefore were used to test if the bacteria and the latex particles were internalised by vimentin expressing cells in culture. Indeed, during early stages of infection *S.* Typhiumurium interacted primarily with vimentin expressing cells in culture (Fig. 3.2.8a D) that also had internalised the latex beads (Fig. 3.2.8a E), a further indication that these cells can be considered M-cells based on previous M-cell characterisation studies (Kerneis *et al.*, 1997). Taken together, this data indicates that primary cells cultured from crypts isolated from bovine rectal FAE do contain a subset of cells with characteristics of M-cells and are capable of taking up particles including bacteria.

To determine the significance *espF* has on the interaction of *E. coli* O157 with these primary cells, translocation across these primary cell cultures was then assessed for the wild type strains, the EHEC O157 Δ*espF* deletion and EPEC Δ*espF* complemented with the three different alleles. In agreement with the co-culture translocation assay, *espF* significantly contributed to inhibition of translocation through these cultured cells; however, in clear contrast to the 'human' co-culture system, in the bovine assay EHEC O157 was significantly better at inhibiting translocation compared to EPEC O127 and EHEC O26 ($P<0.001$). In line with this, the $espF_{O157}$ allele showed the highest activity in restricting transcytosis in the EPEC

Δ*espF* background, although this was not significant (Fig. 3.2.8b C) by comparison with the two other alleles. As an alternative assessment of the function of these alleles on the bovine primary cells, an intracellular kanamycin protection assay was developed that determined the intracellular bacterial numbers at 90 min post infection. The relative percentage of bacteria taken up into cells is low but increased significantly on deletion of *espF* (Fig. 3.2.8c A). EHEC O157 was taken up into cells at significantly lower levels compared to EPEC O127 and EHEC O26 (Fig. 3.2.8c B, $P<0.001$) in agreement with the transcytosis results. This assay was then carried out with the three *espF* alleles in the EPEC E2348/69Δ*espF* strain. All three *espF* alleles reduced uptake significantly (Fig. 3.2.8c C, $P<0.001$) with the $espF_{O157}$ variant showing significantly higher activity than the $espF_{O127}$ (Fig. 3.2.8b E, $P<0.05$) and $espF_{O26}$ ($P<0.001$) alleles.

The fact that the relative activities of the *espF* clones are reversed in the bovine assays indicates that the differences measured between the variants are not due to expression or secretion levels. This is supported by the correlations with the relative activity levels of the parental strains in the different assays. Taken together, it is likely that the relative activities are due to different functional capacities of the EspF variants and in turn these are dependent on the host cell type in which the variants are acting.

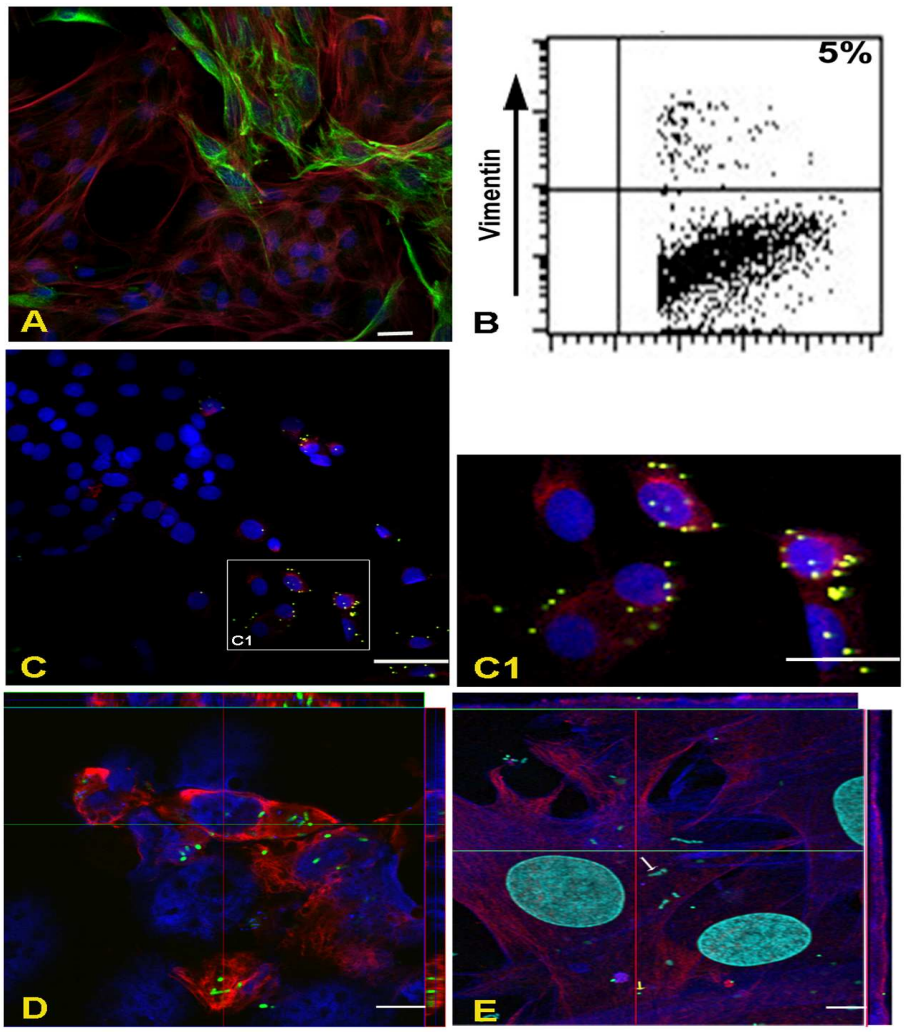


**Fig. 3.2.8a Characterization of bovine primary rectal epithelial cells.** (A) Heterogeneous population of epithelial cells in a primary cell culture from the bovine terminal rectum. A five day old culture was prepared and immuno-labelled to detect: vimentin (green); pan-cytokeratins (red) and nuclei (blue) as described in Materials and Methods. A subset of the cells expressed the intermediate filament protein, vimentin (green), indicative of M-cells. (B) Vimentin expressing cells were quantified using flow cytometry from 4 independent primary cultures (SD+0.3). (C) Micro-particle co11 localization with vimentin expressing cells. A subset of vimentin expressing (red) cells interacted with fluorescent micro-particles (green). C1 inset image digitally magnified by a factor of 4. The primary rectal epithelial cells were incubated with latex particles (green) (0.2µm size) at 37o14 C, 5% CO2 for 45 min; fixed, permeabilized and labeled with anti-vimentin (red) and TO-PRO nuclear stain (blue). (D) Orthogonal section demonstrating *Salmonella* Typhimurium uptake by vimentin expressing cells during early stages of interaction. The cells were infected with mid-log phase *S.* Typhimurium (pUC18GFP-labelled SL1344 strain) at a MOI of 1:100 at 37o18 C, 5% CO2 for10 min. (E) Orthogonal section demonstrating combined uptake of *S.* Typhimurium (white arrow, DAPI-stained) and micro-particles (yellow arrow, green) by vimentin positive cells (red) in a bovine rectal primary culture. The cells were incubated with latex particles (green) for 45 min, washed (3xPBS) and further infected with mid-log phase *S.* Typhimurium at an MOI of 1:100 for 10 min. The infected cells were fixed, labeled with anti-vimentin (red) and DAPI nuclear stain. Confocal images were acquired using Zeiss LSM510 x 63 objective). Scale bar 10µm.

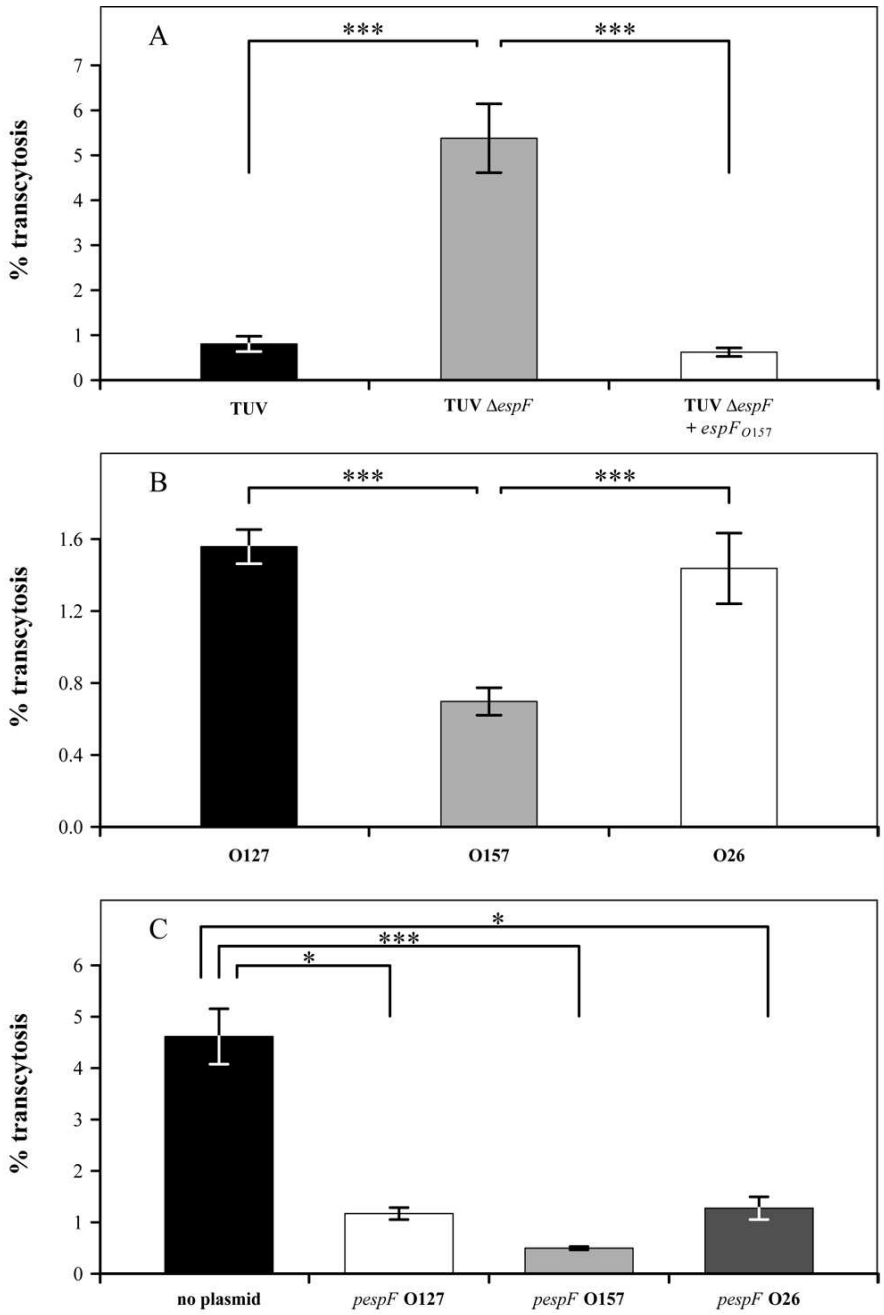


**Fig. 3.2.8b Interaction of EHEC and EPEC strains with cultured epithelial cells from the bovine terminal rectum (Transcytosis).** (A) *espF* limits EHEC O157 TUV93−0 uptake into rectal primary cells. Comparative transcytosis levels (%) of EHEC O157 TUV93−0 compared with an isogenic *espF* deletion mutant and complemented with *espFO157* (pAT1, Table 2.2).(B) Comparative transcytosis levels (%) of wild type strains: EPEC O127:H6 E2348/69, EHEC O157:H7 TUV93−0 and EHEC O26:H11 on interaction with bovine rectal primary cells. (C) Comparative transcytosis levels (%) of an EPEC O127D*espF* mutant complemented with the three defined *espF* alleles. * & *** indicate a statistical significance of $P<0.05$ and $P<0.001$, respectively.

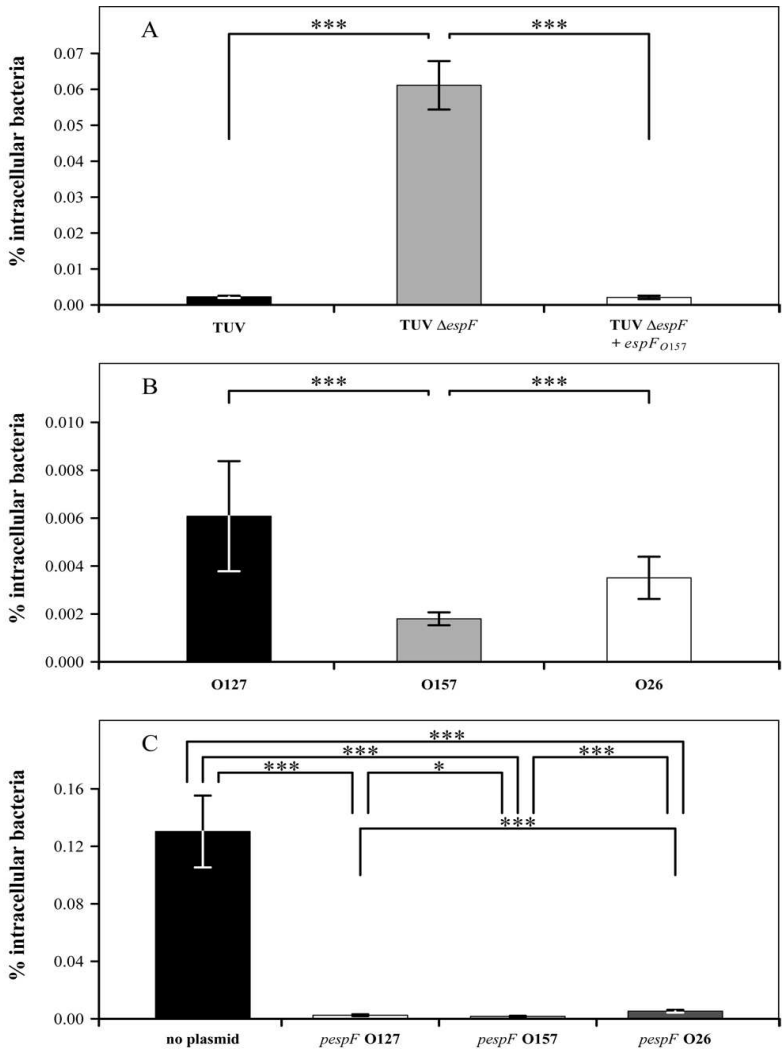


**Fig. 3.2.8c Interaction of EHEC and EPEC strains with cultured epithelial cells from the bovine terminal rectum (internalization).** (A) *espF* limits EHEC O157 TUV93-0 uptake into rectal primary cells. Intracellular levels of EHEC O157 TUV93-0 compared with an isogenic *espF* deletion mutant and complemented with *espFO157* (pAT1, Table 2.2). (B) Comparative intracellular levels of wild type strains: EPEC O127:H6 E2348/69, EHEC O157:H7 TUV93-0 and EHEC O26:H11 on interaction with bovine rectal primary cells. (C) Comparative intracellular levels of an EPEC O127D*espF* mutant complemented with the three defined *espF* alleles. *&*** indicate a statistical significance of $P<0.05$ and $P<0.001$, respectively.

### 3.2.9 Molecular basis to the comparative activity of EHEC and EPEC *espF* alleles

Previous work has established that EspF has a number of interacting partners in eukaryotic cells including interactions with both Sorting Nexin 9 (SNX9) and N-WASP. As SNX9 is important for endocytosis dynamics and N-WASP is central to actin polymerization, we investigated by LUMIER binding assay whether differences in the interactions of the EspF variants with these proteins could account for the differences in functional levels measured in this study. To quantify the binding activity, the three EspF variants as well as their binding partners SNX9 (human) and N-WASP (human) were cloned into plasmids that express either protein A-tagged proteins or Renilla luciferase fusion proteins (Fig. 3.2.9 a&b and Materials & Methods). Cell lysates containing both sets of tagged proteins were generated and then the protein A-tagged complexes removed using antibody-coated beads. Total fluorescence and captured fluorescence were measured and z-scores for each interaction calculated by comparison with a large bank of negative non-interacting controls (Barrios-Rodiles *et al.*, 2005). The results confirmed the interaction of all the EspF variants with both SNX9 and N-WASP (Fig. 3.2.9 c). The EspF O157 variant demonstrated a significantly weaker interaction with luciferase-linked SNX9 compared to the other variants and had the lowest interaction score of the three with respect to binding to N-WASP.

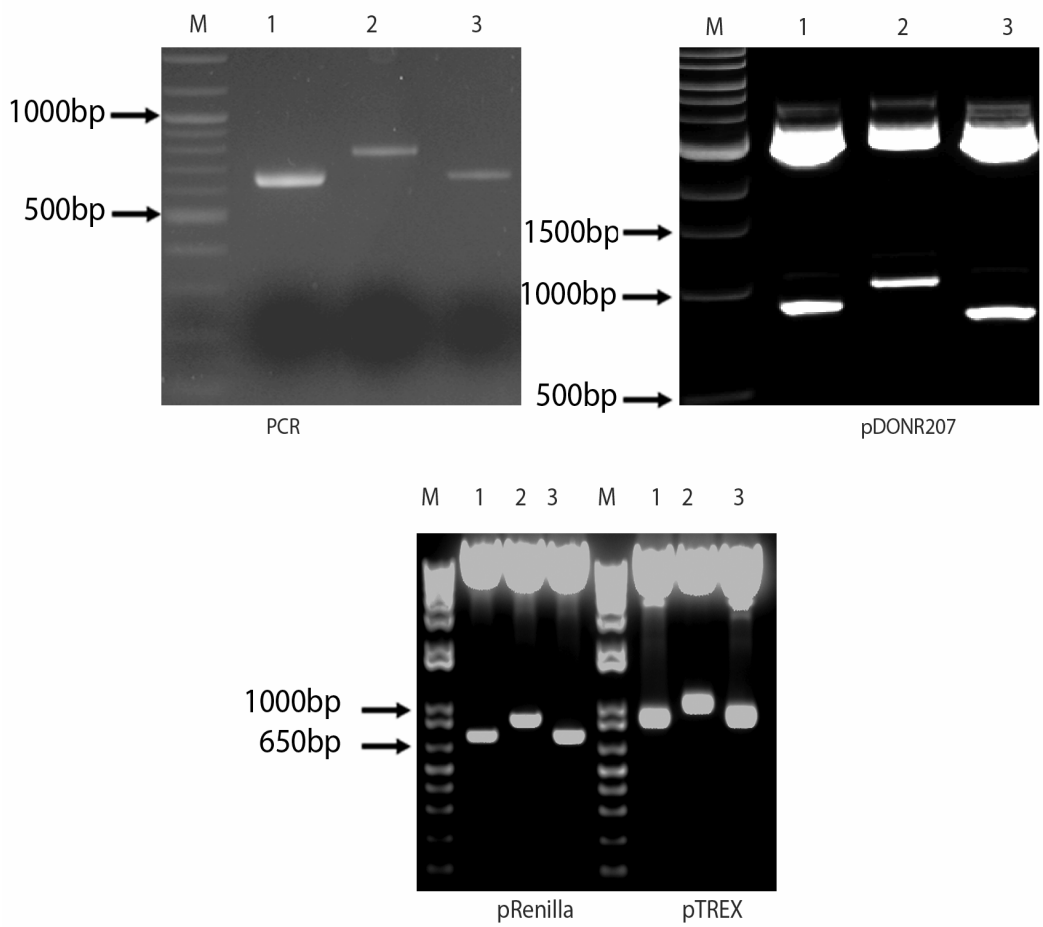


**Fig. 3.2.9a Gate way cloning of different *espF* alleles for protein- protein interaction:** Scanned image of gel red stained 1% TAE agarose gel which shows PCR amplification of of *EPEC* O127:H6 and *EHEC* serotypes O157:H7 and O26:H11 respectively. *Ban*II digests of different *espF* clones in the entry vector pDONR 207 and cloning of these three alleles into different destination vectors to create expression clones. *Xho*I and *Xba*I digests of different ORF clones in the pcDNARenilla. *Xho*I and *Nhe*I digests of different ORF clone into PTREX. Lane M contains 1 kbp plus DNA ladder (Invitrogen). Lane 1-3 *espF* of EPEC O127:H6 and EHEC serotypes O157:H7 and O26:H11respectively.

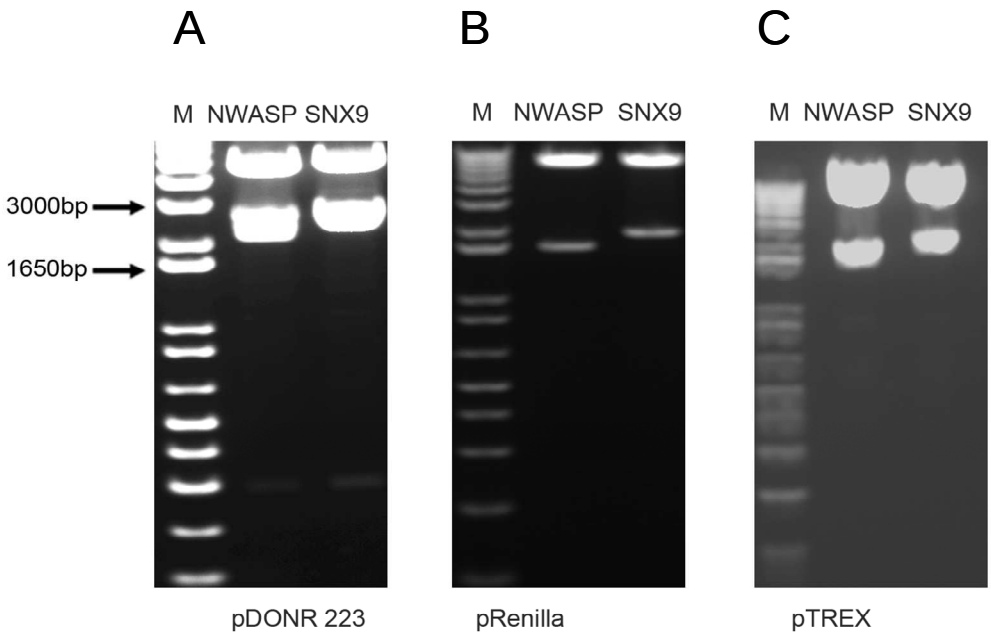


**Fig 3.2.9b Gateway cloning of NWASP and SNX9 alleles for protein- protein interaction studies:** Scanned image of gel red stained 1% TAE agarose gel which shows XhoI and XbaI digests of NWASP and SNX9 clones in the entry vector pDONR 223 and cloning of these two alleles into different destination vectors to create expression clones. *Xho*I and *Xba*I digests of different ORF clones in the pcDNARenilla. *Xho*I and *Nhe*I digests of different ORF clone into PTREX. Lane M contains 1 kbp plus DNA ladder (Invitrogen).

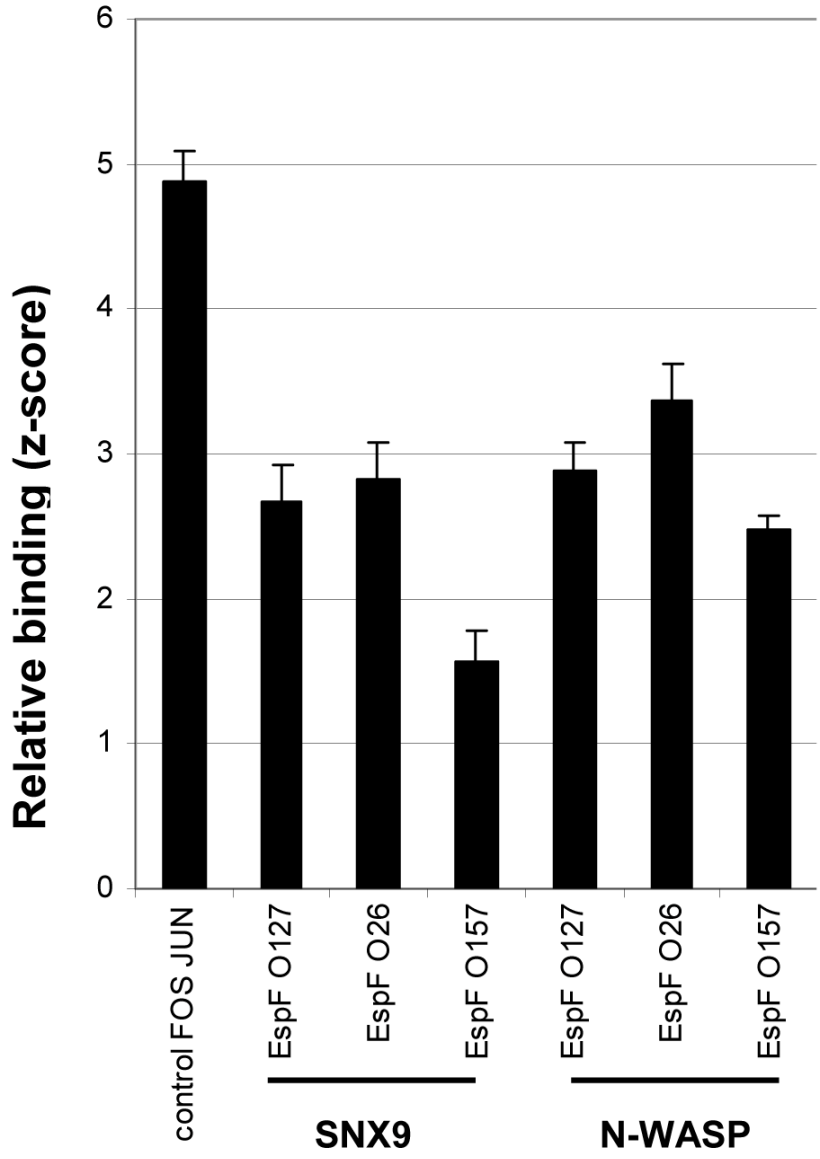


**Fig 3.2.9c EspF and human SNX9 or NWASP interaction in the LUMIER binding assay.**the *espF* alleles were expressed with a protein A tag and then immobilized on immunoglobulin beads. SNX9 and N-WASP were expressed with Renilla luciferase tags and incubated with the immobilized EspF variants. The luminescence signals were measured and z-scores calculated by subtracting the population mean from an individual raw score and then dividing the difference by the population standard deviation. FOS Jun used as control positive.

## 3.3 DISCUSSION

Enteropathogenic *Escherichia coli* is known to inhibit phagocytosis via inhibition of PI 3-kinase activity and this has been shown to be due to the activity of the type III secreted effector EspF (Celli *et al.*, 2001; Quitard *et al.*, 2006). While more recent research has established that a number of secreted proteins can also function to inhibit uptake into cells, including EspB (Iizumi *et al.*, 2007), EspJ (Marchès *et al.*, 2008) and EspH (Dong et *al.*, 2010). The inhibition of EPEC translocation through M-cells has been shown to be dependent on *espF*, presumably in a manner analogous to its activity on macrophages. Comparison of EspF protein sequences from EPEC O127, EHEC O26, and EHEC O157 shows a number of differences in both the localization domain and in the number and sequence of proline-rich repeats. The aim of this study was to investigate if these differences affected the capacity of the EspF variants to: (i) inhibit bacterial phagocytosis into macrophages; (ii) inhibit M-cell translocation in a human-derived Caco-2/Raji-B co-culture system; (iii) inhibit uptake into bovine primary epithelium, containing M-like cells, cultured from the terminal rectum of cattle, the predominant colonisation site of EHEC O157:H7 in cattle.

Strain comparisons demonstrated that both EPEC O127 E2348/69 and *E. coli* O26:H11 had a much greater capacity to block non-opsonized phagocytosis into cultured murine macrophages when compared with EHEC O157:H7. Both the EHEC O157 and EHEC O26 strains adhered to the macrophages at lower levels than the EPEC O127 strain (Fig. 3.2.1 A-C). To determine whether this strain difference could be accounted for by variation in the EspF effector protein, the genes from the three serogroups, EPEC O127, EHEC O157, and EHEC O26 were cloned and expressed in EPEC O127 that was deleted for *espF*. While the *espF* alleles from both O127 and O26 completely complemented the mutant, the $espF_{O157}$ allele showed a significantly reduced ability to block phagocytosis. This indicates that the initial strain observations may be accounted for by differences in the *espF* alleles. This result does not rule out important anti-phagocytic functions for the other effector proteins but does indicate synergistic roles with EspF and a significant dependence

on EspF. The different alleles did not alter the level of attachment of the complemented EPEC strain to Caco-2 cells in line with previous research that indicates EspF has no direct role in attachment as well as attaching and effacing lesion formation (McNamara & Donnenberg, 1998; Naylor *et al.*, 2005b; Shaw *et al.*, 2005).

It was then investigated whether the different *espF* alleles varied in their capacity to inhibit *E. coli* translocation through M-cells. The rationale for investigating this function is the finding that EHEC O157:H7 predominately colonizes the terminal rectum of its main reservoir host, cattle, and that this site is rich in lymphoid follicles (Naylor *et al.*, 2003). Epithelium associated with lymphoid follicles is subject to different signals from the high abundance of B and T cells in these follicles leading to production of M-cells (Guttman *et al.*, 2007) that sample lumen particles from the gut, delivering them to antigen presenting cells that initiate appropriate adaptive immune responses at these sites. M-cells have been postulated to be an important cell for the initial uptake or colonisation by different enteric bacterial pathogens such as *Salmonella*, *Shigella* and *E. coli* ( Kohbata *et al.*, 1986; Jensen *et al.*, 1998). For EHEC O157:H7, it is a cell type that the bacteria will encounter when colonizing the terminal rectum ( Naylor *et al.*, 2003; Mahajan *et al.*, 2005) and consequently inhibiting translocation may promote colonisation for example by providing an initial attachment site and/or limiting bacterial presentation to the host's immune system. The origin of M-cells is unclear as studies either support their derivation from enterocytes via extrinsic stimuli (lumenal antigens and/or lymphoid-follicle derived signals) or conclude that they originate from lineage specific precursor cells (Smith & Peacock, 1980; Savidge & Smith, 1995; Savidge, 1996; Gebert *et al.*, 1999). An M-cell co-culture system was first defined in 1997 (Kerneis *et al.*, 1997) and makes use of the capacity of Raji-B cells to signal differentiation of human colon-derived Caco-2 cells. Initial experiments demonstrated that *espF* from EHEC O157 is required to limit EHEC O157 translocation through M-cells but that the EHEC O157 *espF* allele was again reduced in its capacity to limit translocation of *E. coli* when compared with the O127 and O26 alleles. This finding mirrored the relative capacity of the specific strains to inhibit their translocation, again demonstrating the significance of the *espF* allele for the strain phenotype.

The strains and *espF* alleles were then compared on primary cells cultured from crypts isolated from the bovine terminal rectum. These cultures were further characterised in this study. A subset of the primary cells expressed the intermediate filament protein, vimentin, that is found in rabbit intestinal M-cells (Clark *et al.*, 1993). M-cells in the terminal rectum of cattle have been demonstrated to express vimentin as the predominant intermediate filament protein (Mahajan *et al.*, 2005) and the same staining was apparent in the cultured primary cells. A subset of vimentin expressing cells were also able to take up *S.* Typhimurium (Fig. 3.2.8a D), and *S.* Typhimurium and beads together (Fig. 3.2.8a E). Therefore, the bovine rectal primary cultures contain a subset of cells that express vimentin and have characteristics of M-cells. There was a significant role for *espF* in inhibiting bacterial uptake into cells and translocation through these cells and the activity of the strains correlated with that of the *espF* alleles. An important result was that in contrast to the previous experiments on mouse macrophages and the human-derived M-cell co-culture system, EHEC O157 was the most effective strain at inhibiting uptake and translocation and this correlated with the relative activity of the *espF* alleles (Fig. 3.2.8b). Taken together the results indicate that EHEC O157 and the $espF_{O157}$ allele are more effective at inhibiting uptake into bovine M-cells compared with the O26 and O127 strains and alleles, a reversal of the situation observed for the interactions with murine macrophages and the human co-culture system.

There are multiple phenotypes associated with EspF in addition to inhibition of phagocytosis, including inhibition of water transport and disruption of tight junctions mitochondria and the nucleolus (Viswanathan *et al.*, 2004a). Uptake functions are likely to be related to EspF interactions with N-WASP and Sorting Nexin 9 (SNX9) (Alto *et al.*, 2007). N-WASP stimulates actin filament assembly by direct activation of the Arp2/3 complex and SNX9 is essential for clathrin-coated pits (CCPs) at the late stages of vesicle formation. SNX9 binds to β2-appendages of adaptor protein complex 2 (AP-2) and interacts with clathrin and dynamin-2, two other important molecules in the endocytic process (Lundmark & Carlsson, 2003; Soulet *et al.*, 2005). SNX9 has two lipid interaction domains; a phospholipid-binding region termed the phox (PX) domain followed by a putative Bin/Amphiphysin/Rvs (BAR) lipid-binding domain. The BAR domain is a banana-shaped helical dimer that senses

membrane curvature and can reconfigure lipid vesicles or sheets into membrane tubules (Alto *et al.*, 2007). SNX9 also possesses an N-terminal Src homology-3 (SH3) protein interaction region that was recently shown to bind WASP (Badour *et al.*, 2004) and to functionally activate dynamin at CCPs. Therefore, SNX9 is an important factor in re-modelling the membrane and cytoskeletal during endocytosis (Alto *et al.*, 2007; Soulet *et al.*, 2005). The SH3 domain of SNX9 binds to the PPRRs of EspF (Dean *et al.*, 2010). Despite both direct and indirect (via SNX9) activation of N-WASP, EspF is not considered to have a role in A/E lesion formation (McNamara *et al.*, 2001; McNamara & Donnenberg, 1998). To determine if the different EspF variants have different affinities for SNX9 and N-WASP, the interactions were assayed inside transfected HEK293 cells using a LUMIER assay (Barrios-Rodiles *et al.*, 2005) and described in Materials and Methods. This assay confirmed that all three EspF variants bound to both N-WASP and SNX9, although $EspF_{O157}$ showed a lower affinity for SNX9 and for N-WASP by comparison with the other two variants. A specific region in the PRRs of EspF (shaded grey in Fig. 3.2.2b) interacts directly with SNX9 through its SH3 domain–binding motif. There are only minor differences in this region between the serotypes examined in this study and we cannot determine whether these or their presentation, due to sequence changes in flanking regions, account for the differences observed.

The published *Bos taurus* sequence for SNX9 (NCBI) contains a number of changes over the human variant used in the LUMIER assays, however these differences are mainly present in the amino terminus of the predicted bovine SNX9 and lie outside the SH3 domain. There are also very few sequence differences between the predicted *Bos taurus* N-WASP protein sequence (NCBI) and the human variant. Taken together, our results indicate that there are likely to be other protein interactions involving EspF that could also contribute to the host specificity demonstrated in this study. The greatest region of diversity between the variants lies in the motif adjacent to the first PRR, with no established function for this EspF region. Future work will aim to identify other interacting partners for EspF and address how important M-cell interactions are for colonisation of cattle at the terminal rectum or whether other factors may explain the tropism of the EHEC O157:H7 for this gastrointestinal site.

# *Chapter 4*

## *Identification of host proteins interacting with EspF*

# 4. Identification of host proteins interacting with EspF

## 4.1 INTRODUCTION

EspF, a LEE-encoded effector protein is translocated into host cells in a T3S system dependent manner. $EspF_{O127}$ disrupts tight junctions, causes an increase in mitochondrial membrane permeabilization leading to cell death, inhibits bacterial uptake by macrophages and restricts translocation across antigen sampling M-cells in the gut. From the previous results chapter LUMIER binding assays demonstrated differences in the interactions of the EspF variants with SNX9 and N-WASP; it may be that other yet uncharacterized interactions may contribute to the host-based variation in EspF activity determined in this study.

In this study, protein-protein interactions for three EspF variants from EHEC O157 EHEC O26 and EPEC O127 were analysed using yeast -2- hybrid approaches.

Protein-protein interactions underlie many biological systems and global screening techniques to identify specific protein-protein interactions have been developed. The most established of these is Yeast 2-hybrid screening that, despite limitations, is still an excellent and evolving system for high-throughput screening of interactions. The Y2H approach is based on the Gal4-based yeast-two-hybrid (Y2H) system (Fields & Song, 1989). The bait proteins are fused to the Gal4 DNA binding domain and the 'prey' proteins to the Gal4 activation domain. If the bait and the prey interact then this generates an active transcription factor activity leading to the expression of an easily detected reporter gene. Major advances in the technique have allowed it to be used in yeast mating protocols that can be automated (Fromont-Racine *et al.*, 1997). Another major advance that has facilitated the approach has been the ease with which large libraries can be created and relevant DNA sequences sub-cloned using the Gateway recombinational cloning (Walhout *et al.*, 2000). While these high- throughput techniques have been applied to examine protein interactions within a single species (Rain *et al.*, 2001; LaCount *et al.*, 2005), there is increasing use of the technique to screen individual targets against 'host' organism cDNA libraries.

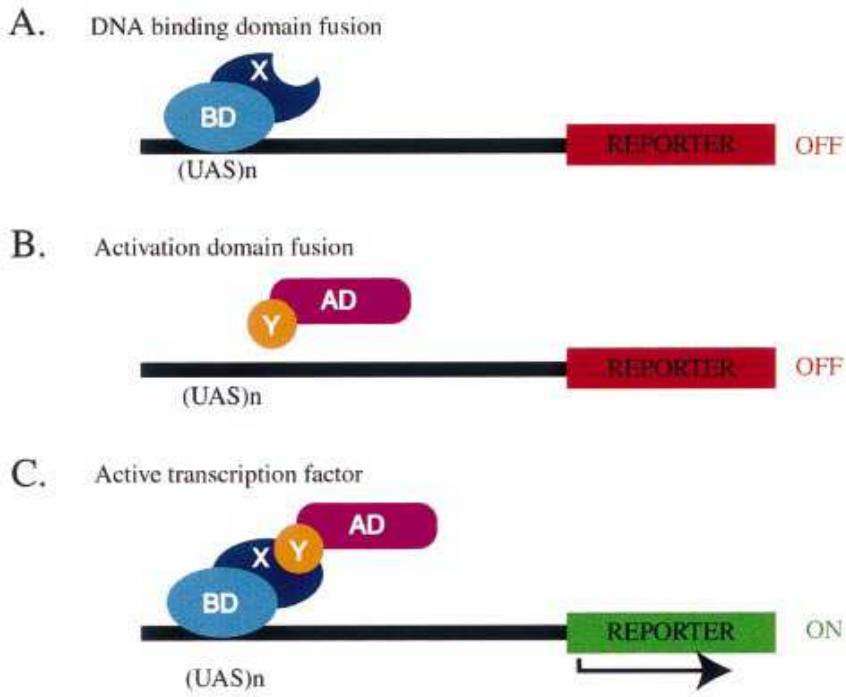


**Figure 4.1 The yeast-two-hybrid system.** The binding domain (BD) of the yeast transcription factor GAL4 fused with the protein to be screened (X) in bait. The protein liberary to be identified as interactors (Y) was fused to activating domain (AD) of the yeast trancretion factor GAL4. If X and Y interact together, the activity of the transcretion factor will be reconstituted and initiating the transcription of a gene under regulation of the up-stream activation sequence (UAS) (Fields & Song, 1989).

Applied to infectious disease research, there have been a number of studies that have taken specific proteins from the infectious agent and identified interacting proteins from human protein libraries.

From this, tremendous progress has been made in understanding the molecular basis of the disease, process, for example in the field of enteropathogenic *E. coli* disease (Hemrajani *et al.*, 2010; Shames *et al.*, 2010).

Furthermore, once libraries are created then the identified interacting partners can easily be sub-cloned into a variety of vectors to allow the interactions to be verified and the biology to be investigated. Several Y2H screens in the recent years have identified very important interactions that have been crucial in understanding EHEC O157:H7 pathogenesis. The type III secretion system of EHEC O157:H7 translocates approximately 50 proteins into host cells that contribute to bacterial persistence and shedding. The functions of these 'effector' proteins are being elucidated in several labs across the world. The Y2H assay has previously been used to identify that EspF interacts with the eukaryotic proteins Cytokeratin 18, N-WASP and Sorting Nexin 9 (SNX9). Subsequent work has aimed to elucidate the importance of these interactions for bacterial colonization of intestinal cells ( Viswanathan *et al.*, 2004b; Marchès *et al.*, 2006; Alto *et al.*, 2007).

The aim of this work is the identification of host specific proteins that interact with the EspF secreted effector protein from EHEC O157:H7 and to confirm any interactions with co-localisation studies.

## 4.2 RESULTS

### 4.2.1 Gateway cloning of different *espF* alleles

#### 4.2.1.1 Cloning of *espF* alleles into the pDONR 207 vector

*espF* alleles were amplified using primers (27&28) for EPEC and EHEC O157:H7 *espF* and primer (25&26) for EHEC O26:H11 *espF*. The PCR products were purified before being cloned into the donor vector according to the manufacturer's instructions to form the entry clones. These entry clones can be easily subcloned into different destination and expression vectors to be used for different purposes. The

cloning into the pDONR 207 vector was initially confirmed by restriction digestion with *Ban*II enzyme (Fig. 4.2a). MAD2L2 and NFIC were cloned into the pDONR 223 vector by Prof. Juergen Haas, University of Edinburgh, UK initially and I confirmed this clones by restriction digest with *Xba*I and *Nhe*I enzymes and sequencing.

#### 4.2.1.2 Sub-cloning of *espF* from entry clones into multiple destination vectors

##### 4.2.1.2a Plasmids for Y2H and Co-IP

In order to use the Y2H system to investigate interactions between EspF proteins and eukaryotic proteins, *espF* entry clones were sub-cloned into the Y2H bait pGBKT7-DEST and the eukaryotic entry clones library was sub-cloned into the prey vector pGADT7-DEST. Both vectors are destination vectors with different resistance genes, ampicillin in case of the prey vector and kanamycin for the bait vector. The prey and bait clones were confirmed by a double restriction digest using *Eco*RI and *BamH*I restriction enzymes (Fig. 4.2a and 4.2b respectively).

##### 4.2.1.2b Plasmids for LUMIER pull-down assay

In order to use LUMIER pull-down assay for validation of interactions, a subset of EspF, and specific identified target eukaryotic proteins (Mad2L2 and NFIC) were sub-cloned from the pDONR 207 or pDONR 223 entry clones into the LUMIER Renilla and protein A-tagged vectors pcDNA-Renilla and pT-REx-A (Fig 4.2a and 4.2b, respectively). Both vectors are expression vectors with an ampicillin resistance. The Renilla-tagged clones were confirmed by a double restriction digest using *Xho*I and *Xba*I restriction enzymes, and the protein A-tagged clones were confirmed by a double restriction digest using *Xho*I and *Nhe*I restriction enzymes (Fig. 4.2a and 4.2b, respectively).

##### 4.2.1.2c Plasmids for eukaryotic transient-expression to validate the Y2H positive interaction

In order examine possible EspF co-localisation with identified eukaryotic targets (Mad2L2 and NFIC) in the cell, the *espF* entry clones were sub-cloned into the N-terminal GFP-tagged peGFP-DEST vector that contain kanamycin resistant cassettes.

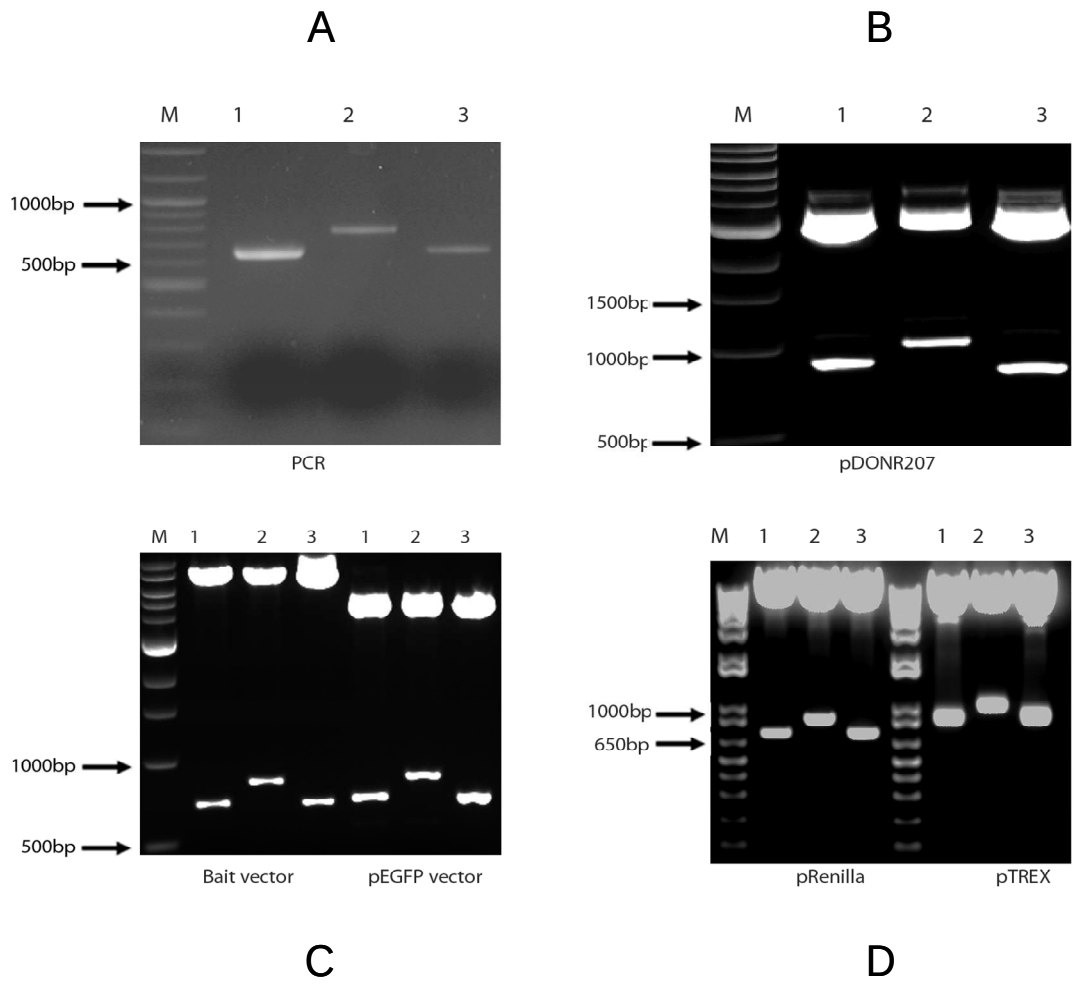


**Fig 4.2a Gateway cloning of different *espF* alleles to study protein-protein interactions.** Scanned image of gel red stained 1% TAE agarose gel which shows PCR amplification of the three different *espF* alleles, from EPEC O127:H6 and EHEC serotypes O157:H7 and O26:H11 respectively (A). *Ban*II digests of different *espF* clones in the entry vector pDONR 207 (B) and cloning of these three alleles into different destination vectors to create expression clones. *Eco*RI and *BamH*I digest of different ORF clones in Bait (C). *Eco*RI and *BamH*I digest of different *espF* clones in the peGFP vector (C). *Xho*I and *Xba*I digests of different ORF clones in the pcDNARenilla (D). *Xho*I and *Nhe*I digests of different ORF clone into PTREX (D). Lane M contains 1 kbp plus DNA ladder (Invitrogen) Lanes 1-3 *espF* of *EPEC* O127:H6 and *EHEC* serotypes O157:H7 and O26:H11 respectively.

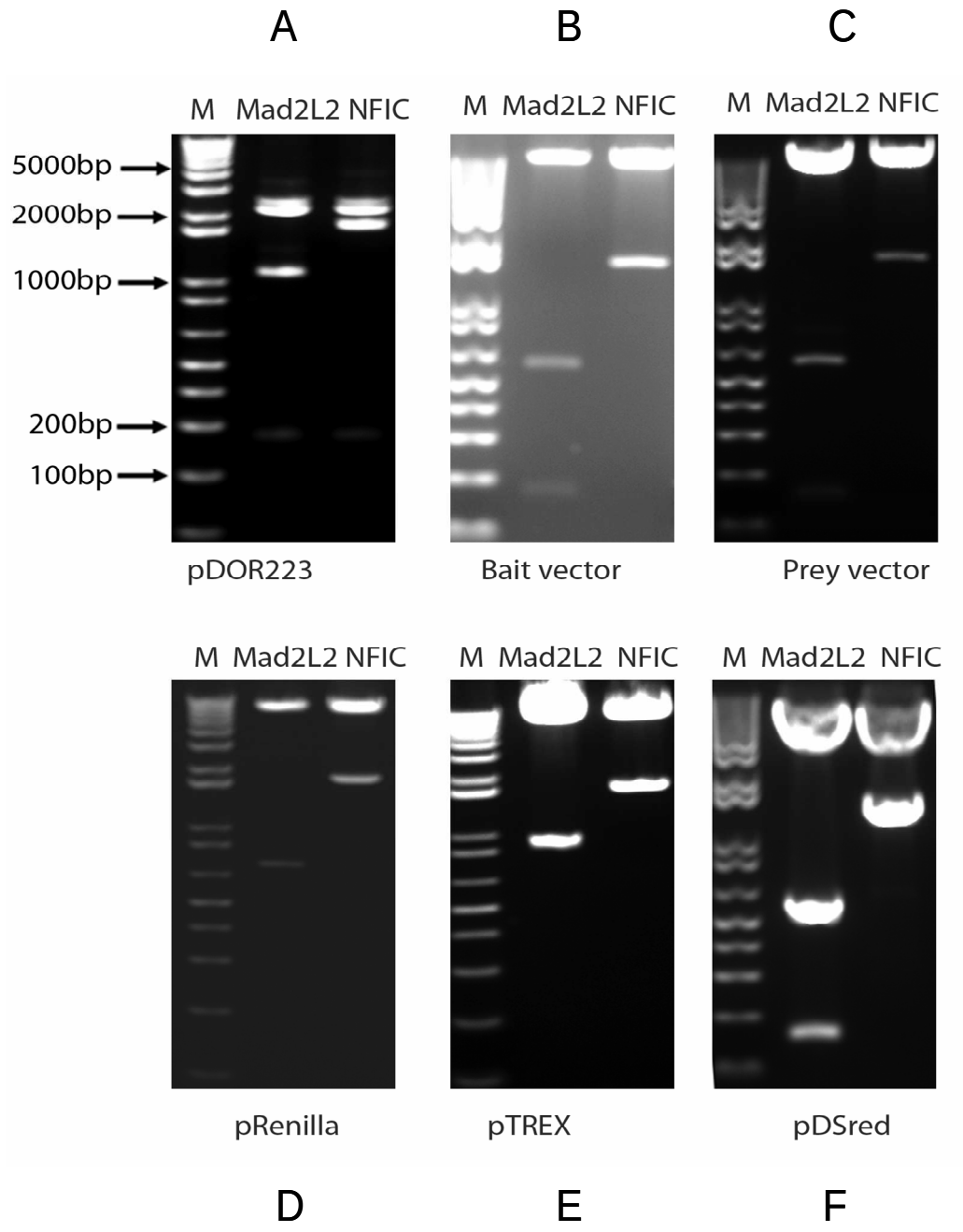


**Fig 4.2b Gateway cloning of Mad2L2 and NFIC genes for protein-protein interaction studies**: Scanned image of gel red stained 1% TAE agarose gel which shows *Xho*I and *Xba*I digests of *mad2L2* and *nfiC* clones in the entry vector pDONR 223 (A)and cloning of these two alleles into different destination vectors to create expression clones. *EcoR*I and *BamH*I digests of different ORF clones in Bait and prey(B&C). *Xho*I and *Xba*I digests of different ORF clones in the pcDNARenilla(D). *Xho*I and *Nhe*I digests of different ORF clone into PTREX (E). *EcoR*I and *BamH*I digests of different ORF clone into PDSred (F). Lane M contains 1 kbp plus DNA ladder (Invitrogen).

The expression clones were confirmed by a double restriction digest using *EcoR*I and *BamH*I (Fig. 4.2b). On the other hand, Mad2L2 and NFIC entry clones were sub-cloned into the N-terminal PD tagged pDSRED2-DEST containing kanamycin resistant cassettes. Clones were confirmed by a double restriction digest using *Xho*I and *Nhe*I restriction enzymes (Fig. 4.2b).

### 4.2.3 Y2H screening to identify eukaryotic proteins that interact with EspF

To identify physical interactions occurring between EspF and different eukaryotic cell proteins, different EspF bait clones were screened against a human cDNA prey library using the yeast two-hybrid system. Both the EspF bait clones and human cDNA prey clones library were transformed into compatible yeast strains. Two different yeast strains were used to generate Y2H prey and bait arrays, AH109 (α mating type) for the preys and Y187 (α mating type) for the baits. The transformed prey clones were grown on plates of mating media lacking leucine and the transformed bait clones were grown on plates of mating media lacking tryptophan. The vectors have the appropriate cassettes that allow the transformed yeast to grow on the selective plate lacking the appropriate amino acid (Prey plasmid pGBKT7 contains Trp gene that enable AH109 to grow on Leu-ve, Trp+ve plates. Bait plasmid pGADT7 contains Leu gene that help Y187 grow on Trp –ve, Leu +ve plates. These two strains were allowed to mate together and if the mating success the new clone can grow on the double-selection –LW SD/5% YPDA; minus Leucin and minus Trptophan. For different EspFs screen: Yeast strain Y187 for Prey and Yeast strain AH109 for Bait). The transformed yeast clones were tested in collaboration with Dr. Peter Uetz and Thorsten Stellberger at the Institute of Toxicology and Genetics, Karlsruhe, Germany, in a robot-assisted Y2H assay in 96- well plate format. In a matrix analysis, all EspF and human cell proteins were tested against each other. Since the Y2H assay can generate a number of false positive interactions, each pairwise interaction was tested in duplicates. If two of the matings were positive the interactions were scored as positive. If only one replicate was positive, the interaction was scored non-reproducible and negative. The positive results from the Y2H array were visualised and analysed using Cytoscape bioinformatics software

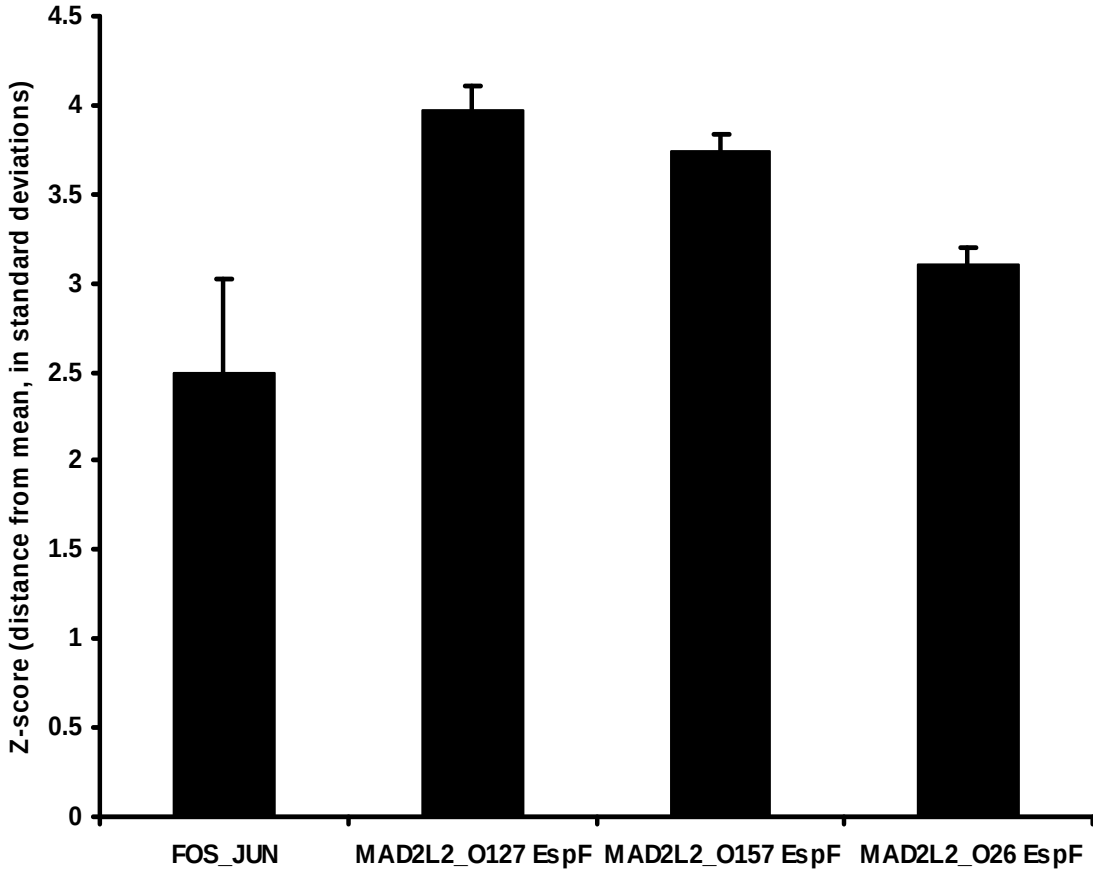


**Fig 4.2.2e Comparative binding analysis between EspF variants and human Mad2L2.** In the LUMIER binding assay the *espF* alleles were expressed with a protein A tag and then immobilized on immunoglobulin beads while Mad2L2 was expressed with Renilla and incubated with the immobilized EspF variants. The luminescence signals were measured and z-scores calculated by subtracting the population mean from an individual raw score and then dividing the difference by the population standard deviation, FOS Jun used as control positive.

Screening of yeast transformants yielded two positive hits for EspF of EHEC O157:H7 and one positive for EspF from EHEC O26:H11, but not interactions for EspF from EPEC O27:H6. The two interactions for $EspF_{O157}$ were with the CCAA-binding transcription factor (NFIC) and actin2. $EspF_{O26}$ interacted with the mitotic arrest-like 2 (MAD2L2) protein.

### 4.2.4 LUMIER binding assays

To confirm the interaction of EspFO26 with Mad2L2 and establish whether the other variants could also interact with it, the three EspF variants and Mad2L2 (human) were cloned into plasmids that produce either protein A-tagged proteins or Renilla luciferase fusion proteins as described in Materials and Methods. Cell lysates containing query luciferase fusions and protein A-tagged proteins were generated and then the protein A-tagged complexes removed using antibody-coated beads. Total fluorescence and captured fluorescence were measured and z-scores for each interaction calculated by comparison with a large bank of negative non-interacting controls (Barrios-Rodiles *et al.*, 2005). The results demonstrated that all the EspF variants were interacting with Mad2L2 (Fig 4.2.2e). The work was done in collaboration with Dr Manfred Koegl and Gibriellia Siszler.

### 4.2.5 Confocal microscopy to validate the interaction of EspF and Mad2L2

To confirm the co-localization of EspF and Mad2L2 proteins. HEK93 cells grown on chamber glass slide were transfected with an EspF N-terminal GFP tagged expression plasmid (pAT11-13), and a Mad2L2 DSred tagged expression plasmid (pAT20). 72 hr after transfection, cells were fixed, stained with DAPI and examined by fluorescence microscopy. The images indicate co-localisation of EspF and Mad2L2 in the nucleus (Fig 4.2.2f).

### 4.2.6 Purification of His-tagged EspF

The EHEC O157:H7 *espF* allele was amplified from genomic DNA using Expand DNA polymerase and the primers (19&20) Table 2.3.

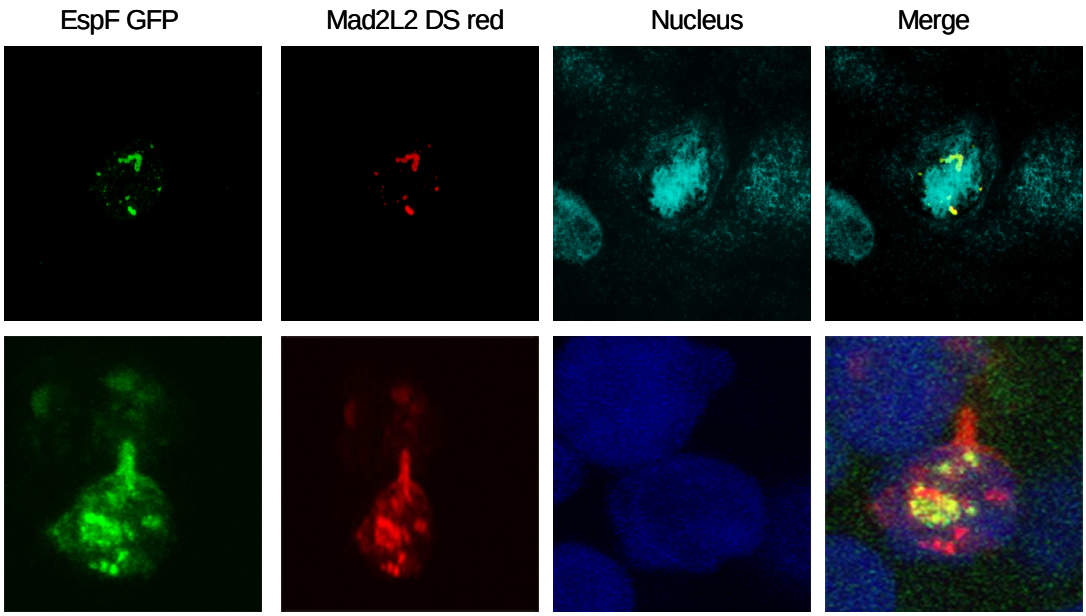


**Fig 4.2.2f Confocal microscopy to validate the interaction of EspF and Mad2L2** Cells were transfected with the expression plasmid peGFP-DEST (Promega) expressing EspF tagged to GFP, and pDSRED2-DEST (Promega) expressing DS red-tagged Mad2L2. After 72 hr, the cells were fixed and permeabilized with 2% (w/v) formalin / 0.25% (v/v) Triton X-100. The cells were incubated with DAPI 1/5000 to stain the nucleus (blue). The slides mounted in fluorescence mounting medium (DAKO). The pictures were acquired using a Leica TCS NT confocal system (x 63 objective).

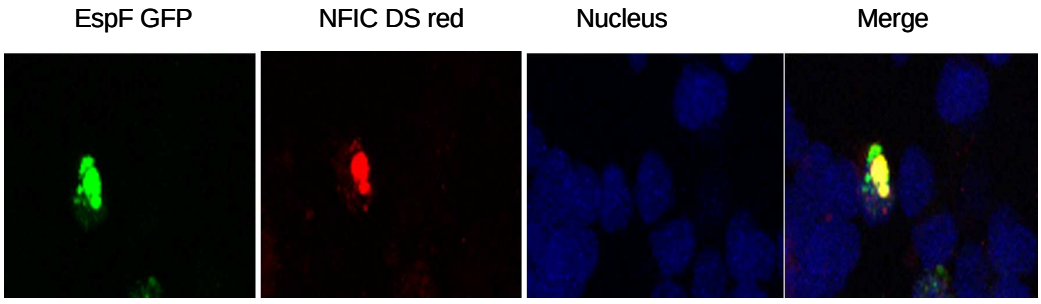


**Fig 4.2.2g Confocal microscopy to validate the interaction of EspF and NFIC.** Caco-2 cells were transfected with pAT11 expressing GFP-tagged EspF expression plasmid peGFP-DEST (Promega), and DS red -tag NFIC expression plasmid pDSRED2-DEST (Promega). After 72 hr, the cells were fixed and permeabilized with 2% (w/v) formalin / 0.25% (v/v) Triton X-100. The cells were incubated with DAPI 1/5000 to stain the nucleus (blue). The slides mounted in fluorescence mounting medium (DAKO). The pictures were acquired using a Leica TCS NT confocal system (x 63 objective).

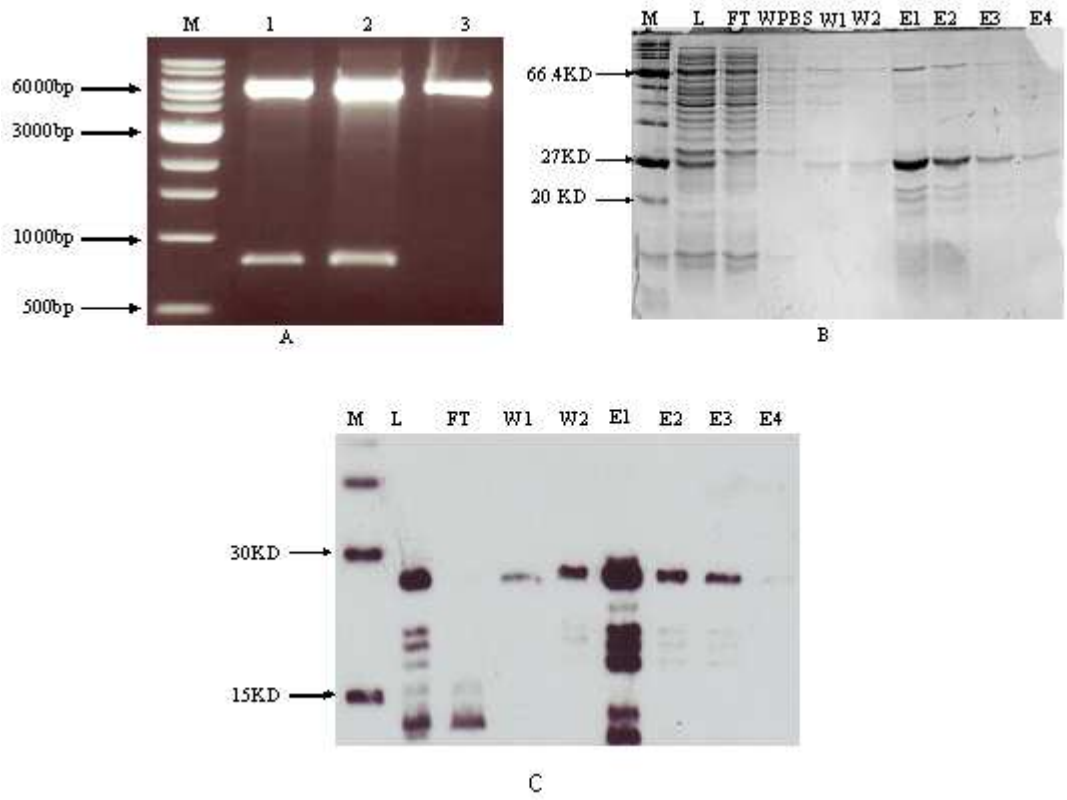


**Fig 4.2.2h Purification of *E. coli* O157:H7 histidine-tagged EspF** (A) Diagnostic digests of pET21-d clones of EHEC O157:H7 *espF* obtained by digestion with *Xba*I and *Xho*I as described in Materials and Methods. Electrophoresis was carried out through a 0.8% agarose gel. Lane M, 1Kb DNA ladder. Successful clones (lanes 1 & 2) contain a 700bp band. (B) SDS PAGE gel of whole cell lysate from BL21 with pET21-D-$espF_{O157}$ before and after IPTG induction. (C) Western blot analysis of the same samples using anti polyhistidine antibody.

The PCR product was purified and cloned into pET-21d by digesting both PCR and vector with *Xba*I/*Xho*I (New England Biolabs, Inc.) to obtain a plasmid that can express a polyhistidine-tagged EspF fusion protein-EspF-His. *E. coli* BL21/DE3 was transformed with pespF-His to optimize expression of EspF-His induction with IPTG. The culture was centrifuged and the lysate was obtained by sonication in lysate buffer. The EspF-His was purified using a nickel column resin, dialyzed in PBS and then stored at -20 °C.

### 4.2.7 Discussion

Both enteropathogenic and enterohemorrhagic *E. coli* persist in human or animal hosts by attachment to epithelial cells that line the gastrointestinal tract. Type III secreted effector proteins manipulate the actin cytoskeleton and innate responses of host cells to promote intimate bacterial attachment and persistence The well characterized translocated effector protein EspF is associated with tight junction disruption, water channel dysregulation and inhibition of bacterial uptake into M-cells. It localizes to both mitochondria and the nucleolus and is known to interact with Sorting Nexin 9, N-WASP and Abcf2. In this study yeast-2-hybrid screening was used to identify additional host proteins that may interact with EspF. The anaphase promoting complex inhibitor, Mad2L2, was identified from this screen. Mad2L2 was then demonstrated to interact with EspF variants from EHEC O157:H7, O26:H11 and EPEC O127:H6 by Lumier assays in which Protein A-tagged EspF was recovered from transfected cell lysates attached to luciferase linked Mad2L2, the reciprocal approach also confirmed the specificity of the interaction. EspF was also shown to co-localise with Mad2L2 in cells and the impact of EspF on the cell cycle will be investigated in the future work. While Mad2L2 has been shown to be targeted by the non homologous *Shigella* effector protein IpaB to limit epithelial cell turnover, I hypothesis that the interaction of EspF with MAD2L2 might have a similar function to promote EPEC and EHEC colonization.

MAD2L2 is also known as Mad2-related protein and is an inhibitor of the anaphase promoting complex (APC)-Cdh1 (Pfleger *et al.*, 2001). Activation of APC during interphase (G1/S) leads to cell cycle arrest at G2/M. MAD2L Cdh1 binding inhibits the activity of the Cdh1-APC complex and, thus, functions as a mitotic spindle

assembly checkpoint protein, preventing the onset of anaphase until all chromosomes are properly aligned at the metaphase plate. Mad2L2 plays a pivotal role in translational DNA synthesis (TLS) in S phase (Cheung *et al.*, 2006) and it also participates in the spindle assembly checkpoint, similarly to Mad2 (Reimann *et al.*, 2001) .

*Shigella* IpaB was shown to sequester MAD2L2 away from the APC-Cdh1 complex, leading to unregulated APC activity during interphase and subsequent cell cycle arrest (Table 1.1 Introduction & Iwai *et al.*, 2007). Cell division plays an essential role in the innate immune defense against infection as the rapid turnover of epithelial cells limits bacterial colonization. It is possible that slowing this turnover could prolong bacterial colonization by inceraese the ability of bacteria to multiply (Iwai *et al.*, 2007).

MAD2L2 has been shown to bind to TCF4-catenin complex that inhibit cell proliferation and enhance differentiation of epithelium cells to mesenchymal cells EMT (Naishiro *et al.*, 2001; Hong *et al.*, 2009).The binding of MAD2L2 to TCF4-catenin transcription factor also maintain the cell cycle and prevent the celluler transformation.

# *Chapter 5*

## *Interaction of Salmonella Typhimurium with intestinal epithelial cells*

# 5. Interaction of *Salmonella* Typhimurium with intestinal epithelial cell

## 5.1 Introduction

### 5.1.1 The intestinal immune system

The intestinal immune system must defend against the many infectious and toxic assaults that may breach the epithelium and cause intestinal injury. The intestinal immune system must simultaneously ignore the multitude of commensal organisms and dietary antigens that are not threats to the host.

To deal with this challenge, the gut-associated lymphoid tissue has evolved several important modifications of antigen sampling and processing, humoral immunity, and cellular immunity. These include flattened epithelial "microfold cells" that are a kind of intestinal epithelial cell in the follicle-associated epithelium of Peyer's patches. These cells can transport antigens and micro-organisms into underlying lymphoid tissues. The basal membrane of M cells is invaginated to form a pocket where cells of haematopoietic origin such as dendritic cells, macrophages, and lymphocytes in intimate contact with extruded antigen or intact organisms (Gebert, 1997). All of these cell types play a fundamental role in M cell differentiation (Ermak & Owen, 1986). M cells actively pinocytose luminal antigens but do not possess lysosomes, so antigens are not degraded or processed (Czerkinsky *et al.*, 1999). Although intact commensal bacteria are rarely engulfed, numerous pathogens, including human immunodeficiency virus (Knoop *et al.*, 2009), reoviruses, *Vibrio cholerae*, and *Shigella* species, selectively adhere to M cells, possibly through specialized carbohydrate- binding mechanisms, and are transported without degradation (Owen *et al.*, 1986a; Owen *et al.*, 1986b). M-cells generally lack the distinct microvilli and thick filamentous brush border glycocalyx (Owen *et al.*, 1977; Frey *et al.*, 1996) and instead have variable microfolds. That morphological change on M cells surface is regulated by the distribution of microvillar proteins such as actin and villin (Kerneis *et al.*, 1997; Kanaya *et al.*, 2008). Therefore, the disorganization of microvilli in M cells is not only a typical morphology of this cell type but also essential for the transcytosis of macromolecules. In particular, the

crucial role of lymphocytes in M cell differentiation has been demonstrated *in vitro* by M cell models using human colon adeno-carcinoma cell line Caco-2 cells and murine intestinal crypt-derived cell-line mICcl2 cells ( Kerneis *et al.*, 1997; El Bahi *et al.*, 2002). Caco-2 cells differentiate into cells resembling M cells during co-culture with Peyer's patches (PPL) or Raji B cells. These M-like cells have some of the in *vivo* characteristics, such as transcytosis activity, a decrease of sucrase-isomaltase and villin expression, disorganization of microvilli and a change of β-1 integrin localization (Kerneis *et al.*, 1997). Murine intestinal epithelial cell line (MIE) is able to differentiate into M cells following co-culture with intestinal lymphocytes or PPL co-stimulated with anti-CD3/CD28 mAbs. Following this, these cells display features typical of M cells, such as transcytosis activity, the disorganization of microvilli, enhanced expression of annexin V and Sgne-1, which are thought to be markers for M cells (Verbrugghe *et al.*, 2006; Kanaya *et al.*, 2008).

### 5.1.2 M cell development

M cells arise from pluripotential epithelial stem cells in adjacent crypts and migrate to the follicle-associated epithelium, where they differentiate into their distinctive phenotypes under the influence of the lymphotoxin β receptor (Kraehenbuhl & Neutra, 2000; Debard *et al.*, 2001). Interaction of Peyer's patch lymphocytes, particularly B cells, facilitates M cell differentiation, (Kerneis *et al.*, 1997) but M cells develop in Rag- 1-/- mice, which lack T and B lymphocytes, indicating that lymphotoxin can arise from other sources (Debard *et al.*, 2001).

Certain microbes appear to exploit the innate plasticity of epithelial cells to trigger their transformation into a cell phenotype that suits their habitat or life style. *S.* Typhimurium can activate different pathways of cellular differentiation to transform a subset of epithelial cells to cells that phenotypically and functionally resemble to specialized antigen sampling microfold/M cells in the gut (Savidge *et al.*, 1991).
Other numerous studies have reported an increased M cell number and/ or enhanced trancytosis activity at the FAE following microbial challenge. *In vivo* studies in rabbit demonstrated an increased the number of cells which is morphologically and

functionally resembles to M cell phenotype following microbial challenge. The increased transcytosis of microspheres across FAE to PP tissues after challenge with *Streptococcus pneumoniae* could either be explained by induction of the M cell phenotype and/or an increased transport rate in already present M cells (Meynell *et al.*, 1999b). Similar results have been shown in various *in vitro* models, for example, *Y. pseudotuberculosis* induced a substantial increase in transcytosis of nanoparticles across Caco-2 monolayers, with only a very moderate effect on paracellular translocation (Ragnarsson *et al.*, 2008). *Campylobacter jejuni* increased cells expressing the M cell-specific marker, galectin-9, in Caco-2 monolayers and reduced numbers of enterocytes that stained with the absorptive enterocyte marker, Ulex europaeus agglutinin-1, and reduced activities of enzymes typically associated with absorptive enterocytes (alkaline phosphatase, lactase, and sucrase). These cells are also functionally similar to m cell phenotype as these cells were able to internalized commensal *E. coli* (Kalischuk *et al.*, 2010).

### 5.1.4 The wnt (β-catenin) pathway

Wnt is very important cell signalling pathway that activates target genes in the nucleus. Wnts are secreted glycoproteins that bind to frizzled seven transmembranespan receptors. under normal conditions, Wnt is not activated, which lead to a degradation of cytosolic β-catenin(Jamora *et al*., 2003; Waterman, 2004).

Normally, β-catenin is phosphorylated by casein kinase Iα (CKIα) and/or CKIϵ at Ser45. This in turn enables glycogen synthase kinase 3β (GSK3β) to phosphorylate serine/threonine residues 41, 37 and 33 of β-catenin. Phosphorylation of these last two residues triggers ubiquitylation of β-catenin by βTrCP and degradation in proteosomes (Jamora *et al*., 2003; Waterman, 2004).

Phosphorylation of β-catenin occurs in a multi-protein complex containing the scaffold protein axin, which can form a homodimer or a heterodimer with the related protein conductin/axin2. In the presence of Wnts, dishevelled (Dsh) blocks β-catenin degradation possibly via phosphorlation of GSK3β.

Stabilised β-catenin enters the cell nucleus and associates with LEF/TCF transcription factors, which leads to the transcription of Wnt, target genes (Hülsken & Behrens, 2000).

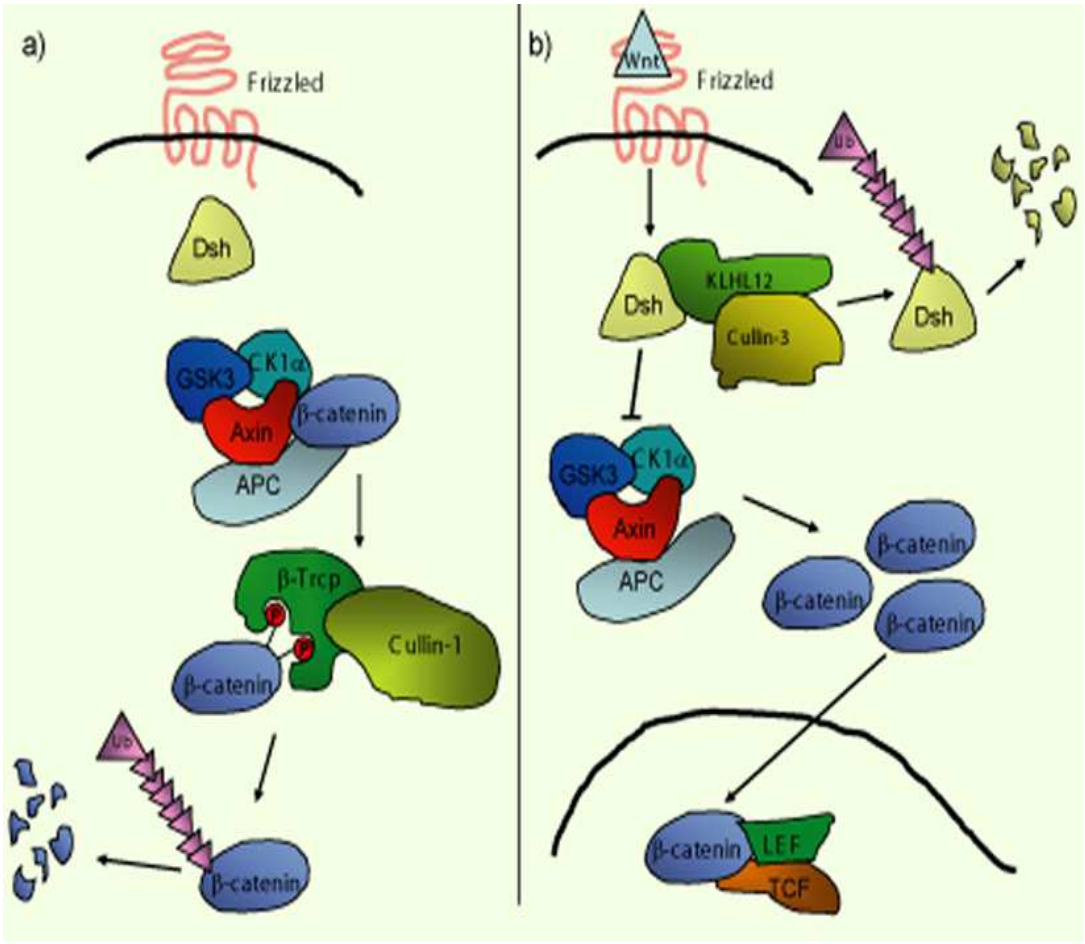


**Fig. 5.1.4 Activation of Wnt pathway stimulates EMT.**

(a) under normal conditions Wnt is not expressed and Dsh dose not phosporylate GSK3 β. β-catenin is phosphorylated by GSK3 β leading to its degredation by the proteosome. (b) Wnt is activated and binds to receptor frizzled that activate Dsh lead to phosphorylation of GSK3 β (inactivation) and the β-catenin increase in the cytoplasm and transolocated to the nucleus and ac as co factor for transcription factor TCF/LEF. This figure is derived directly from phm.utoronto.ca/angers/research.html

### 5.1.3 Epithelial-mesenchymal transition or transformation (EMT)

EMT is physiologically important for the development especially in embryogenesis, in which epithelial cells acquire properties of mesenchymal cells (Thiery *et al.*, 2009). Pathologically, it may lead to metastasis and cancer formation (Geiger & Peeper, 2009). The most important cell signalling pathway involved in EMT are peptide growth factors, Src, Ras, Ets, integrin, Wnt/beta-catenin and Notch. The activation of one or more of these cell signalling activates the transcription factors Snail and Slug. Both of these proteins are transcriptional repressors of E-cadherin and their expression induces EMT (Larue & Bellacosa, 2005). The activation of the phosphatidylinositol 3' kinase (PI3K)/AKT also act as a central feature of EMT which is important during embryonic development (Larue & Bellacosa, 2005). The activated Akt induces a transcription factor, Snail, which is known to repress expression of the E-cadherin gene through inactivation of GSK3β. this leads to the accumulation of β-catenin in the cytoplasm which acts as a cofactor for the transcription factor LEF/TCF (lymphoid enhancer factor/T cell factor) (Grille *et al.*, 2003).

The *H. pylori* injects the effector protein cytotoxin associated gene A (CagA) via a T4ss into epithelial cells, which stimulates multiple signal transduction pathways associated with EMT. *H. pylori* selectively activates β-catenin pathway that lead to accumulation of β-catenin in the nucleus. the translocated β-catenin work with TCF/LEF as transcription factor that mediate the EMT and consequently lead to gastric ulcer (Murata-Kamiya *et al.*, 2007)..

Although many bacterial pathogens have been associated with increased numbers of M cells, there is no work that elucidates the underlying molecular mechanisms. Current work set out to test if bacterial pathogens can drive EMT mediated cellular transformation of epithelial cells in to M cell phenotype and hence their increased number following bacterial infection. We have used a combination of cellular and molecular approaches to establish this unique feature of host-pathogen interaction using *S.* Typhimurium a model organism.

## 5.2 Results

We have identified both the key bacterial effector protein and the eukaryotic signal molecules that orchestrate epithelial cell transformation. *S.* Typhimurium preferentially targets antigen sampling M cells to enter the mucosal epithelium. Our results show that *S.* Typhimurium targets a subset of epithelial cells in intestinal crypts, associated with lymphoid follicular tissue, to a cell type that phenotypically and functionally resembles M cells in the gut.

### 5.2.1 *S.* Typhimurium transforms intestinal epithelial cells in to M cell phenotype in a RANKL dependent manner

Previous *in vivo* studies in mice reported that a short term exposure (1-3 hours) to certain bacterial pathogens induced dramatic alternations of FAE, including a marked increase in operational M cells in topographically restricted areas of the FAE (Borghesi *et al.*, 1996; Borghesi *et al.*, 1999; Meynell *et al.*, 1999). To investigate molecular basis to these observations we expanded on our findings about the role of RANKL in M cell biology/differentiation, and set out to test whether following bacterial challenge the increased M cell activity is RANKL mediated. To these end primary intestinal epithelial cells from terminal rectum were infected with S. Typhimurium, a bacterium known to preferentially target M cells, and analysed for the expression of RANKL. In time course infection studies with *S.* Typhimurium, it was observed a temporal increase both in protein and transcript levels of for RANKL as well as vimentin and Slug, markers associated with M cell phenotype and EMT, respectively (Fig. 5.2.1). In addition, the gradual decrease in E-cadherin expression (Fig. 5.2.3 A & B) a feature associated with EMT (Larue & Bellacosa, 2005), that further confirms *S.* Typhimurium mediated epithelial cellular transformation in infected cultures.

Since β-catenin/TCF can directly trans-activate RANKL (Shin *et al.*, 2005), vimentin (Gilles *et al.*, 2003) and Slug (Vallin *et al.*, 2001), I explored the possibility if increased levels of these three proteins correspond to the levels of β-catenin.

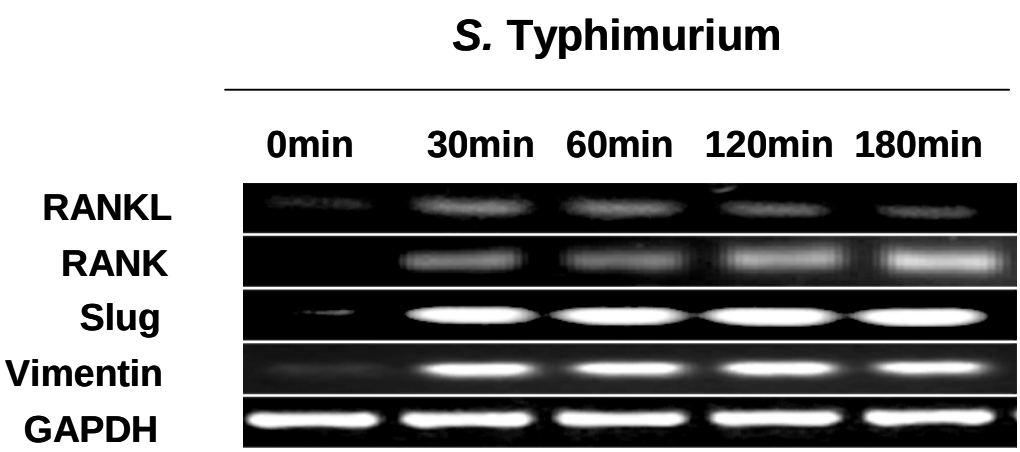


**Fig. 5.2.1 *S.* Typhimurium induced temporal increase in mRNA expression of RANKL, RANK, Slug and Vimentin.** Terminal BRE cells were infected with *S.* Typhimurium strain (SL1344) at 37$^{o}$C, 5% $CO_2$ for 10 min, extracellular bacteria were removed by washing and cells were incubated further for indicated time points. Following *S.* Typhimurium infection of epithelial cells, cell lysates were processed for preparation of RNA. CDNA was prepared from each sample and the PCR were performed using primer for RANKL, RANK, Slug and Vimentin. (Table 2.4 Materials & Methods) GAPDH were used as a control for equal loading. Results are representative of three independent experiments.

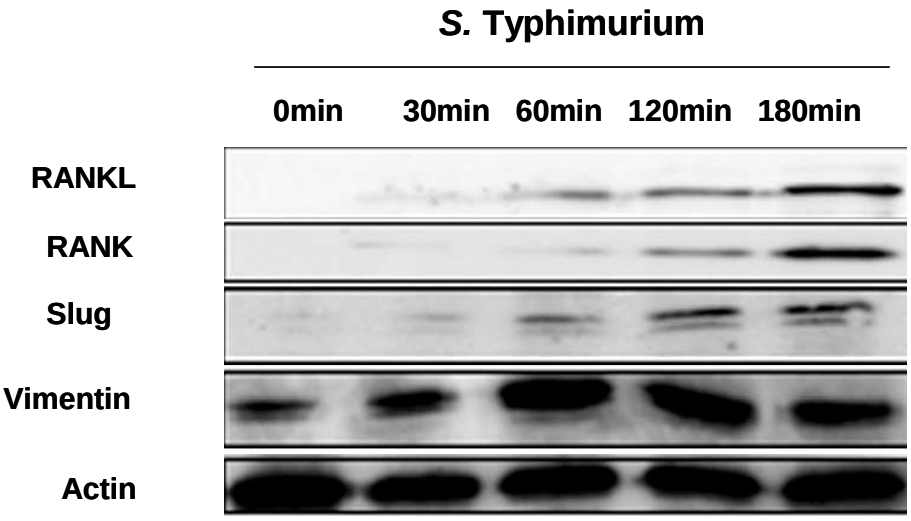


**Fig. 5.2.2 *S.* Typhimurium induced temporal increase in protein level of RANKL, RANK, Slug and Vimentin.** Terminal BRE cells were infected with *Salmonella* Typhimurium strain (SL1344) at 37$^{o}$C, 5% $Co_2$ for 10 min, extracellular bacteria were removed by washing and cells were incubated further for indicated time points. Following *S.* Typhimurium infection of epithelial cells**,** cell lysates were processed for immunoblotting. Immunblots were stripped and re-probed with panel of antibodies as indicated. Molecular mass standards (kDa) are indicated. actin were used as a control for equal loading. Results are representative of three independent experiments.

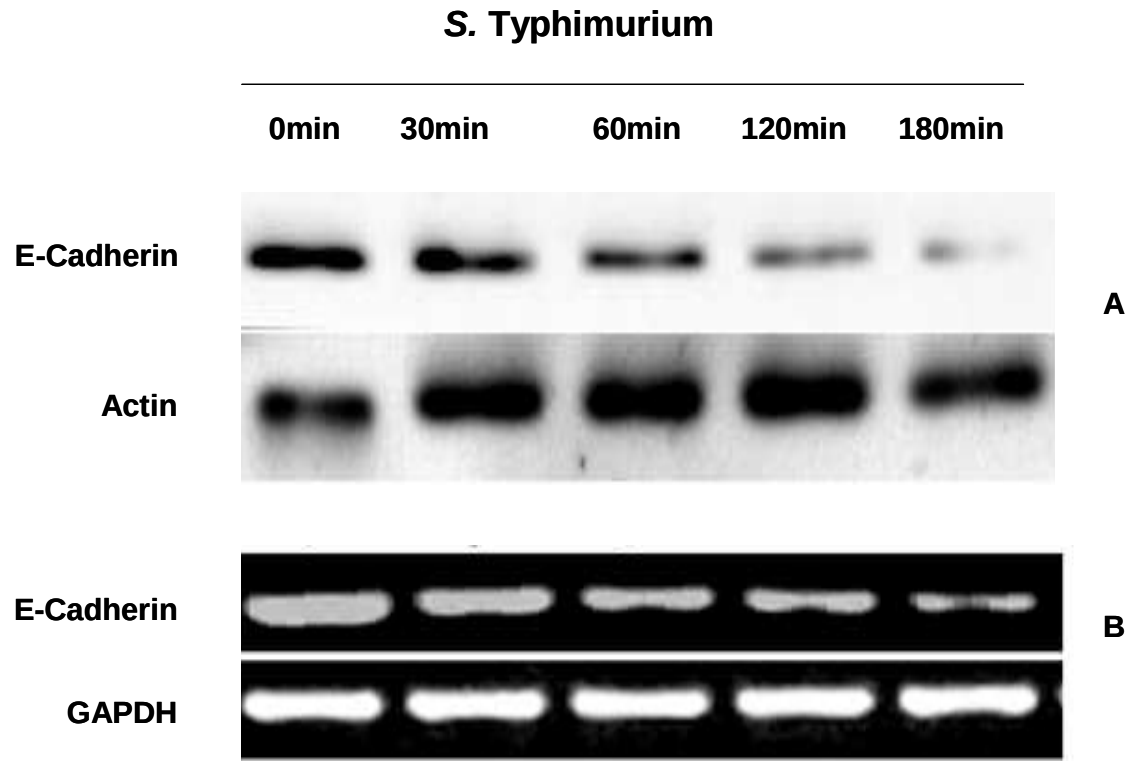


**Fig. 5.2.3 *S.* Typhimurium induces transformation of primary intestinal epithelial cells.** Heterogeneous population of epithelial cells in a primary cell culture from the bovine terminal rectum were isolated and cultured on 6 well plates (Materials and Methods). Five day old cultures were co-cultured with *S.* Typhimurium strain SL1344 for different time points. Suppression of tight junction protein E-cadherin was induced gradually and this was detected using immunobloting (A) and semiquantitative RT-PCR (B). Results are representative of three independent experiments.

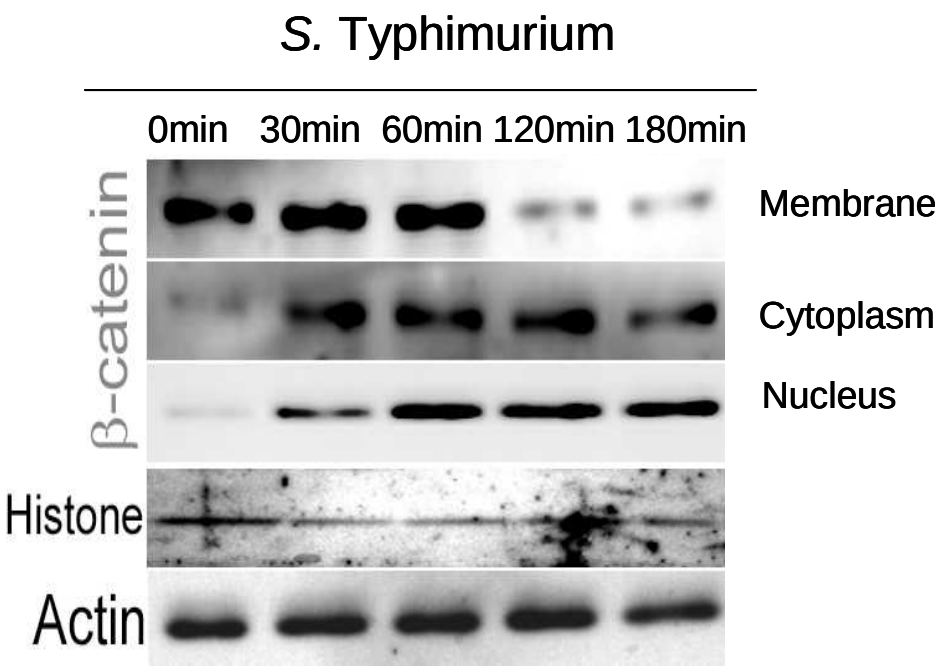


**Fig. 5.2.4 *S.* Typhimurium affects subcellular localisation of beta-catenin.** Heterogeneous population of epithelial cells in a primary cell culture from the bovine terminal rectum were isolated and cultured on 6 well plates (Materials and Methods). Five day old cultures were co-cultured with *S.* Typhimurium strain SL1344. Subcellular protein fractions were separated using a ProteoExtract kit (Materials and Methods).The samples were analysed by immunoblotting using anti- β catenin or anti-β-actin primary antibodies followed by HRP-conjugated secondary antibodies. Histone and actin were used as a control for equal loading. Results are representative of three independent experiments.

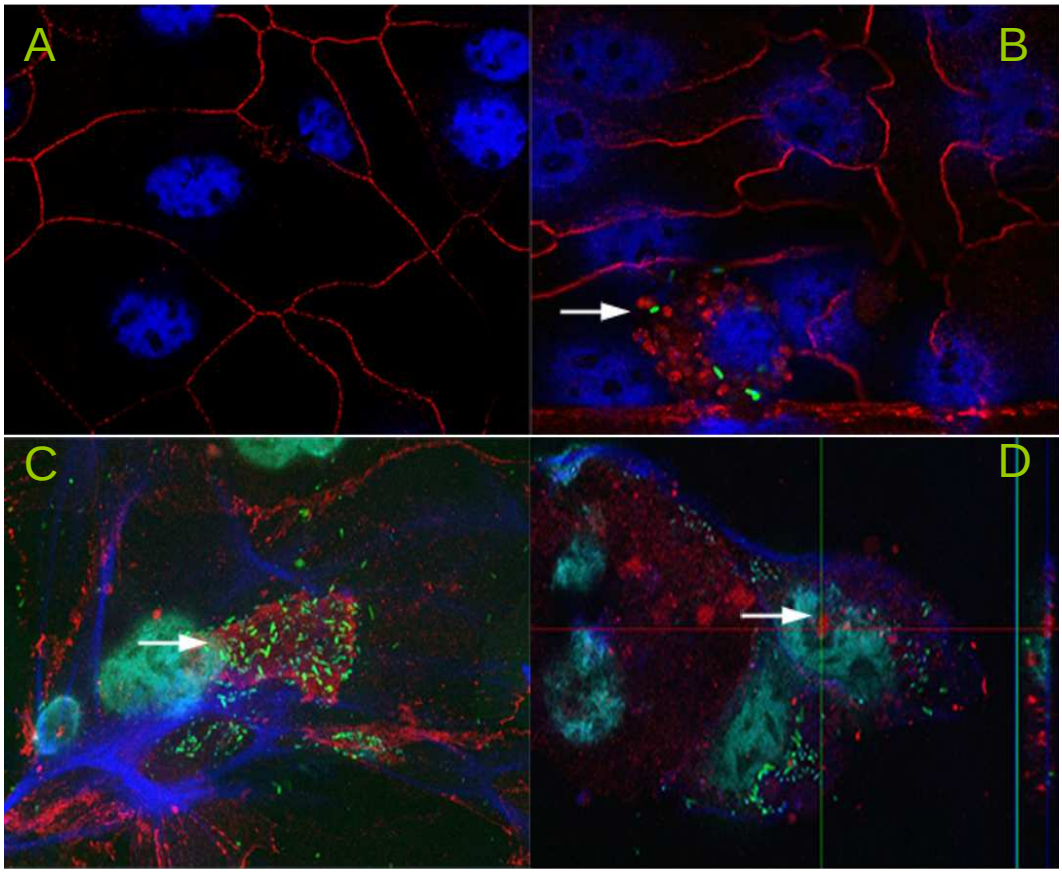


**Fig. 5.2.5 *S.* Typhimurium challenge redistributributed β-catenin in Bovine Rectal epithelial cell.** Heterogeneous population of epithelial cells in a primary cell culture from the bovine terminal rectum were isolated and cultured on glass cover slips (Materials and Methods). Five day old cultures were co-cultured with *S.* Typhimurium strain SL1344 for different time points. The cells were fixed and permeabilised in 2 %( v/v) formalin/ 0.2% (v/v) Triton X-100. (A) control cells were stained for beta-catenin red in the tight junctions. (B-D) Infected cells were analysed for beta-catenin localisation in cellular compartments by immuno-fluorescence staining. Beta-catenin (red) primarily localised at the membranes in uninfected cells and during the course of infection (B) 10 min, (C) 30 min and (D) 60 min it was translocated to cytoplasm (arrow) and nucleus (blue). Images were acquired using Zeiss Axiovert, objective xx63. Results are representative of three independent experiments.

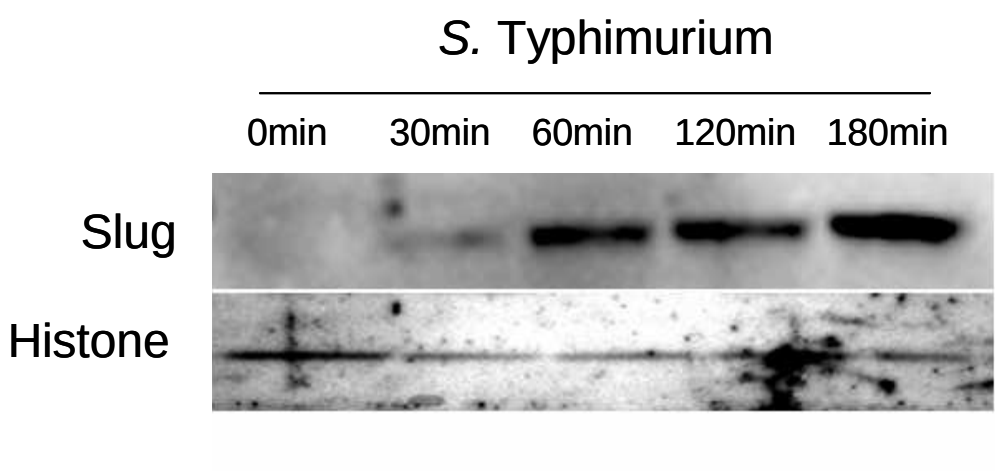


**Fig. 5.2.6 *S.* Typhimurium enhances Slug expression and its localisation in the nucleus.** Heterogeneous population of epithelial cells in a primary cell culture from the bovine terminal rectum were isolated and cultured on 6 well plates (materials and methods). Five day old cultures were co-cultured with *S.* Typhimurium strain SL1344. Subcellular protein fractions were separated using ProteoExtract kit (materials and methods).The samples were analysed by immunoblotting using antibodies as indicated. *S.* Typhimurium infection increased nuclear loclization of slug. Histone was used as a control for equal loading. Results are representative of three independent experiments.

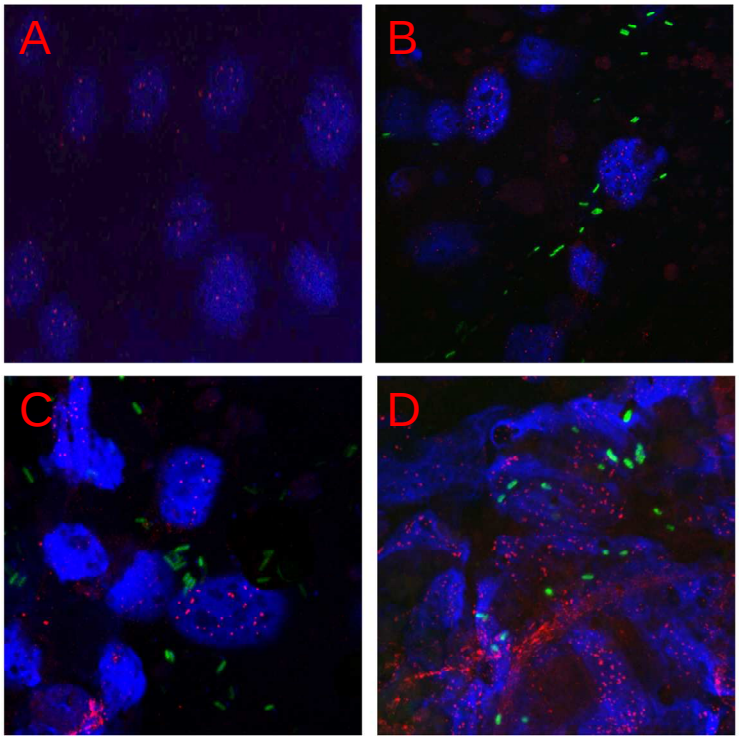


**Fig. 5.2.7 *S.* Typhimurium up-regulates the intra-nuclear transcription factor Slug in a Primary Bovine Rectal epithelial cell.** Heterogeneous populations of epithelial cells in a primary cell culture from the bovine terminal rectum were isolated and cultured on glass cover slips (Materials and Methods). Five day old cultures were co-cultured with *S.* Typhimurium strain SL1344 for different time points. The cells were fixed and permeabilised in 2 %( v/v) formalin/ 0.2% (v/v) Triton X-100. (A) Cells expressed slug (red) in nucleus (blue), immunolabelling was done as described in Materials and Methods. (B-D) Gradual increase in Slug localisation following infection with *S.* Typhimurium (GFP- labelled) for the times shown. Images were acquired using Zeiss Axiovert microscope, (objective x 63). Results are representative of three independent experiments

Indeed, we observed a temporal increase in β-catenin levels that coincided with increased RANKL, Vimentin, RANK and Slug in time course *S.* Typhimurium infection experiments (Fig. 5.2.1). Following *S.* Typhimurium challenge, β-catenin was redistributtributed to the nucleus as seen both via immunoblotting and immunofluorscence staining of infected epithelial cells Fig. 5.2.4 &5.2.5). The cytoplasmic and nuclear redistribution of β-catenin and the *de novo* expression of vimentin are frequently involved in the epithelial-to-mesenchymal transition associated with increased invasive/migratory properties of epithelial cells.

Slug, a member of a zinc finger family of transcriptional/ repressors, is an established downstream mediator of the β-catenin/TCF pathway. Increased intranuclear accumulation of Slug functions as both a transcription repressor, for example E-cadherin and activator, for example vimentin to promote EMT. Following *S.* Typhimurium challenge during initial stages of infection Slug was redistributed from the nucleus to the cytoplasm and during time course experments its levels significantly increased in the nucleus as seen both by immunoblotting and immunofluorscence staining of infected epithelial cells (Fig. 5.2.6 & 5.2.7).

### 5.3.1 S. Typhimurium but not *E. coli* K12 activates EMT

To investigate if the EMT is activated specifically in the presence of pathogenic *S.* Typhimurium or it is non specific processes that can be induced by other non pathogenic bacteria such as *E. coli* K12. Terminal BRE cells were infected with wild-type *S.* Typhimurium (SL1344) or *E. coli* K12 (Zap 1167) strain and at incubated at $37^{o}$C, 5% $Co_2$ for 3 h. The western blot results showed that the level of Vimentin was higher only with *S.* Typhimurium infection indicating that the EMT process is specific for *S.* Typhimurium infection but not for *E. coli* K12 (Fig. 5.3.1).

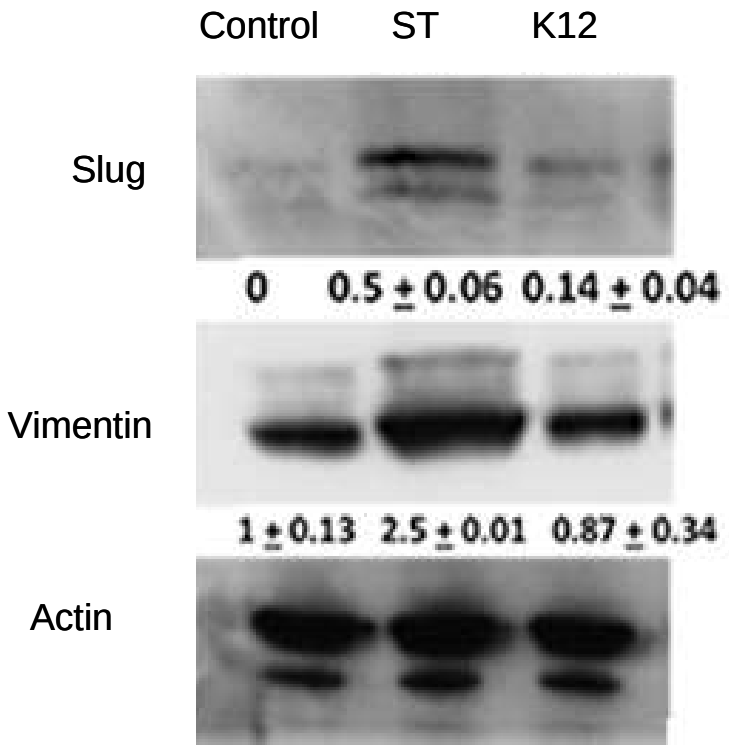


**Fig. 5.3.1 *S.* Typhimurium but not *E. coli* K12 activates the Wnt signalling pathway.** Heterogeneous population of epithelial cells in a primary cell culture from the bovine terminal rectum were isolated and cultured on 6 well plates (Materials and Methods). Five day old Terminal BRE cells were infected wild-type *S.* Typhimurium (SL1344) or *E. coli* K12 (Zap 1167) strain and at incubated at 37$^{o}$C, 5% $Co_2$ for 3 hrs. The cell lysates were processed for Western blotting as indicated. Immunblots were stripped and re-probed with panel of antibodies as indicated. Molecular mass standards (kDa) are indicated. Actin was used as a control for equal loading. Graph represent mean -fold changes of band intensity from two separate experiments ± S.E. with the value of control as 1 arbitrary. Western blot films were quantified using Quantity One software (Bio-Rad, Richmond, CA), and each sample was normalised using the actin signal.

## 5.4 *S.* Typhimurium induced ultra-structural changes and EMT in the epithelial architecture

To examine if the observed changes in EMT markers corelate with morphogenic modifications, the *S.* Typhimurium infected intestinal epithelial cells were processed for ultrastructural studies. Unaffected control epithelial cells were marked by the presence of dense microvilli as seen in the Fig. 5.4.1. Following *S.* Typhimurium challenge we observed a stark loss of microvilli, seen as "baldy patches" on infected cells. Some of the acutely infected cells showed a complete loss of microvilli. Time course infection studies demonstrated acute morphological and structural changes in infected cells.

To further investigate whether *S.* Typhimurium mediated increase in number of functional M cells was due to rapid re-differentiation of enterocytes into M cells or rather arise from pre-determined undifferentiated stem cells from FACs changing phenotype that contribute towards increased number M like cells in culture. *S.* Typhimurium can induce vimentin, a typical marker of M cells; induce molecular and phenotypic alterations typical of cellular transformation (5.4.2), suggest a possibility that *S.* Typhimurium can drive a subset of lineage specific epithelial cells towards M cell phenotype.

## 5.5 *S.* Typhimurium transforms epithelial cell in to M cell phenotype in a SopB dependent manner

To identify bacterial factors that have a role in initiating epithelial cell transformation into M cell phenotype, infection studies were done with a panel of wild type and mutant *S.* Typhimurium strains. For initial testing we targeted *Salmonella* pathogenicity island-1 (SPI-1) encoded type 3 secretion system (T3SS) that mediate early bacterial invasion (Galan, 1996), membrane ruffling (Hardt *et al.*, 1998), apoptosis (Guiney, 2005), and tight junction disruption (Jepson *et al.*, 1996; Tafazoli *et al.*, 2003).

BRE cells were infected with various bacterial mutants and the expression of target proteins, identified with EMT signalling, was examined by Western blotting.

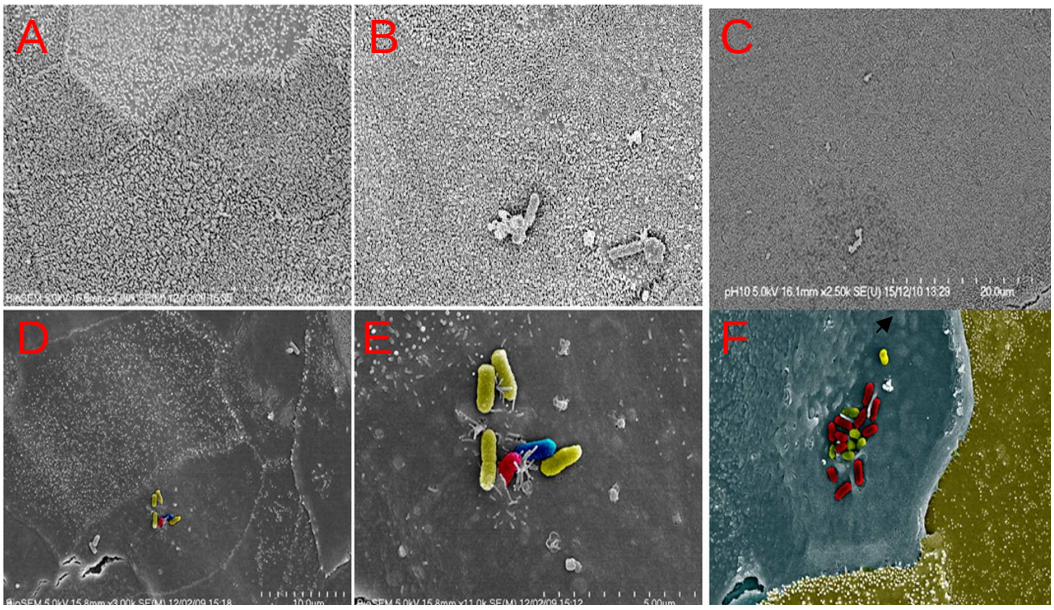


**Fig. 5.4.1 *S.* Typhimurium induced ultra-structural changes in the epithelial architecture**. Heterogeneous population of epithelial cells in a primary cell culture from the bovine terminal rectum were isolated and cultured on thermonex cover slips (Materials and Methods). Five day old cultures were co-cultured with *Salmonella typhimurium* strain SL1344 for different time points. (A) a typical epithelial monolayer with dense apical microvilli. Early stages of *S.* Typhimurium induced changes in cell morphology at 60 min of infection (C), seen as electron dense spots around attached bacterium (arrow). During course of infection epithelial cells undergo pronounced, sequential morphological and molecular changes characterised by partial loss of apical microvilli at 120 min (D&E) or complete loss of microvilli at 180 min (F). A typical *S.* Typhimurium infected cell with internalized bacteria seen as silhouettes (arrow head), microvilli lost and surrounded by normal epithelial cells. The specimens were examined using a Hitachi 4700 Field Emission Scanning Electron Microscope. Results are representative of three independent experiments.

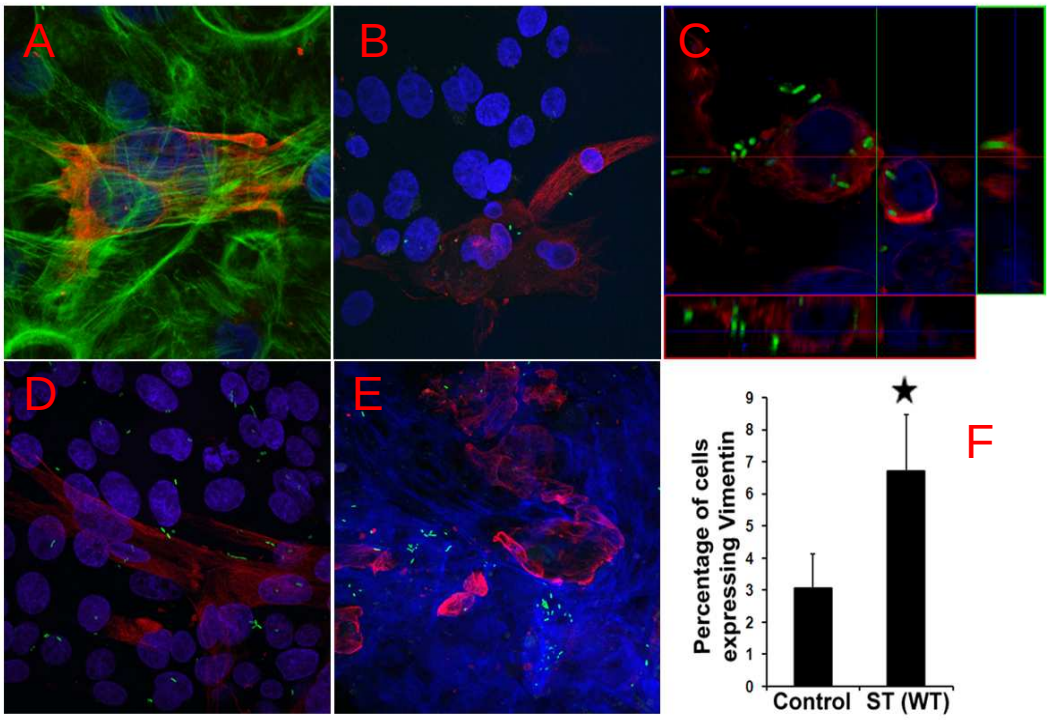


**Fig. 5.4.2 *S.* Typhimurium induces transformation of primary intestinal epithelial cells.** Heterogeneous population of epithelial cells in a primary cell culture from the bovine terminal rectum were isolated and cultured on glass cover slips (Materials and Methods). Five day old cultures were co-cultured with *S.* Typhimurium strain SL1344 for different time points. The cells were fixed and permeabilised in 2 %( v/v) formalin/ 0.2% (v/v) Triton X-100. (A) A subset of primary epithelial cells in culture expressed intermediate filament protein vimentin (B) *S.* Typhimurium gets attached and internalized in vimentin expressing cells within 10 min of infection (B-C). With the progression of infection *S.* Typhimurium start to attack the epithelial cells (D), and after 180 min the number of vimentin expressing cells starts to increase in reponse to *S.* Typhimurium infection (E). Images were acquired using Zeiss Axiovert, objective xx63. Internalized bacteria are shown in x-y view from orthogonal z-section images. In (F) the cells were removed from plates by trypsinisation and stained with a vimentin antibody (Material & Methods), Flow cytometry results from 5 independent experiments expressed as mean ± SEM measured on the FACS Calibur.

Initial experiments with heat killed *S.* Typhimurium and *invA* mutant strain that lack a functional SPI-I T3SS, showed no change in protein expression of RANKL, β -catenin, Slug and Vimentin when compared with wild type *S.* Typhimurium (Fig. 5.5.1), confirming that the observed changes in protein expression are indeed SPI- 1 dependent. Next, we investigated the role of specific SPI-1 effectors. We focused on three SPI-1-secreted effectors that activate Rho family GTPases (i.e. SopB, SopE and SopE2) and another that directly interacts with actin (i.e. SipA) (Lilic *et al.*, 2003b). We hypothesised for these effectors to be crucial in triggering signal cascades underlying cellular architecture/ cytoskeleton modifications during transformation of epithelial cell phenotype. SPI-1 and *sopB* mutants showed a significant reduced adherent phenotype comparable with other effector mutants (*sipA*, *sopE* and *sopE2*) and wildtype *S.* Typhimurium (Fig. 5.5.2). As shown in Fig. 5.5.3 & Fig. 5.5.4 the enhanced protein levels of RANKL, beta-catenin, Slug and Vimentin following infection with wild type *S.* Typhimurium were suppressed in presence of a *sopB* deletion. This data provide evidence that *S.* Typhimurium can trigger events similar to EMT perhaps in a SopB dependent manner. To further confirm the role of SopB in Wnt/ β-catenin signalling regulation and RANKL expression, we analyzed transcriptional activity using specific reporter gene assays. Caco-2 cells were co-transfected with luciferase reporter plasmid containing wild type (TOP Flash) or mutated (FOP Flash) LEF/TCF binding sites and PCR3 plasmid containing SopB. The firefly luciferase activity was normalised relative to renilla luciferase activity. As shown in Fig. 5.5.6, SopB directly regulates luciferase activity with in 48 h post-transfection (fold compared with FOpflash). LiCl, a known activator of β catenin signalling (Stambolic *et al.,* 1996), was used as a positive control.

Recent studies on the transcriptional regulation of the rankl gene have identified several transactivators that cooperate to activate its transcription including NFκB and β-catenin /TCF signaling, (Fan *et al.,* 2004; Fu et *al.*, 2002; Mori *et al.,* 2006). To further confirm the role of SopB in regulation of RANKL expression and determine underlying mechanisms, we performed RANKL-reporter gene assays in the presence of pharmacological inhibitors specific to *S.* Typhimurium mediated signalling in intestinal infected cells.

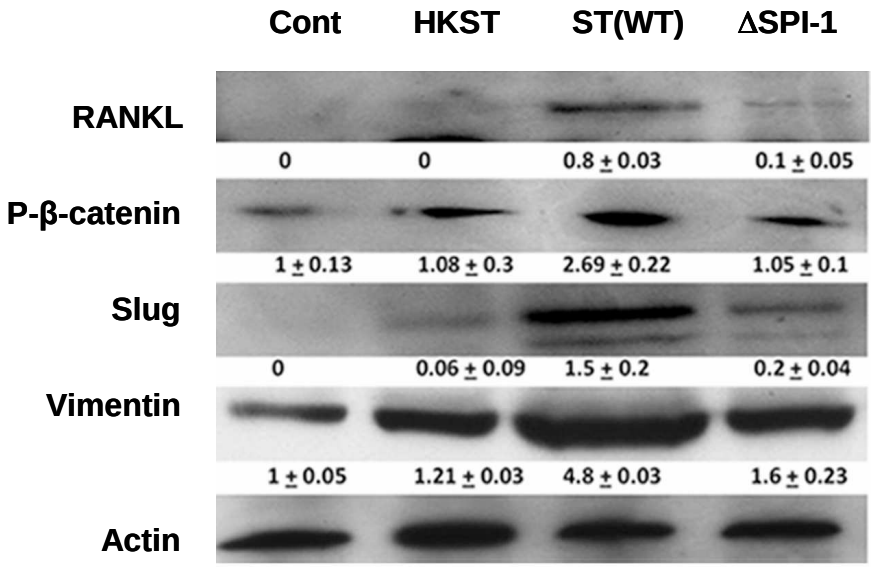


**Fig. 5.5.1 *S.* Typhimurium activates Wnt pathway in a SPI-1 dependent manner.** Heterogeneous population of epithelial cells in a primary cell culture from the bovine terminal rectum were isolated and cultured on 6 well plates (Materials and Methods). Five day old terminal BRE cells were infected with, heat killed *S.* Typhimurium (HKS), wild-type (*S.* Typhimurium) or SpI-1 mutant of *S.* Typhimurium strain (SL1344) and incubated at 37$^{o}$C, 5% $Co_2$ for 3 hrs. The cell lysates were processed for western blotting as indicated. Immunblots were stripped and re-probed with panel of antibodies as indicated. Molecular mass standards (kDa) are indicated. Actin was used as a control for equal loading. Results are representative of two independent experiments.

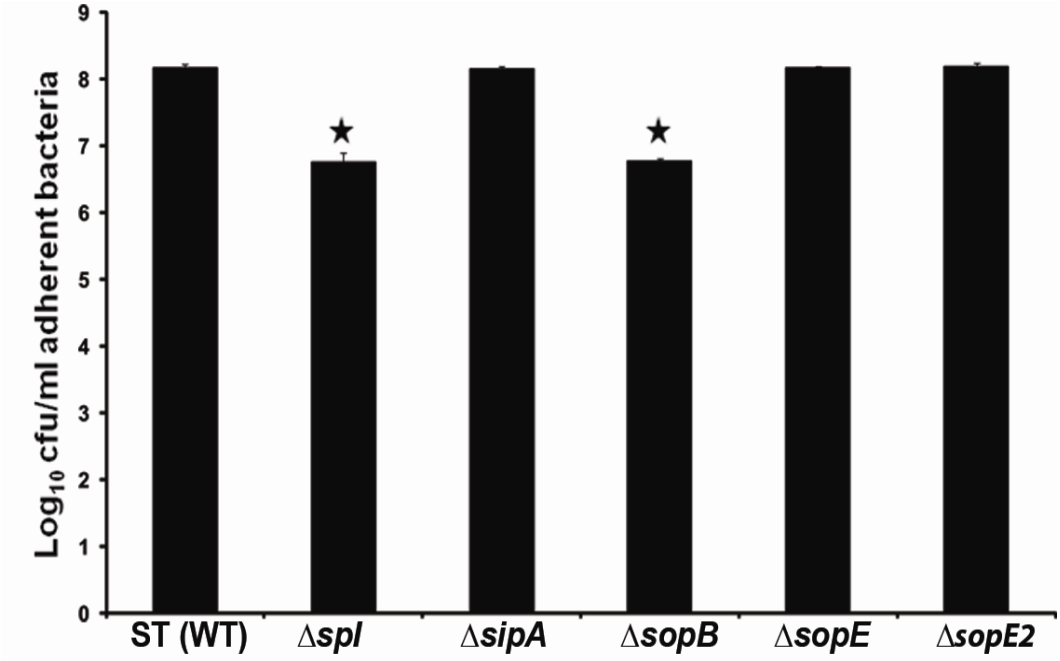


**Fig. 5.5.2 Comparative adhesion of wildtype *S.* Typhimurium and SPI-1 mutant strains to bovine terminal rectal epithelial primaries.** Heterogeneous population of epithelial cells in a primary cell culture from the bovine terminal rectum were incubated with *S.* Typhimurium and SPI-1 mutant strains at 37$^{o}$C, 5% $Co_2$ for 30 min. *spI*-1 and *sopB* mutants showed a significant reduction in adherence in comparison to other the effectors mutants or wildtype *S.* Typhimurium. Results are representative of three independent experiments and presented as * $p<0.05$ relative to wildtype *S.* Typhimurium.

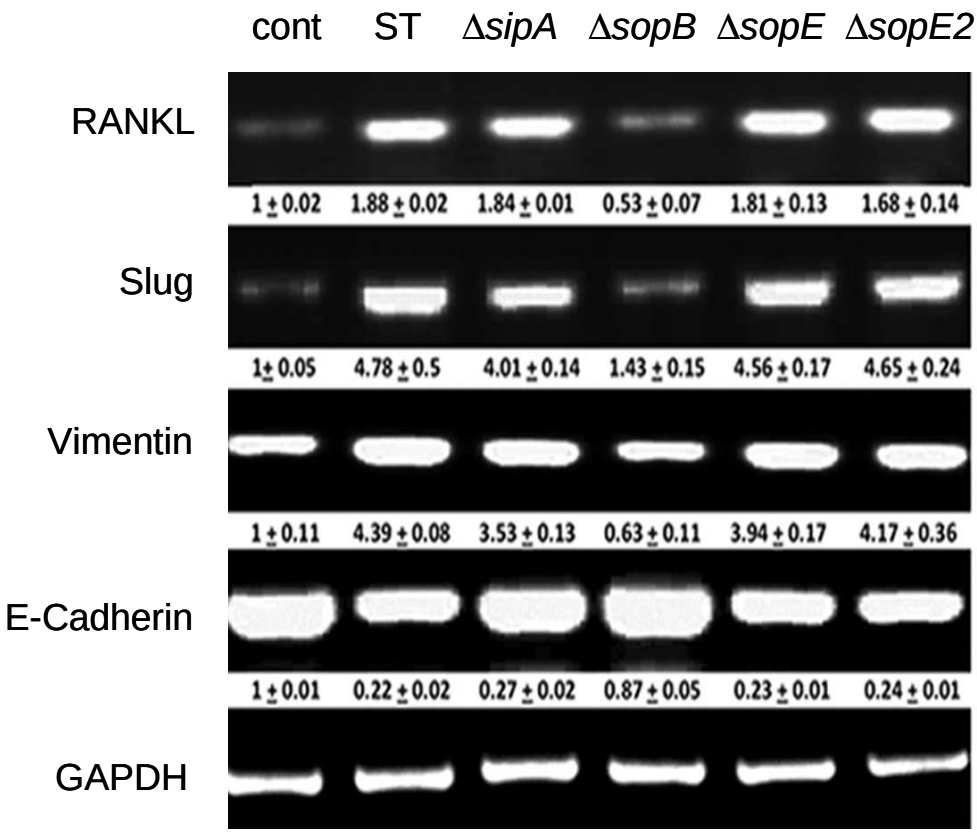


**Fig. 5.5.3 Role of SPI-1 encoded T3S effectors on transcription of EMT specific gene targets.** Heterogeneous population of epithelial cells in a primary cell culture from the bovine terminal rectum were isolated and cultured on 6 well plates (Materials and Methods). Five day old cultures were infected with *S.* Typhimurium strain SL1344 at 37$^{o}$C, 5% $Co_2$ for 2 h. The cells were lysed at different time points and RNA were collected (Materials and Methods). The samples were analysed by PCR using primers (Table 2.4 Materials and Methods). The values represent mean - fold changes of band intensity from two separate experiments ± S.E. PCR films were quantified by using Quantity One software (Bio-Rad, Richmond, CA), and each sample was normalised using the GAPDH signal.

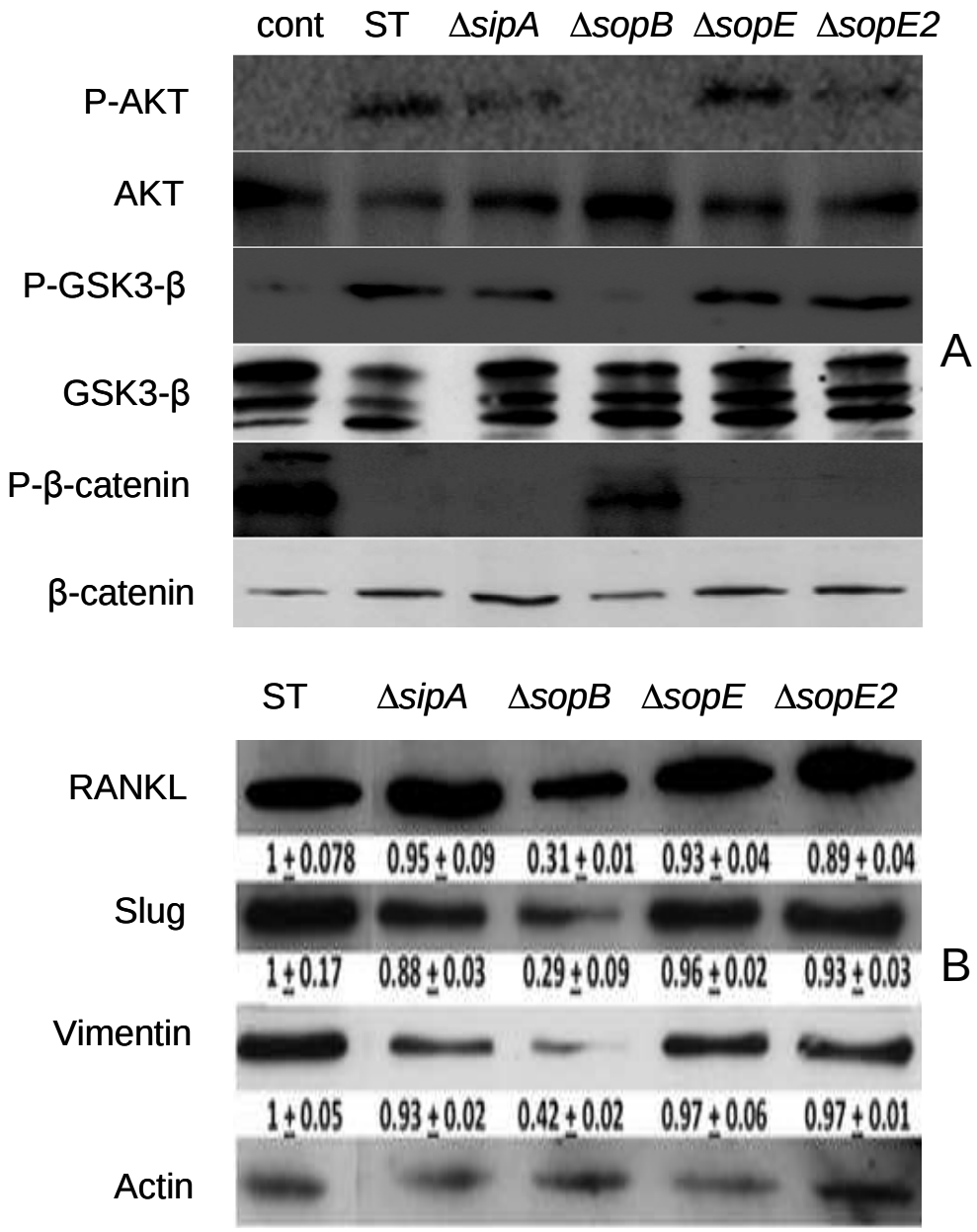


**Fig. 5.5.4 *S.* Typhimurium activates Wnt pathway in a SopB-dependent manner.** Bovine rectal epithelial cells were infected wild-type *S.* Typhimurium or mutant derivative of *S.* Typhimurium strain (SL1344) at 37$^{o}$C, 5% $Co_2$ for 3 h. The cell lysates were processed for western blotting studies as indicated. Immunblots were stripped and re-probed with panel of antibodies specific to Wnt pathway signalling molecules (A) and its EMT specific targets (B) as indicated. Molecular mass standards (kDa) are indicated. Actin was used as a control for equal loading. The values represent mean -fold changes of band intensity from two separate experiments ± S.E. with the value of control as 1 arbitrary. Western blot films were quantified by using Quantity One software (Bio-Rad, Richmond, CA), and each sample was normalised using the actin signal.

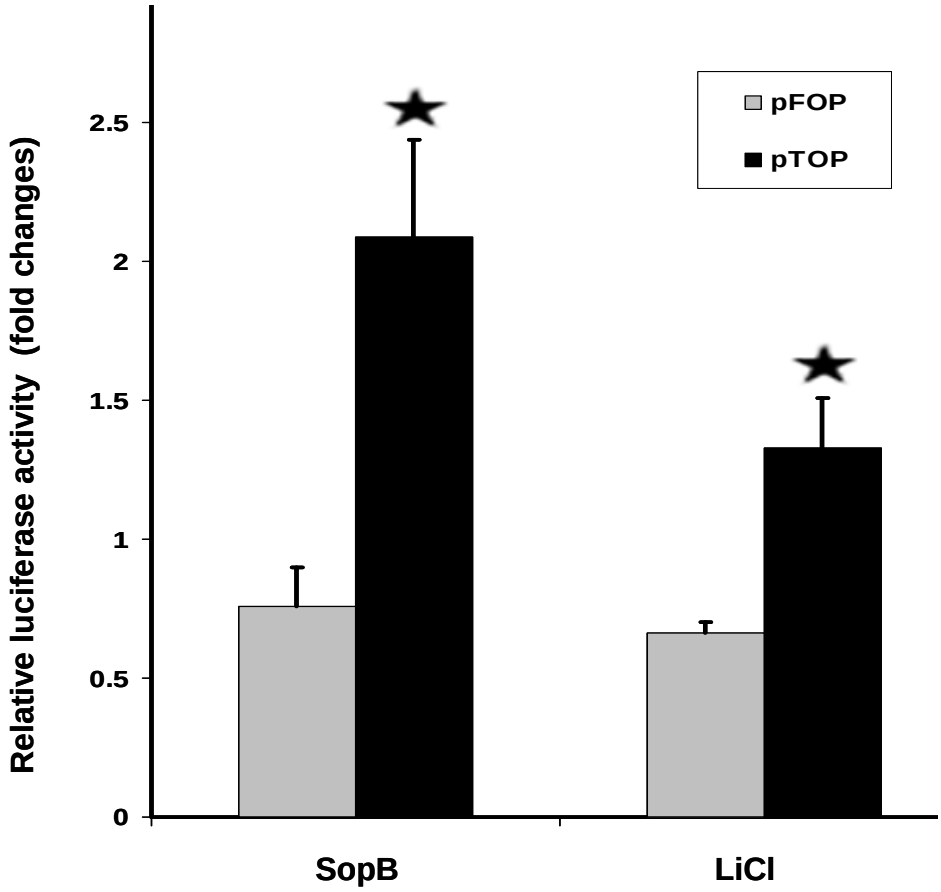


**Fig. 5.5.6 SopB mediated increased β -catenin promoter transcriptional activity.** Caco-2 cells were transiently co-transfected with a plasmid encoding for SopB and β catenin-promoter constructs, inserted in pGL3 plasmid. Cells co-transfected with PCR3 and pGL3 plasmids were used as negative control. 48 h post transfection LiCl (10 mM) was added to the promoter co-transfected with PCR3 as control positive. Cells were harvested and lysed 15 h later and Firefly luciferase (Luc) and Renilla Luciferase (RLuc) activities were measured by using the dual luciferase reporter assay system (Promega). Data are represented as relative luciferase activity (normalized to RLuc activity), with *, $p<0.05$ relative to non-infected cells, and are representative of two independent experiments.

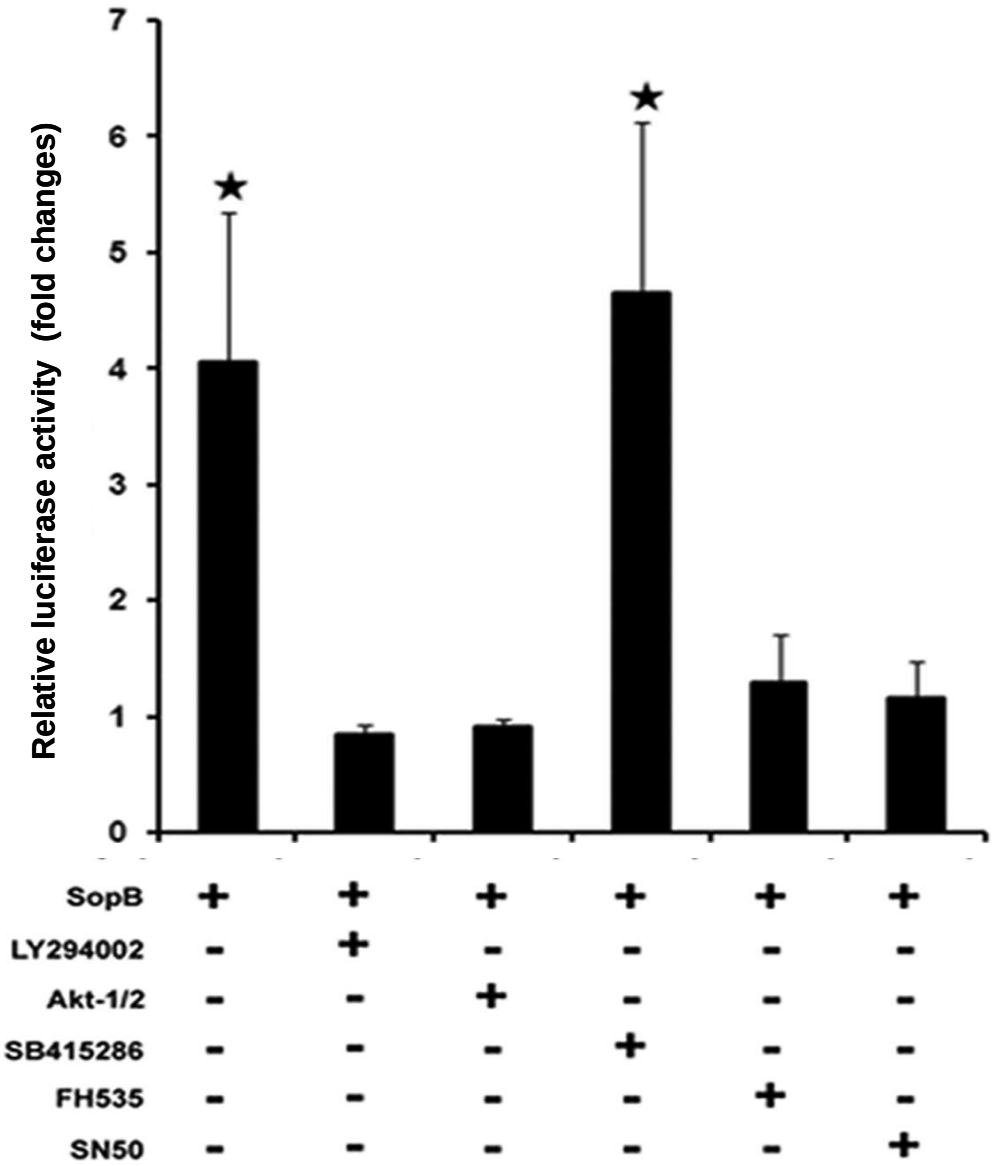


**Fig. 5.5.7 SopB mediated increased RANKL - promoter transcriptional activity was suppressed in presence of NF-κB peptide inhibitor SN50 and Wnt pathway.** Caco-2 cells were transiently transfected with a plasmid encoding SopB and a RANKL - promoter construct, 48 h post transfection, Akt-1/2 inhibitor 20 μM; (PI3 kinase inhibitor) LY 29400250 μM, SB 415286 (GSK-3 β inhibitor), SN50 (NFκB inhibitor), or FH535 (β -catenin/Tcf inhibitor), 10 μM were added to SopB transfected cell to study the pathway. Cells were harvested and lysed 15 h later and Firefly luciferase (Luc) and Renilla Luciferase (RLuc) activities were measured by using the dual luciferase reporter assay system (Promega). Data are represented as relative luciferase activity (normalized to RLuc activity), with *, $p<0.05$ relative to non-infected cells, and are representative of two independent experiments.

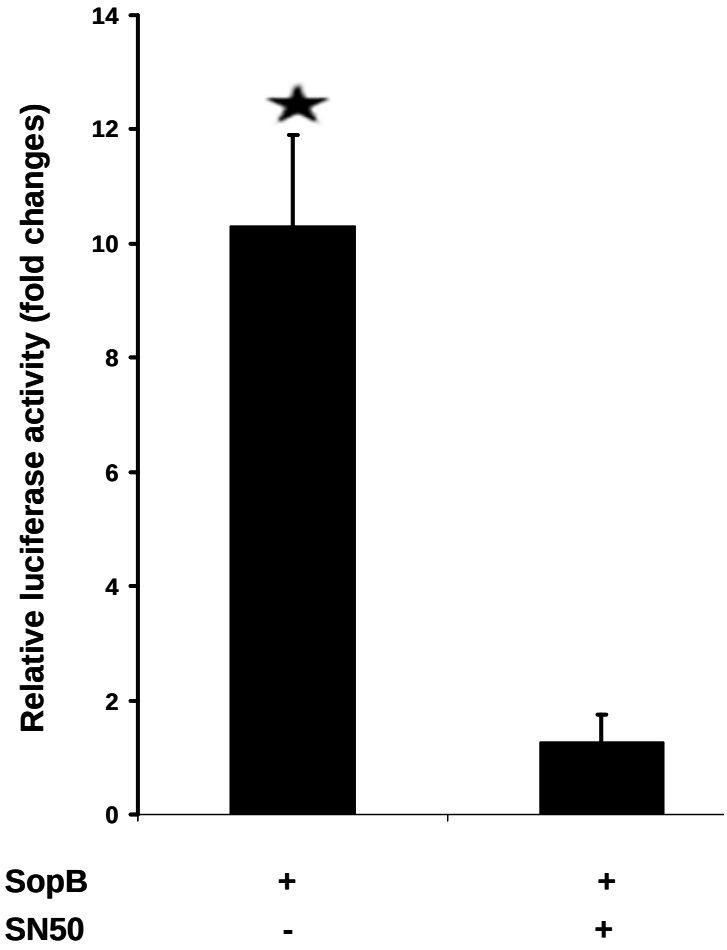


**Fig. 5.5.8 SopB mediated increased SLUG - promoter transcriptional activity was suppressed in the presence of the NF-κB peptide inhibitor SN50.** Caco-2 cells transiently co-transfected with SopB-PCR3 and SLUG- pGL3 or their respective negative controls. Transcriptional activity was measured in presence or absence of NFκB inhibitor (SN50; 50 μM). Cells were harvested and lysed 15 h later and Firefly luciferase (Luc) and Renilla Luciferase (RLuc) activities were measured using the dual luciferase reporter assay system (Promega). Data are represented as relative luciferase activity (normalized to RLuc activity), with *, $p<0.05$ relative to control cells that co-transfected with PCR3 and Slug promotor, and are representative of two independent experiments.

The bovine RANKL promoter sequence was cloned upstream of the Luc reporter gene in the pGL3 basic vector, and the construct, (named RANKL luc-construct), was assayed after Caco-2 cells transfections performed with SopB expression plasmid. As shown in Fig. 5.5.7, transient transfection with SopB resulted in an increase in RANKL luciferase activity relative to the control (cells co-transfected with PCR3 and RANKL promoter), promoter less pGL3-basic vector, demonstrating for a direct role for SopB in RANKL transcription. In presence of inhibitors: AKT inhibitor 20 μM; 50 μM final concentration from PI3 kinase inhbitor LY 294002, SB 415286 (GSK-3 β inhibitor), SN50 (NFκB inhibitor), or FH535 (β -catenin/Tcf inhibitor), 10 μM, the RANKL luciferase activity was significantly reduced. However, the RANKL luciferase activity was significantly increased following challenge with SB415286, GSK-3 inhibitor. Taken together, these results suggest that SopB mediated PI3 kinase, NF-κB and β-catenin signalling regulates RANKL expression at the transcriptional level. In order to investigate the effect of SopB SLUG - promoter transcriptional activity, Caco-2 cells were transient transfected with SopB in the presence or absence of NF-κB peptide inhibitor SN50. The result showed that SopB mediated increased SLUG - promoter transcriptional activity in NF-κB dependent manner (Fig. 5.5.8)

## 5.6 Identification of SOPB domain required to induce Wnt Pathway

SopB protein following translocation in to host cell performs multiple functions including its role in actin rearrangements, phagocytosis and biogenesis of the *Salmonella*-containing vacuole in infected cells (Jepson *et al.,* 1996; Zhou *et al.,* 2001; Terebiznik *et al.,* 2002). SopB phosphatase activity is required for the activation of the serine threonine kinase Akt, also known as protein kinase B, in infected epithelial cells (Steele-Mortimer *et al.,* 2000). Earlier we identified Akt activation as one of the early and essential steps in SopB mediated Gsk3 beta inactivation central to Wnt/beta-catenin signalling pathway (Fig. 5.5.5). We hypothesised for SopB phosphatase activity to be critical to its role in activation of wnt pathway. To this end we infected BRE cells with SopB deficient strains complemented with either catalytically active (psopB) or inactive SopB point mutant (psopB C460S) plasmids.

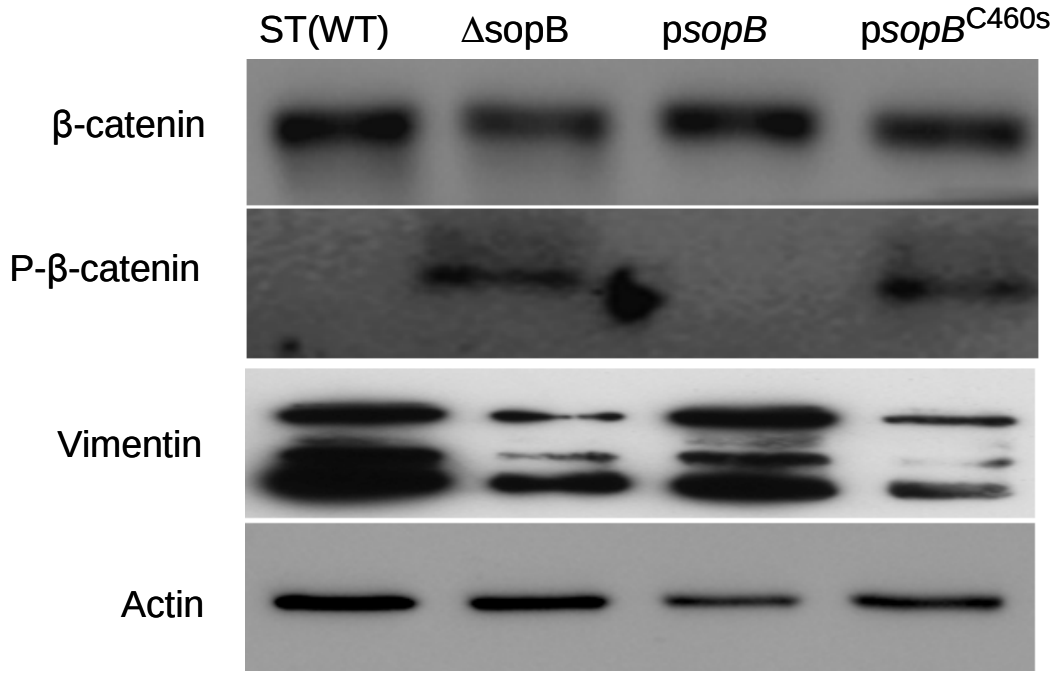


**Fig. 5.6.1 SopB activates Wnt pathway in a phosphatase dependent manner.** Heterogeneous population of epithelial cells in a primary cell culture from the bovine terminal rectum were isolated and cultured on 6 well plates (materials and methods). Five day old terminal BRE cells were infected with WT *S. Typhimurium*, ΔsopB S. Typhimurium, Δ*sopB* S. Typhimurium complemented with WT *sopB* or *sopB* mutated in C460S at 37$^{o}$C, 5% $Co_2$ for 3 h. The cell lysates were processed for western blotting studies as indicated. Immunblots were stripped and re-probed with panel of antibodies as indicated. Molecular mass standards (kDa) are indicated. Actin was used as a control for equal loading. This figure is representative of two experiments.

The cell lysates were immuno-detected for β-catenin and β-catenin –phosphorylation, as indicators of Wnt/ β-catenin signalling and vimentin and EMT indicator (Fig. 5.6.1).

As shown earlier, *S.* Typhimurium mediated Akt-phosphorylation was dependent on SopB, because an isogenic sopB deletion strain (Δ*sopB*) showed the presence of significant phospho- β-catenin when complemented with psopB, the phospho- β-catenin disappeared (Fig. 5.6.1). Furthermore, complementation of sopB with a catalytically inactive mutant, SopB C460S, show more phospho- β-catenin. This indicates that the conserved cysteine residue Cys-460, which is essential for the inositol phosphatase activity required for Akt activation by SopB (Steele-Mortimer *et al.*, 2000), is also necessary for activating the Wnt/ β-catenin signalling pathway.

## 5.7 GSK3β is central to *S.* Typhimurium-induced up-regulation of β-catenin

To confirm GSK3β role in *S.* Typhimurium-induced up-regulation of β-catenin and thus activation of Wnt/ β-catenin pathway, the cells were infected in the presence of pharmacological inhibitors specific to signalling pathways important in regulation of GSK3β activation and its target substrates. Bovine rectal epithelial cells before the *S.* Typhimurium infection were pre-treated with a panel of following inhibitors: LY294002, (an inhibitor of PI3K pathway) 50 μM; SN50 (NFκB inhibitor), 50 μM; Akti-1/2, an allosteric inhibitor of Akt1 and Akt2 isozymes 20 μM; MG132 (proteosome inhibitor) 10 μM, and SB415286 (GSK3 inhibitor) 50 μm. Pre-treatment with SN50, LY294002 and Akti-1/2, inhibitors suppressed β-catenin, RANKL, SLUG and Vimentin, while SB415286 and MG132 elevated their levels in *S.* Typhimurium infected epithelial cells (Fig. 5.7.1 A). Pre-treatment of cells with LY294002 and Akti-1/2 completely inhibited *S.* Typhimurium induced GSK3β phophorylation and inactivation (Fig. 5.7.1 B). Together, these results suggest that *S.* Typhimurium in a SopB dependent manner induces PI3K activity leading to Akt activation that phophorylates and inactivates GSK3β, and thus the increased cytosolic β-catenin and the downstream Wnt/ β-catenin mediated Slug and vimentin proteins.

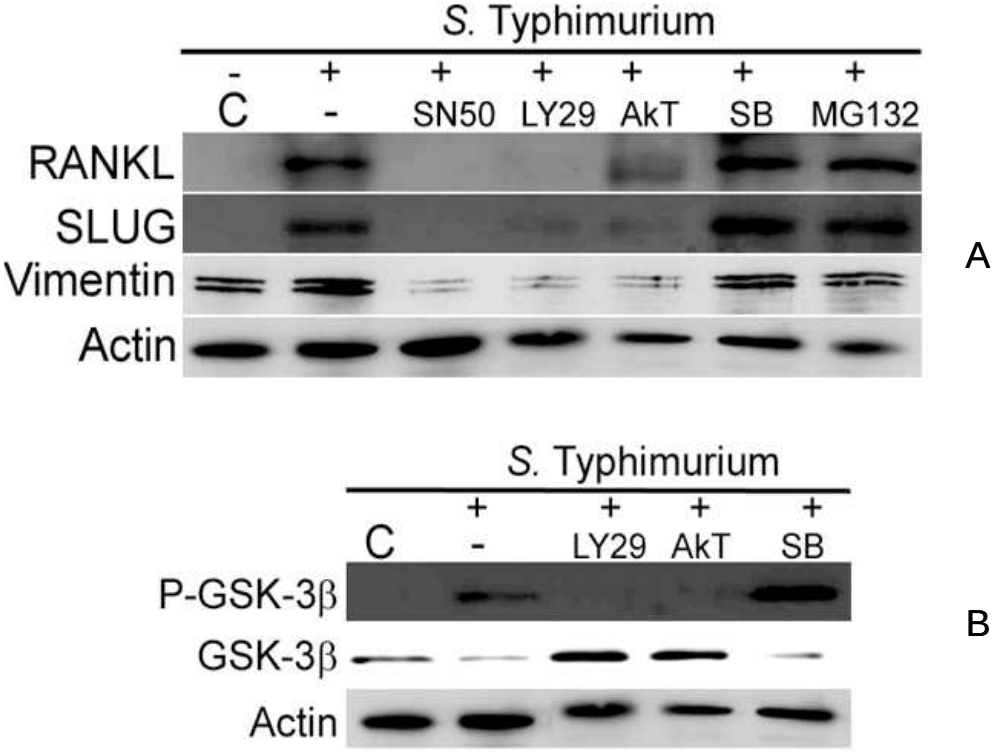


**Fig 5.7.1 *S.* Typhimurium activates NFkB and Wnt signalling pathways.** Heterogeneous population of epithelial cells in a primary cell culture from the bovine terminal rectum were isolated and cultured on 6 well plates (Materials and Methods). Five day old cultures were treated with DMSO, SN50, (NFκB inhibitor), 50 μM; LY294002, (AKT inhibitor) 20 μM, SB415286 (GSK3 inhibitor) 50 μM or MG132 (proteosome inhibitor) 10 μM for 5 h before being infected with *S.* Typhimurium strain (SL1344) at 37$^{o}$C, 5% $Co_2$ for 10 min, extracellular bacteria were removed by washing and cells were incubated further for 3 h. Cells were processed for western blotting using anti-RANKL, Slug, vimentin, total P-GSK & P-GSK-3B. Actin was used as a control for equal loading. Results are representative of two independent experiments.

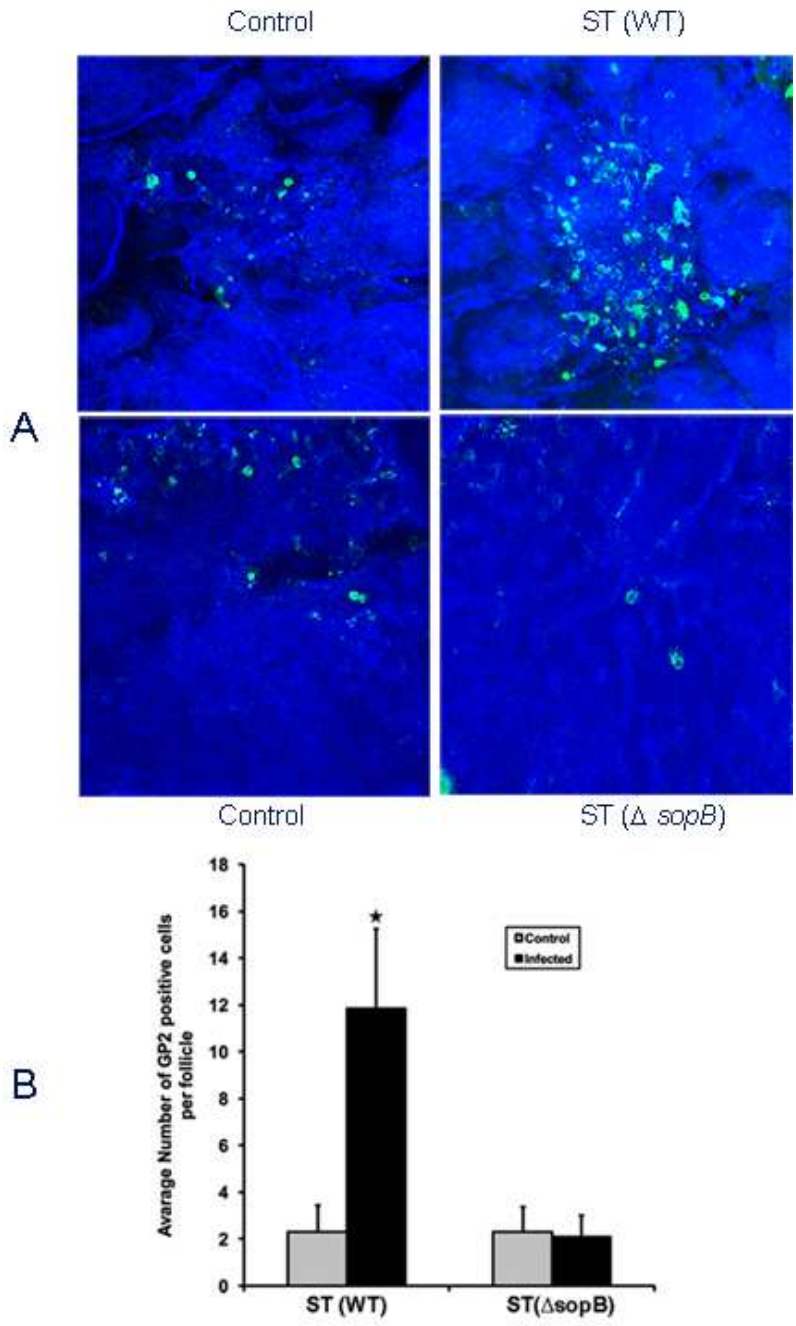


**Fig. 5.8.1 Confocal laser microscopy image of whole-mount staining of mouse Peyer's patch FAE (n = 10).** Five C57/BL6 mice were infected with *S.* Typhimurium SL1334 and C57/BL6 mice with a *sopB* mutant of *S.* Typhimurium SL1334 for 90 min. (A) the Peyer's patches were isolated from the rest of the intestine washed, fixed, stained for GP-2 (green) and actin (far red 647nm (blue). Z-stack images of the FAE of individual PP follicles were obtained using LSM5 confocal microscope (Zeiss). (B) The number of GP-2 expressing cells per follicle were increased significantly in wildtype *S.* Typhimurium infection whereas there was no increase with the Δ sopB *S.* Typhimurium mutant.

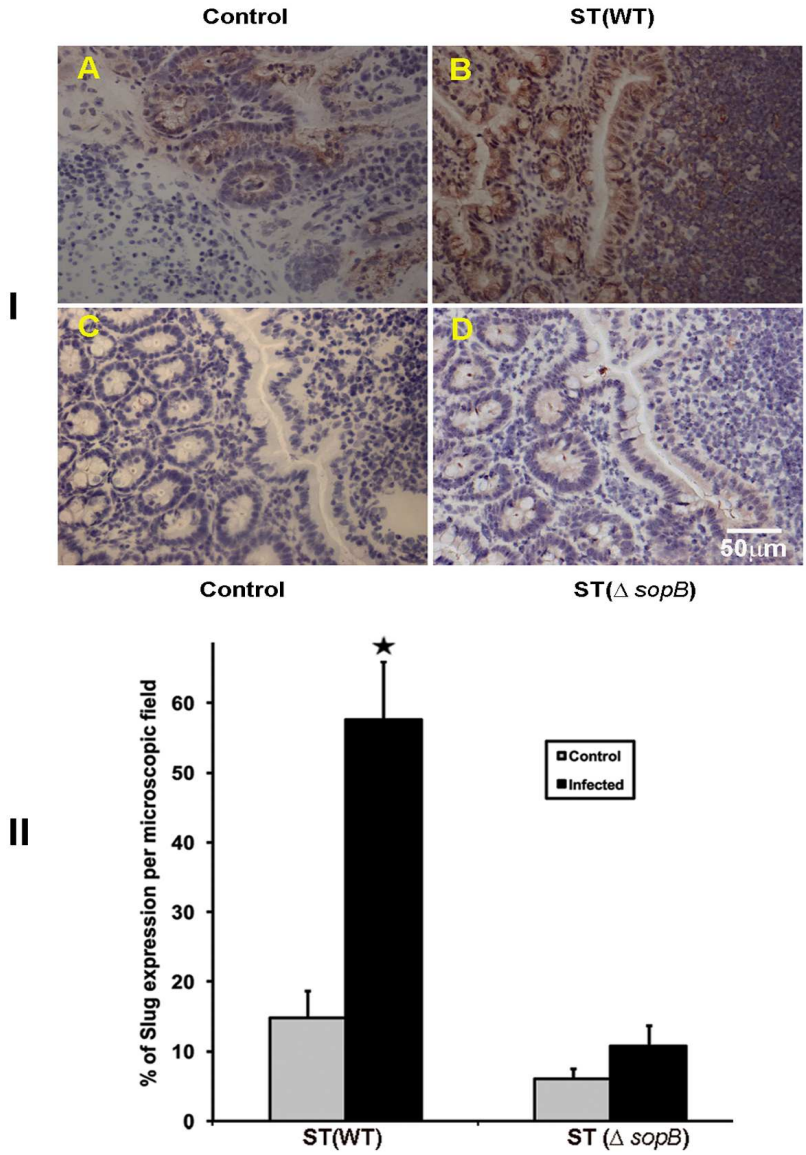


**Fig. 5.8.2 Elevated diaminobenzidine (DAB) enhanced Prussian blue staining in mice Peyer's patch FAE sections (n = 10). (**A) Five C57/BL6 mice were infected with S. Typhimurium SL1334 and other Five C57/BL6 mice with *sopB* mutant, S. Typhimurium SL1334 for 90 min. The Peyer's patches were isolated from the rest of the intestine. The cryospecimens were cut into 3-µm-thick sections and subjected to DAB staining using anti-slug antibody (Materials & Methods). Slug expression was increased significantly with wildtype S. Typhimurium infection where as there was no effect when Δ*sopB* S. Typhimurium was used. The Slug expression was measured as % of area of DAB staining compared to the total staining on the slide using imagJ software. The images were acquired using a laser-scan Nikon E800 Microscope.

## 5.8 *S.* Typhimurium increases the number of M cell in mice through activation of slug expression

To investigate whether SopB is important for *S.* Typhimurium mediated *in vivo* induction of M cells; murine ligated loops were infected with wildtype *S.* Typhimurium or a *sopB* mutant strain (Δ*sopB*). To quantify any change in M cell numbers PPs from respective infected mice were immuno-stained as whole tissue mounts and counted for expression of GP2 positive cells. Unlike SopB mutant strain the number of GP2 expressing cells significantly increased during infection with the WT *S.* Typhimurium strain (Fig. 5.8.1 A & B)**.**

Following on from *in vitro* studies that identified transcription factor Slug to be central to epithelial - M cell transformations, we examined for any change in its expression in infected murine tissue sections of PPs. Immunohistochemistry analysis demonstrated a statistically significant increase in Slug expression in tissue sections from mice infected with WT strain but not with that of SopB mutant strain (ΔsopB) (Fig. 5.8.2 I & II).

## 5.9 Discussion

Studies have suggested that microbes such as *S.* Typhimurium and *Streptococcus pneumoniae* can induce either increased number of M cells in FAE (Savidge *et al.*, 1991; Borghesi *et al.*, 1999; Meynell *et al.*, 1999), or increased trancytosis activity with constant number of M cells (Gebert *et al.*, 2004) raising the possibility that microbes may drive enhanced M cell number/ activity in a RANKL dependent manner. To follow up this hypothesis I set out to investigate molecular basis to microbial driven increase in M cell number. To this end time course conducted infection studies with wild type *S.* Typhimurium and examined for expression of RANKL and members of Wnt pathway including, β-catenin, Slug and vimentin, associated with EMT. The finding confirmed a temporal increase in expression of all these factors both at the transcript and protein levels.

To this end I compared the effect of viable versus heat inactivated and wild type versus SPI-1 mutant *S.* Typhimurium strains on the expression of RANKL, and Wnt pathway proteins including, β-catenin, Slug and vimentin, associated with EMT. This

work results confirmed that the fact that *S.* Typhimurium up-regulates β- catenin and its target genes, I set out to test up-stream components that could have direct increased β-catenin expression. Cytosolic β-catenin, a known target for GSK3β - phosphorylates and thereby marks the protein for ubiquitinylation and subsequent proteosomal degradation (Behrens, 2000), resulting in its inability to stimulate TCF/LEF transcription factors. GSK3β inhibition, through its phosphorylation at Ser-9 by Akt kinase (Cross *et al.,* 1995), could therefore, lead to increased β-catenin. To test if *S.* Typhimurium mediated increased β catenin expression depended on GSK3β activity, protein lysates from *S.* Typhimurium infected epithelial cultures were compared for active and inactive forms of GSK3β. Western blot analysis of protein lysates revealed an induction of GSK3β Ser-9 phosphorylation by the wildtype strain but not by *sopB* mutant strain (Fig. 5.5.5). These data suggests that *S.* Typhimurium in a SopB-dependent manner inhibits GSK3β, and hence the increased β-catenin and its downstream TCF/LEF mediated target genes, including vimentin and Slug, both of which are critical in EMT. To investigate whether SopB can directly control β-catenin activity, central to EMT, Caco-2 cells were co- transfected with luciferase reporter plasmid containing wild type (TOP Flash) or mutated (FOP Flash) LEF/TCF binding sites and PCR3 plasmid expressing SopB. The firefly luciferase activity was normalised relative to renilla luciferase activity. As shown in Fig. 5.5.6, SopB directly regulates luciferase activity with in 48 h post-transfection (fold compared with Fop Flash). LiCl, a known activator of β-catenin signalling (Stambolic *et al.,* 1996), was used as a positive control.

The presence of putative *cis* elements for Lef-1, in bovine SLUG gene promoter raised the possibility that Lef-1 may be implicated in the modulation of SLUG expression as previously demonstrated in other species such as human and chicken (Lambertini *et al.,* 2010). We hypothesised for SopB to activate SLUG transcription perhaps in NF-κB dependent manner as shown previously (Fig. 5.5.8). The bovine SLUG promoter sequence was cloned upstream of the Luc reporter gene in the pGL3 basic vector and tested in Caco-2 cells on activation by SopB. As shown in Fig. 5.5.8, transient transfection with SopB resulted in an increase in SLUG luciferase activity relative to the cell co-transfected with PCR3 and SLUG luc-construct, demonstrating for a direct role for SopB in activation of transcription factor SLUG.

GSK3β a known target for PI3K/Akt kinases (Doble & Woodgett, 2003), phosphorylates and marks cytosolic β-catenin for ubiquitinylation and subsequent proteosomal degradation (Behrens, 2000), as well as repress NF-κB dependent expression of certain target genes, like Snail, associated with EMT (Bachelder *et al.*, 2005). To identify up-stream regulators and to confirm that GSK3β plays a role in *S.* Typhimurium-induced up-regulation of β-catenin, we infected cells in the presence of pharmacological inhibitors specific to signalling pathways, nuclear factor -κB, phosphoinositide 3-kinase (PI3K)/Akt pathways, important in regulating GSK3β and its target substrates. Bovine rectal epithelial cells before the *S.* Typhimurium infection were pre-treated with a panel of following inhibitors: LY294002 (50 μM), an inhibitor of PI3K pathway; SN50, an inhibitor of NF-κB pathway (50μM), Akti-1/2, an allosteric inhibitor of Akt1 and Akt2 isozymes (20 μm) ; MG132 (10 μm), proteosome inhibitor and SB415286 (50 μM) GSK-3 inhibitor. None of these inhibitors showed any effect on viability of *S.* Typhimurium. As seen in Fig. **5.7.1** pre-treatment with SN50, LY294002 and Akti-1/2 inhibitors suppressed β-catenin, while SB415286 and MG132 elevated its levels in *S.* Typhimurium infected epithelial cells suggesting a role for GSK3β in *S.* Typhimurium mediated regulation of β-catenin. The results suggest that the suppressed GSK3β activity had an enhanced cascade effect on downstream β-catenin/TCF/LEF mediated targets, including RANKL, vimentin and Slug (Fig. 5.7.1), which are defining proteins in EMT.

These findings provide evidence for a unified signalling mechanism driven by convergence of GSK3β regulated signalling molecules that confer acquisition of the mesenchymal phenotype and in this instance trigger epithelial to M cell transformation. *S.* Typhimurium mediated GSK-3 beta suppression is central to EMT and *S.* Typhimurium regulates beta-catenin in a GSK 3 beta dependent manner.

# *Chapter 6*

## *Receptor activator of nuclear factor kappa-B ligand (RANKL) induces transformation of intestinal epithelial cells to antigen-sampling M cells*

# 6. Receptor activator of nuclear factor kappa-B ligand (RANKL) induces transformation of intestinal epithelial cells i to antigen-sampling M cells

## 6.1 INTRODUCTION

Mucosal-associated lymphoid tissue, a major compartment of the immune system that operates at the mucosal level, is present throughout the intestine either as isolated lymphoid follicles or as lymphoid follicle aggregates such as Peyer's patches in the small intestine. The lymphoid tissue is separated from the intestinal milieu by the follicle-associated epithelium (FAE) composed of numerous enterocytes and a relatively small number of highly specialized M cells.

Epithelial cells usually exist as sheets of immotile, tightly packed, well-coupled, polarized cells with distinct apical, basal and lateral surfaces. Remarkably, these cells can dramatically alter their morphology to become motile, fibroblast-like mesenchymal cells in a process of epithelial-mesenchymal transition (EMT). This process and the reverse, mesenchymal-epithelial transition, occur repeatedly during normal embryonic development. A phenomenon similar to physiological EMT occurs during the pathophysiological progression of some cancers (Geiger & Peeper, 2009) .

The TNF superfamily member receptor activator of NFk-B ligand (RANKL) recptor RANK is selectively expressed by subepithelial stromal cells in PP domes (Knoop *et al.*, 2009). It has also been reported that it could be secreted within villus epithelium (Knoop *et al.*, 2009). it is essential for the formation of follicle associated M cell as the mice mutant in RANKL or using of Ab-mediated neutralization of RANKL in adult wild-type mice markedly eliminated most PP M cells. RANKL also has been shown to be essential for M cell survival (Knoop *et al.*, 2009). RANKL acts through its specific receptor (RANK) and plays an important developmental role in multiple tissues. it is important for development of lymph nodes and osteoclast so its deficiency lead to absence of lymph node and malformed skeleton (Kong *et al.*, 1999).

## 6.2 RESULTS

Following recent publication on RANKL as one of the critical factors controlling differentiation of M cells *in vivo*, we set out to test if RANKL could be used as a supplement to induce M cell phenotype in a primary epithelial cell culture model system. With the knowledge that interaction of intestinal crypts with their specialized microenvironment or "niche" is critical for their maintenance and differentiation *in vivo*, we hypothesized that primary intestinal epithelial cells isolated from different regions of the gut, may vary in their responses to RANKL. To test these hypotheses, epithelial crypts isolated from lymphoid follicle (LF) dense bovine terminal rectum, follicular associated crypts (FACs) or LF deficient proximal rectum were cultured in the presence of purified RANKL. RANKL-treated epithelial cells in these two culture systems were compared for phenotypic and functional characteristics of M cells.

Ultrastructural studies on proximal and terminal bovine rectal tissue revealed morphologically distinct crypt populations at these two sites (Fig**.** 6.2.1 B & C). Unlike crypts with apical round fissure in proximal rectum, most crypts in the follicle dense terminal rectum, exhibited longitudinal fissure morphologically alike "Dome Associated Crypts" previously identified as source for M cell progenitors in mice Peyer's patches (Gebert *et al.*, 1999). Gebert *et al.* (1999) demonstrated that "Dome Associated Crypts" associated with lymphoid follicles are distinct from ordinary intestinal crypts in size, shape, cellular composition, and location and represent a sub-population of cells predetermined as M cells before attaining their morphological and functional features. We hypothesized that the FACs in our culture model system represent "Dome Associated Crypts" composed of distinct subset of predetermined FAE and M cell precursors or cells in transitional stages, that following appropriate immune stimuli, like RANKL, can differentiate into functional M cells.

Immunofluorscent staining for vimentin, a known marker for FAC and FAE in cattle revealed that a subset of crypts and epithelial cells were stained positive for vimentin in the primary cell cultures from terminal rectum (Fig. 3.2.8a chapter 3). The cells in culture express pan-cytokeratin, as a marker for epithelium (Fig. 3.2.8a chapter 3)

By western blotting the cells cultured from both FAC and OC were comparable in expression for RANKL receptpr RANK, vimentin and epithelial specific markers, tubulin and E-cadherin (Fig. 6.2.1D).

RANKL-treated epithelial cells in these two culture systems were compared for phenotypic and functional characteristics of M cells. Intermediate filament protein vimentin is a known marker for follicle associated epithelium (FAE) and M cells in cattle and rabbit (Meynell *et al.*, 1999) and cattle (Mahajan *et al.*, 2005).

*S.* Typhimurium typically targets M cells as portal of entry to translocate across intestinal epithelium. Bacterial trafficking across intestinal epithelial cells is applied as an *in vitro* functional assay to measure M cell activity in culture. RANKL- treated epithelial cells that were specifically cultured from FACs demonstrated an increased (i) expression of vimentin protein (Fig. 6.2.2) as well as number of vimentin expressing cells (Fig. 6.2.3), *S.* Typhimurium translocation across epithelial cells and their intracellular uptake in kanamycin protection assays (Fig. 6.2.4) confirming a change in both the cellular phenotype and the functional attributes that resemble to M cells in the bovine host (Czerkinsky *et al.*, 1999).

Following a recent work that reported RANKL association with epithelial-mesenchymal transition (EMT) (Odero-Marah *et al.*, 2008).We hypothesised that RANKL mediated increase in M cell specific marker and function expression of M cell specific marker, vimentin, and increased *S.* Typhimurium translocation across RANKL-treated cells might as well be due to RANKL dependent transformation of a specific subset of epithelial cells in culture in to a cell type that phenotypically and functionally resembles specialised antigen sampling M cell. To follow this hypothesis we analysed RANKL-treated cultures for the expression of the transcription factor Slug that has previously been associated with RANKL mediated epithelial transformation (Knoop *et al.*, 2009). Indeed, in our culture models RANKL induced expression of Slug (Fig. 6.2.2), specifically in epithelial cells cultured from FACs is suggestive of similar mechanisms underlying RANKL mediated transition of a subset of intestinal epithelial cells in to M cell phenotype.

To determine molecular mechanisms underlying RANKL mediated cellular transformation we hypothesised for a potential role of NFκB and Wnt/beta-catenin, the known upstream signalling activators of EMT transcription factor Slug (Saegusa *et al.,* 2009). To test this hypothesis BRE cells, treated with RANKL in presence of pharmacological inhibitors specific to each of these signalling pathways, were examined for expression of vimentin. Indeed, in the presence of NFκB inhibitor SN50 and beta-catenin inhibitor FH535, the RANKL-mediated enhanced Vimentin expressions were suppressed whilst in presence of proteosomal inhibitor (MG132) its protein levels were elevated (Fig. 6.2.5). To examine whether RANKL regulates slug and vimentin promoter activity as well to determine the underlying signalling mechanisms, intestinal epithelial cells transfected with respective lucifrase reporter constructs were tested for their activity in presence of pharmacological inhibitors specific to NFκB and β-catenin signalling pathways. As seen in Fig. 6.2.6, pre-treatment of RANKL-treated epithelial cells with inhibitors SN50 and FH535 suppressed while GSK-3β inhibitors (SB415286 and LiCl), enhanced SLUG and Vimentin promoter activity. Together, these results suggest a role for both NFκB and Wnt/beta-catenin signalling during RANKL mediated increase in Slug and vimentin expression, the classical EMT markers.

Further, we reasoned that the enhanced uptake or translocation of *S.* Typhimurium across RANKL-treated epithelial cells is due to a change in an epithelial cell subset to M-like cell phenotype that *S.* Typhimurium is known to preferentially target during invasion of intestinal epithelium (Clark *et al.*, 1994). RANKL signals through its receptor RANK (receptor activator of NFκB) and a downstream pathway that involves tumer necrosis factor (TNF) receptor associated factor 6 (TRAF6) and the activation of NFκB. RANKL-RANK interaction can be blocked by Osteoprotegerin (OPG), a decoy receptor for RANKL. To determine specificity of RANKL as well the consequent NFκB signalling in inducing M cell phenotype, *S.* Typhimurium translocation studies, *in vitro* functional assay for M cell activity were done in presence of OPG and NFκB inhibitor SN50. Indeed, in presence of both these inhibitors, the RANKL mediated enhanced translocation of *S.* Typhimurium was significantly reduced (Fig. 6.2.7). Thus, suggesting a role for RANKL mediated

NFκB dependent factors in enhancing *S.* Typhimurium translocation across epithelial cells.

The results so far suggest a role for RANKL in inducing epithelial transformation which is restricted to a sub set of cells cultured specifically from FACs and not from the OCs. We reasoned that the observed distinct effects of RANKL on epithelial cells cultured from these two crypt types may be because of differential expression of epithelial molecules critical to RANKL mediated signalling. To this end we screened epithelial cells from both culture systems for expression of RANK, the RANKL receptor, and Relb, one of the NFκB transcription factors, required for RANKL mediated cellular/ osteoclast differentiation (Vaira *et al.*, 2008). Although RANK was expressed by epithelial cells in both culture model systems, the RelB expression was limited to cells cultured from FACs only.

Unlike other members of NFκB, RelB has a much more restricted tissue distribution (Fig. 6.2.8) including FAE (Yilmaz *et al.*, 2003). RelB plays a critical role in constitutive expression of NFκB regulated genes in lymphoid organs and its deficiency has been associated with multiple lymphoid organ defects (Vaira *et al.*, 2008) including the absence of Payer's patches (Yilmaz *et al.*, 2003). We postulate that a subset of epithelial cells in FAC cultures, that express RelB, on exposure to RANKL differentiate into fully operational M cells.

To further investigate role of RelB in inducing RANKL-mediated cellular transformation, RelB was depleted by siRNA knockdown. As speculated RelB K/O epithelial cells were only partly responsive to RANKL and as a consequence showed limited effect on expression of Slug and vimentin (Fig. 6.2.9a) and *S.* Typhimurium translocation across epithelial cells (Fig. 6.2.9b). These experiments confirm RelB as one essential factor critical to RANKL- mediated

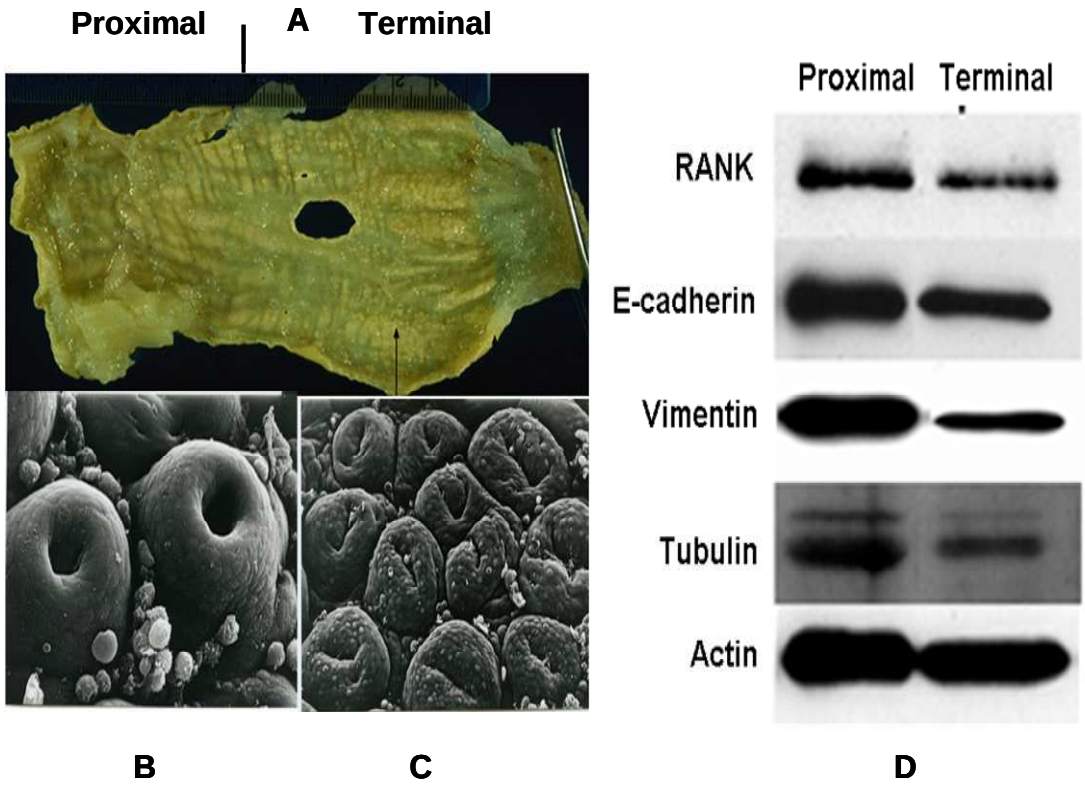


**Fig. 6.2.1 Characterisation of proximal and terminal rectal epithelial cells.**
Bovine rectal tissue fixed in 70% (V/V) acetic acid to show lymphoid dense area in the terminal rectum (A). SEM images of intestinal crypts at the bovine rectum (B-C). Proximal rectal crypts (ordinary crypts, OC) with central round fissure are structurally distinct from crypts with longitudinal fissure (follicular associated crypts, FAC) at the lymphoid follicle (grey nodules) dense bovine terminal rectum. (D) Immunblot analysis of cells cultured from proximal and terminal rectal crypts. Five day old cultures were lysed using protein lysate buffer and protein lysates were collected (materials and methods).The samples were analysed by immunoblotting using anti-RANK, e-cadherin, vimentin and Tubulin antibodies as indicated. Actin was used as a control for equal loading.

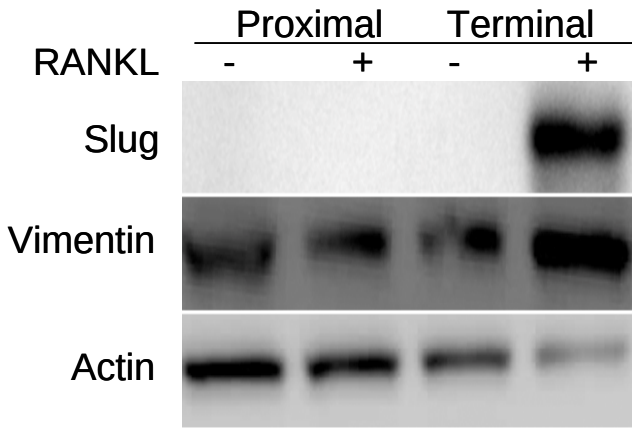


**Fig. 6.2.2 Effect of RANKL on primary intestinal epithelial cells.** Proximal and terminal rectal epithelial cells primed with RANKL at100 ng/ml for 5 days were stained for Slug and Vimentin. The immunoblotmembranes were stripped and re-probed with an anti-actin antibody. The results are representative from one of two independent experiments**.**

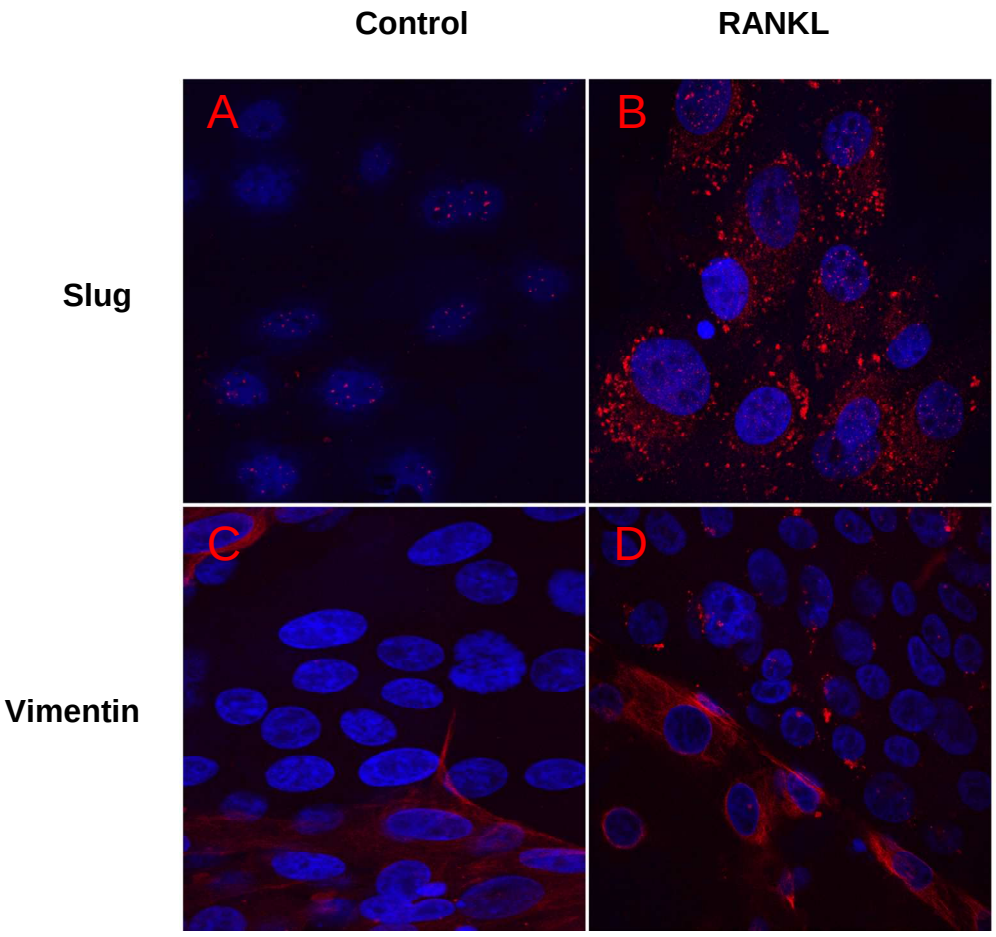


**Fig. 6.2.3 RANKL induces the expression of Slug and Vimentin in bovine rectal epithelial cell.** Heterogeneous population of epithelial cells in a primary cell culture from the bovine terminal rectum were isolated and cultured on glass cover slips (materials and methods). Epithelial cells were incubated overnight with RANKL (100ng/ml). The cells were fixed and permeabilised in 2 % ( v/v) formalin/ 0.2% (v/v) Triton X-100. The cells were analysed for Slug and vimentin expression and localisation in cellular compartments by immuno-fluorescence staining. Slug (A&B) (red) primarily localised at the nucleus in control cells and following exposure to RNKL, Slug expression was increased and localised in the cytoplasm. (C&D)Vimentin (red) and nucleus (blue). Increased expression of Vimentin in RANKL treated cells is seen as speckled staining. Images were acquired using Zeiss Axiovert, objective xx63. The results are representative from one of 2 independent experiments**.**

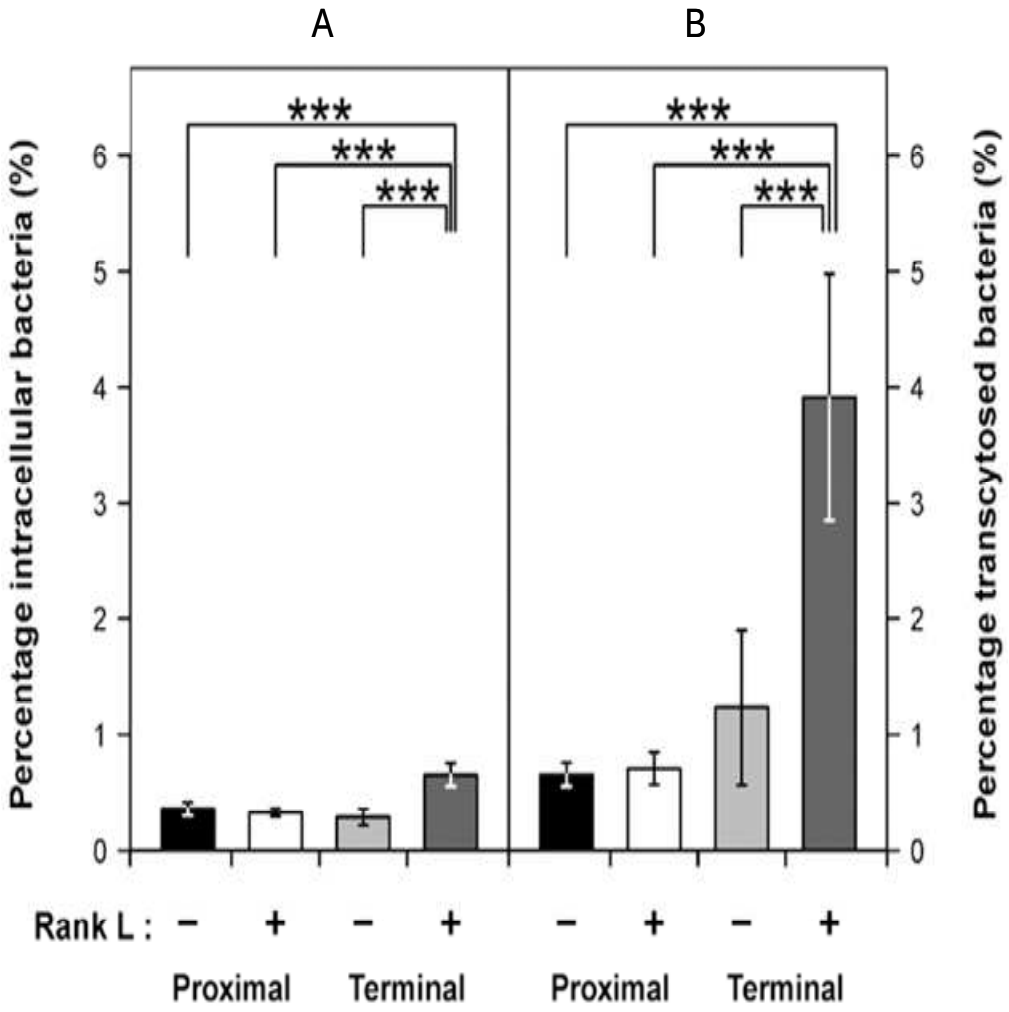


**Fig. 6.2.4 RANKL primed cells were used for *S.* Typimurium internalization (A) and trancytosis (B) studies – in vitro M cell functional assays.**
Heterogeneous population of epithelial cells in a primary cell culture from the bovine proximal & terminal rectum were isolated and cultured on 6 well and transwell plates (materials and methods). Cells were challenged with *S.* Typhimurium as explained in materials and mehthods. (A) The bacteria was incubated with the cells at 37$^{o}$C, 5% $CO_2$ for 30 min; the bacteria was removed and the cells were washed 3 times with PBS and treated with kanamycin (750 μg/ml) for 3 h. (B) Epithelial cells in the upper chamber of the transwell were challenged with *S.* Typhimurium as explained in materials and mehthods for 30 min and the bacteria were collected from the lower chamber, serially diluted and plated.*** $p<0.01$ relative to the control. The results are representative from one of 3 independent experiments**.**

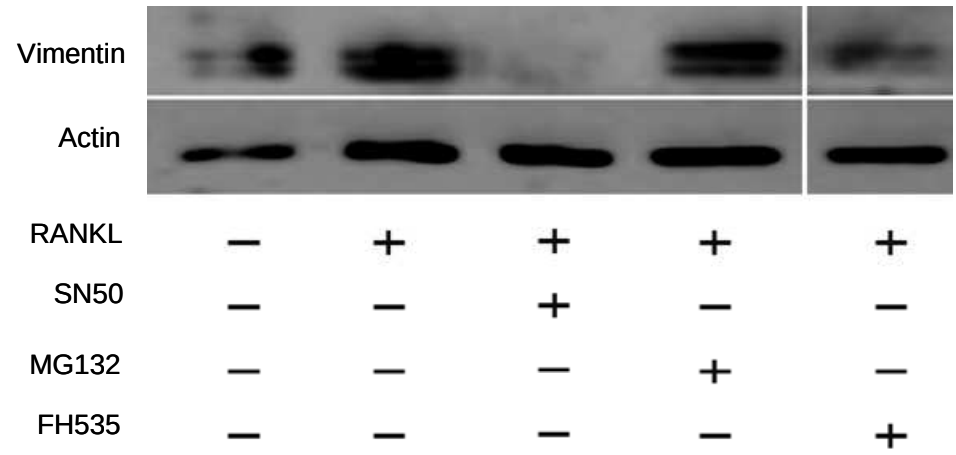


**Fig. 6.2.5 RANKL induces vimentine expression involves NFkB and Wnt signalling pathways.** Terminal rectal epithelial cells were treated for 24 hours with RANKL in the presence or absence of SN50 (NF-κB peptide inhibitor), MG132 (proteosomal inhibitor) 10 μM and β -catenin inhibitor (FH35) 10 μM. The protein concentrations of vimentin were analysed by Western blotting using antibodies as indicated (Materials & methods). The results are representative from one of 2 independent experiments**.**

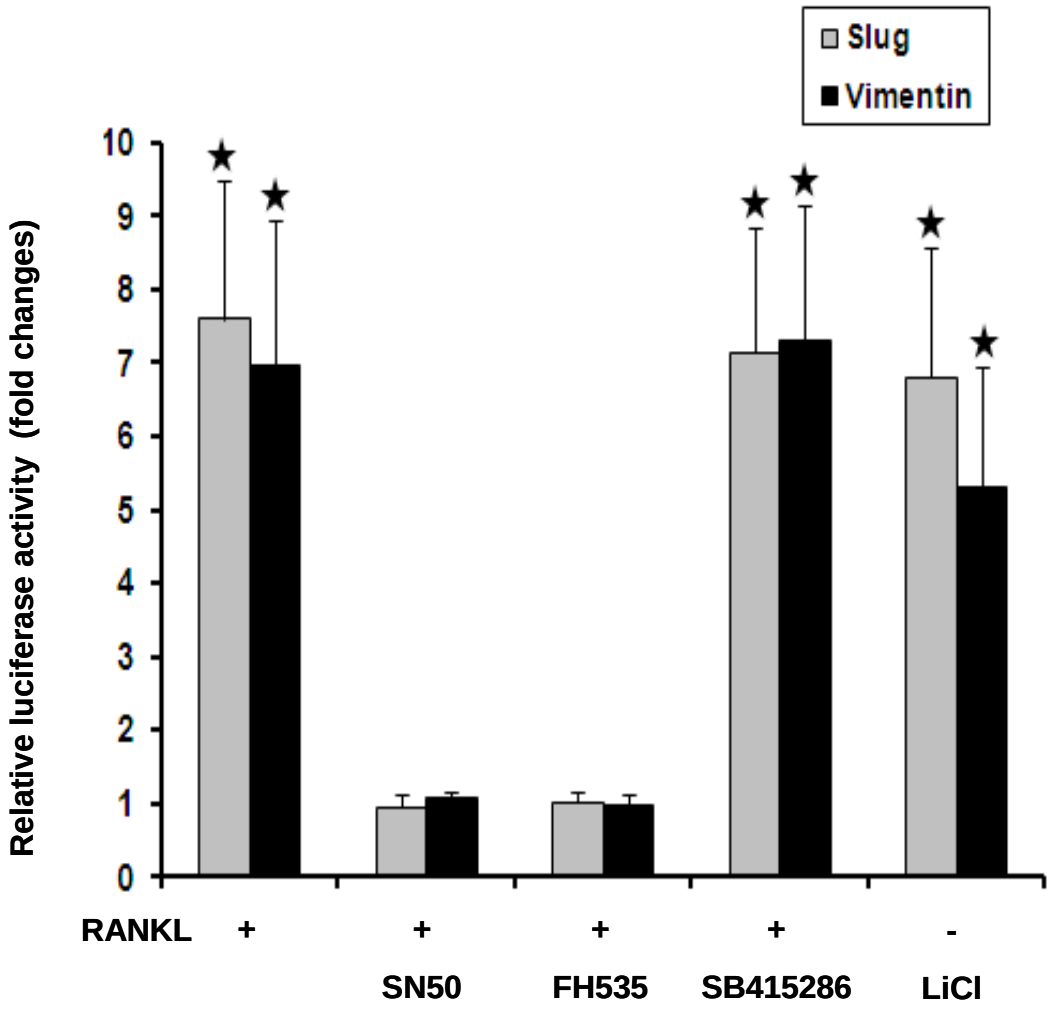


**Fig. 6.2.6 Vimentin and Slug promoter transcription activity is suppressed in the presence of NF-κB peptide inhibitor (SN50), Beta catenin inhibitor (FH535), but not by the GSK-3 inhibitor SB 415286.** SLUG and Vimentin promoter constructs, inserted in pGL3 plasmid, were co-transfected in to the Caco-2 cell lines with a pRTK-Luc (Promega), to normalize transfection efficiency. 100ng/ml of RANKL was added 48 hr post transfection, together with 50 μM for SN50 NF-κB peptide inhibitor and GSK-3 β inhibitor, SB 415286. β -catenin/Tcf inhibitor, FH535 10 μM or LiCl 10 mM final concentration as control positive. Cells were harvested and lysed 15 hr later and Firefly luciferase (Luc) and Renilla Luciferase (RLuc) activities were measured by using the Dual Luciferase Reporter Assay System (Promega). Data are represented as relative luciferase activity (normalized to RLuc activity), and are representative of two independent experiments. * $p<0.05$ relative to the control The grey bars represent the slug promoter a while the black bars represent the Vimentin promoter transcriptional activity.

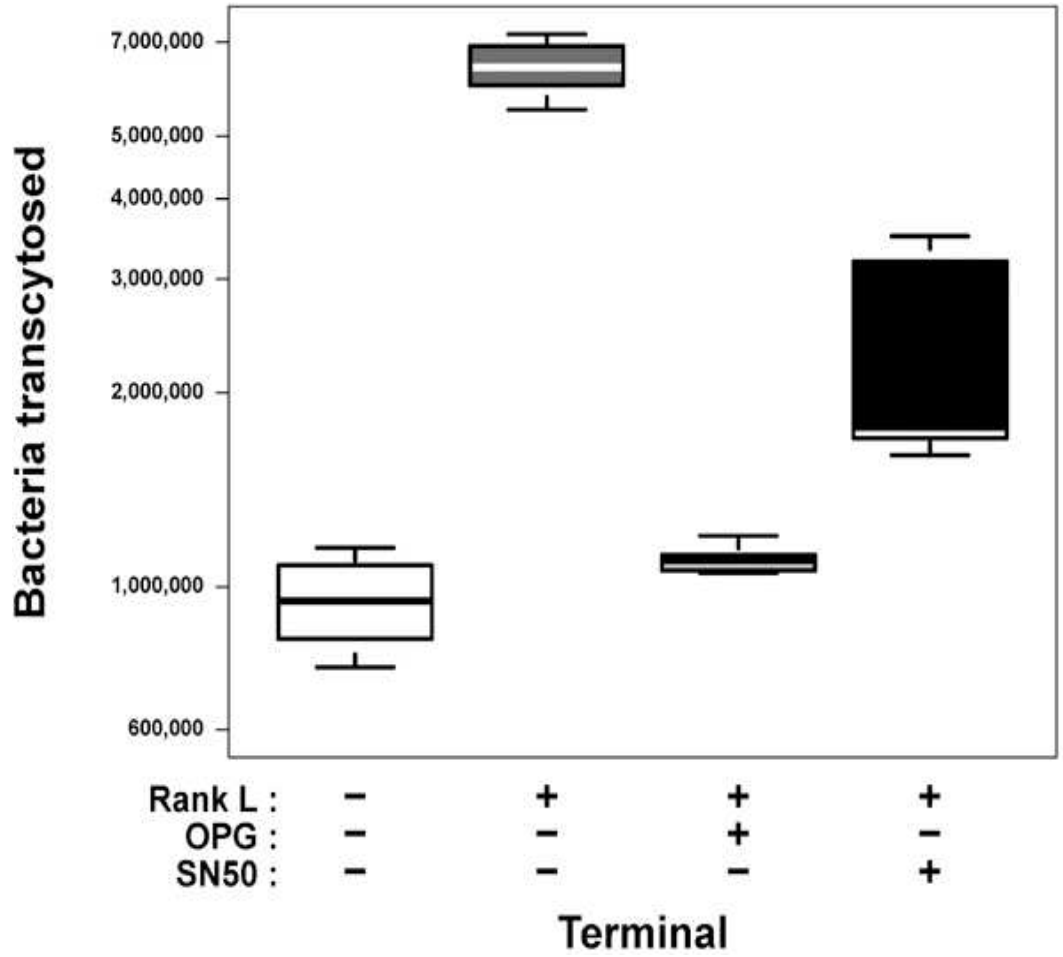


**Fig. 6.2.7 RANKL-dependent induced increased *S.* Typimurium trancytosis in terminal rectal epithelial cells was significantly reduced in presence of RANKL decoy receptor OPG and NF-κB peptide inhibitor SN50.** Heterogeneous population of epithelial cells in a primary cell culture from the bovine terminal rectum were isolated and cultured on 6 well plates (materials and methods). OPG 50ng/ml and 50 μM of SN50 (NF-κB peptide inhibitor) was added to five day old Terminal BRE cells 15 hour before challenging with *S.* Typhimurium. The bacteria was incubated with the cells at 37$^{o}$C, 5% $Co_2$ for 30 min. in presence of both these inhibitors, the RANKL mediated enhanced translocation of *S.* Typhimurium was significantly at $p<0.05$ relative to the control and are representative of three independent experiments.

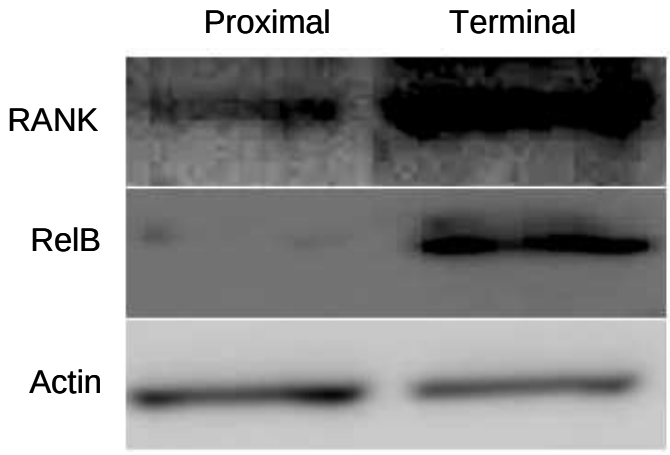


**Fig. 6.2.8 RelB expression is limited to terminal rectal epithelial cells.** Heterogeneous population of epithelial cells in a primary cell culture from the bovine proximal and terminal rectum were isolated and cultured on 6 well plates (Materials and Methods). Five day old cultures were lysed using protein lysate buffer. The protein lysates were collected (Materials and Methods). The samples were analysed by immunoblotting using anti-RANK, RelB primary antibodies. RelB expression is limited to terminal rectal epithelial cells. The immunoblot-membranes were stripped and reprobed with anti-actin antibody. The results are representative from one of 2 independent experiments**.**

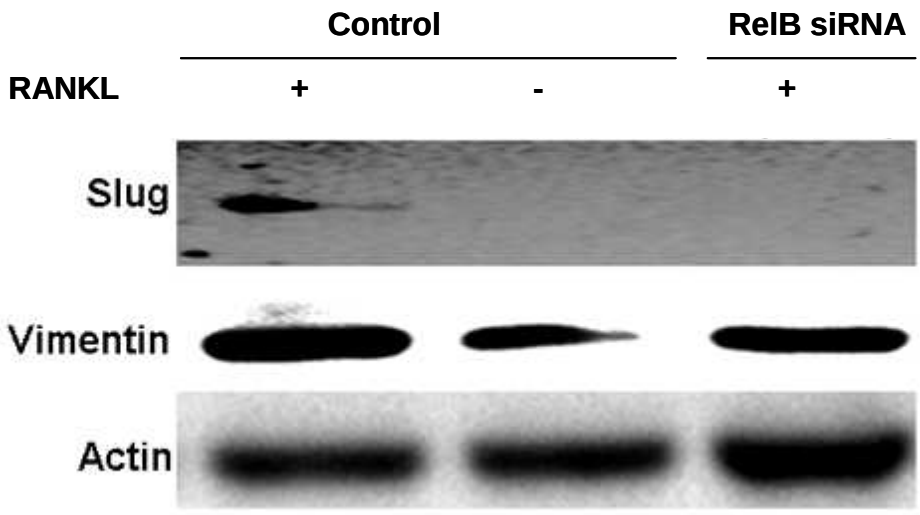


**Fig. 6.2.9a RANKL induced expression of Slug and Vimentin in bovine rectal epithelial cell through activation of transcription factor RelB.** Heterogeneous population of epithelial cells in a primary cell culture from the bovine terminal rectum were isolated and cultured on 6 well plates (Materials and Methods). Five day old the cells were double transfected with two sets of relB siRNA (Materials and Methods). 48 h later control and *relB* (siRNA) transfected rectal epithelial cells were treated with RANKL (100ng/ml) for overnight. Whole cell extracts were immuno-detected for Slug and Vimentin. The same membrane was stripped and reprobed with anti-actin antibody. The results are representative from one of 2 independent experiments.

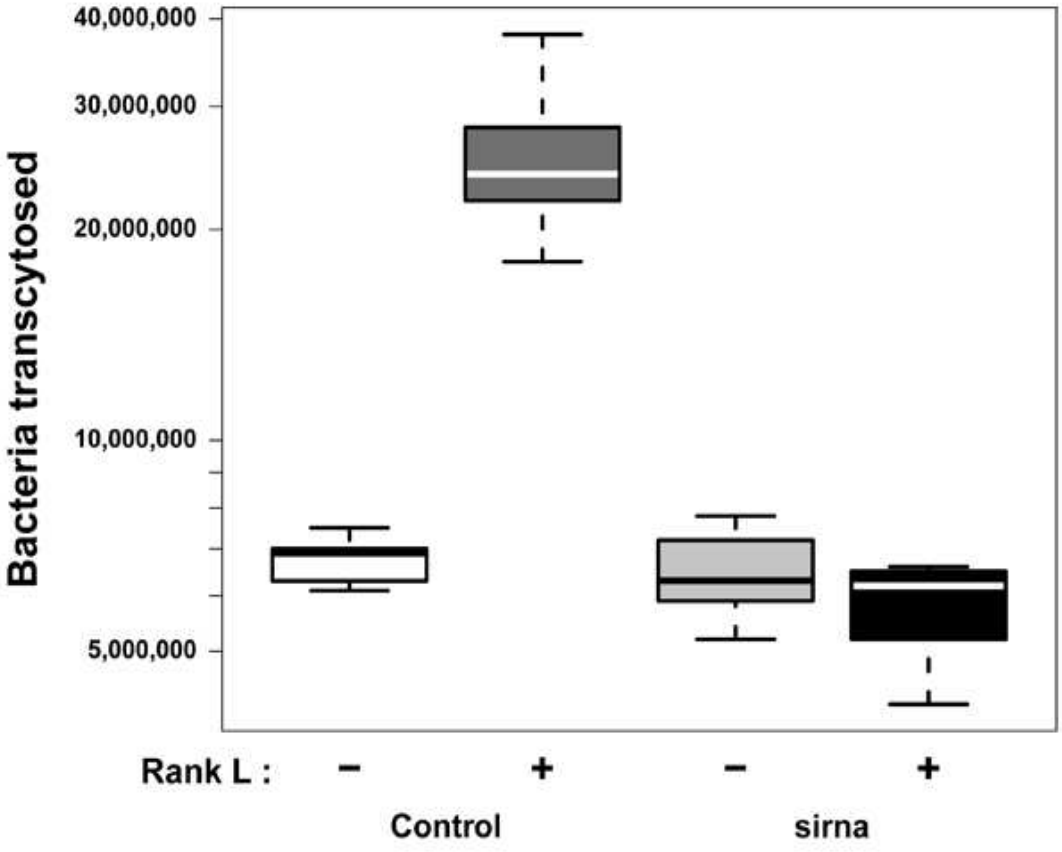


**Fig. 6.2.9b RANKL dependent increased *S.* Typimurium trancytosis was significantly reduced in the presence of *relB* (siRNA) rectal epithelial cells**. Heterogeneous population of epithelial cells in a primary cell culture from the bovine terminal rectum were transfected with RelB siRNA. 48 h later it was incubated with RANKL 100ng/ml overnight. The cells were challenged with *S.* Typimurium at 37$^{o}$C, 5% $Co_2$ for 30 min. RelB siRNA treated and control non transfectd cells showed a significant reduced trancytosis activity for *S.* Typhimurium comparable with RANKL treated cells. Results are presented as $p<0.05$ relative control. The results are representative from one of 3 independent experiments.

signalling as it has been shown previously during its role in RANKL-dependent osteoclast differentiation (Vaira *et al.*, 2008).

Tissue specific expression of RelB may explain for distinct effects of RANKL on primary epithelial cells cultured specifically from FACs. Earlier demonstrated that "Dome Associated Crypts" associated with lymphoid follicles are distinct from ordinary intestinal crypts in size, shape, cellular composition, and location and represent a sub-population of cells predetermined as M cells before attaining their morphological and functional features (Gebert and colleagues1999).
Together these results suggest that the FACs in our culture model system perhaps represent "Dome Associated Crypts" composed of distinct sub-sets of predetermined FAE and M cell precursors or probably cells in transitional stages, that following appropriate immune stimulus, in this instance RANKL, can differentiate in to functional M-cell phenotype. RANKL in NFκB and Wnt/ β-catenin dependent manner activate transcription factor Slug, typically associated with EMT that transforms a specific subset of epithelial cells in to M like cells.

## 6.3 Discussion

Despite the basic functional and ultra structural features of M cells known for over 30 years (Owen & Jones, 1974), some basic aspects of M cell biology, including their origin and differentiation, are still unclear. Debate continues on whether M cells are a distinct lineage arising directly from crypt stem cells or can instead arise from normal FAE enterocytes with the plasticity to transform in to M cells in presence of certain stimuli, whether they could arise from ordinary intestinal crypts or their formation is restricted to specialised follicle/dome-associated crypts within the lymphoid dense intestinal tissue. M cells have been documented primarily in the FAE and occasionally on villous epithelium (Borghesi *et al.*, 1999; Jang *et al.*, 2004). Specific factors released from lymphoid tissue beneath FAE have the potential to induce M cell differentiation in the FAE and promote their function of antigen uptake (Kerneis *et al.*, 1997; Mach *et al.*, 2005). Recent work by identified RANKL as one such critical factor controlling differentiation of M cells (Knoop and colleagues

2009). M cell deficit in RANKL-null mice was corrected by systemic administration of exogenous RANKL and antibody-mediated neutralisation of RANKL in adult wild-type mice significantly reduced M cell numbers. However, specific mechanism(s) underlying RANKL mediated M cell differentiation has not yet been elucidated. Using in *vitro* epithelial cell culture model system it has been confirmed seminal work of (Knoop *et al.*, 2009), and confirmed a role for RANKL in inducing M cell activity. This study hypothesised a role for RANKL, a known inducer of EMT, in transforming a subset of epithelial cells in culture to a cell type that functionally and phenotypically is identical to M cell. This work provides evidence that RANKL induces transcription factor Slug, central to EMT, which perhaps drives a subset of epithelial cells to a cell phenotype typical of M cell. Slug belongs to a family of zinc-finger transcription factors that are essential in promoting EMT, both during defined stages of embryonic development and during early stages of cancer progression (Cano *et al.*, 2000; Thiery *et al.*, 2009). Slug in addition to many target genes like *mucin-1*, *collagen IIaI* or *MMP-2* (matrix metalloproteinase-2) (Guaita *et al.*, 2002; Yokoyama *et al.*, 2003; Martínez-Estrada *et al.*, 2006), up-regulates the intermediate filament protein vimentin and C-myc (Cano *et al.*, 2000) and represses tight junctional proteins including E-cadherin and Claudin. Thus it can drive major morphogenic changes in cellular architecture needed for establishment of a modified cell phenotype with altered functional attributes. here it is shown that RANKL transforms a subset of epithelial cells to display typical features of M cells, such as transcytosis activity, the disorganization of microvilli, and the expression of M cell specific marker vimentin.

The finding of this study also demonstrate that the effect of RANKL in promoting M cell phenotype is restricted only to intestinal crypts cultured from LF dense terminal rectum but not to the ones cultured from LF deficient proximal rectum. Perhaps crypts cultured from LF dense rectum are the specialised Follicle associated crypts, that previously have been associated with origin and development of M cells (Gebert *et al.*, 1999). One possibility is that FACs may have either been already programmed or primed with specific cues from surrounding lymphoid follicles /microenviornment to yield cell subsets alike M cells or FAE (Neutra *et al.*, 2001). Possibly RANKL

acts as a final trigger for these cells in transient to acquire a fully differentiated and functional M cell phenotype in culture. We identified RelB as a distinct, NF-κB family member, protein that was constitutively expressed only in FAC cell culture. RelB as one of the critical down stream mediators of the lymphotoxin-beta recptor (LTβR) signalling is indispensable for PP development (Yilmaz *et al.*, 2003). Earlier lymhotoxin has been reported as one essential cytokine required for development and differentiation of FAE and M cells (Kraehenbuhl & Neutra, 2000; Debard et al., 2001), as well as for inducing expression of FAE specific chemokine CCL20 (Anderle *et al.*, 2005). RelB- deficient mice display a complex phenotype including lack of lymhnodes, splenomegaly and multiple defects in immune responses (Weih *et al.*, 2001). The fact that RelB is constitutively expressed in FAC and FAE (Yilmaz *et al.*, 2003; Wang *et al.*, 2009) and is critical to RANKL-mediated alternative NF-κB signalling that is essential in inducing cellular differentiation (Vaira *et al.*, 2008), I tested if RelB is indispensable for RANKL mediated M cell differentiation in culture in this work. Indeed, FAC epithelial cultures depleted of RelB showed no effect of RANKL on either expression of molecular markers or functional phenotype of cells. Taken together, these results suggest that RelB is critical for RANKL mediated induction of epithelial - M cell transformation/ M cell differentiation and perhaps selective expression of RelB in FACs/FAE is one such basis for M cell formation is restricted to specialised follicle/dome-associated crypts within the lymphoid dense intestinal tissue. However, this work does not define whether RANKL/RelB interaction provides lineage specificity to M cell precursors/progenitors in FACs or it drives phenotypic and functional maturation of cells already, committed to be M cells.

# *Chapter 7*

## *Final Discussion and Future Work*

# 7. Final discussion and future work

EPEC and EHEC constitute a significant risk to human health. EPEC is the cause of severe infantile diarrhoeal disease in the developing world, while EHEC is an emerging zoonotic pathogen primarily in industrialized countries that causes sporadic outbreaks of severe disease in humans. The most important are haemorrhagic colitis (HC), and haemolytic uremic syndrome (HUS). The chromosomal locus of enterocyte effacement (LEE) pathogenicity island encodes a type III secretion system (TTSS) that translocates multiple effector proteins into host cells. TTSS and the multiple effectors are important colonisation factors and therefore presents as potential targets for vaccine interventions. In this study, I have tried to further our understanding of the role of EspF, a proline-rich repeat (PPRRs) effector protein, in EHEC and EPEC pathogenesis, focusing on allelic variants between strains.

I have studied the role of EspF in the interaction of EHEC and EPEC strains with murine macrophages and human-derived M cells. From this work, it was demonstrated that in comparison to $EspF_{O127}$ and $EspF_{O26}$, the $EspF_{O157}$ variant was significantly less effective at inhibiting phagocytosis and also had a reduced capacity to inhibit *E. coli* translocation through a human-derived in vitro M-cell co-culture system. One explanation for this might be an increased ability of both the EPEC O127 and EHEC O26 EspF variants to interact with Human Sorting Nexin-9 (SNX9) and neuronal Wiskott - Aldrich syndrome protein (N-WASP) that are known to play an important role in endocytosis and phagocytosis. However, these interactions are unlikely to account for the finding that the EHEC O157 EspF was the most effective variant at restricting bacterial uptake into and translocation through primary epithelial cells cultured from the bovine terminal rectum, the predominant colonisation site of EHEC O157 in cattle and a site containing M-like cells. As there are no significant differences in the sequences of these two eukaryotic proteins, SNX9 and N-WASP, between human and bovine hosts, it is possible that other unknown interactions with bovine host cell proteins may play a role in phagocytosis

and endocytosis and that these may differ between host species leading to specific evolution of the $EspF_{O157}$ variant for function in cattle.

To examine other possible interactions, Y2H screening with a human expression library was initiated in collaboration with Prof. Juergen Haas at the University of Edinburgh. The results from this study revealed that EspF may interact with both MAD2L2 and NFIC and future work needs to examine how important such interactions could be for *E. coli* pathogenesis. It is known that the *Shigella* effector protein, IpaB, interacts with MAD2L2 the (Mitotic spindle assembly checkpoint protein MAD2B). MAD2L2 normally interacts with an anaphase-promoting complex/cyclosome (APC) to progress the cell cycle normally as MAD2L2 inhibits APC functions. IpaB binding to MAD2L2 in this case APC will stop the cell cycle and prevent epithelial cell turnover. As cellular turnover is a key way that bacteria can be removed from the epithelium, then this activity will promote bacterial colonisation. I anticipate that EspF, through its interaction with MAD2L2, may have a similar function so future work should be aimed at understanding the biological importance of this interaction. Additional future work could involve the generation of a protein expression library from bovine intestinal epithelial cells and a study of the interaction of these bovine host proteins with EspF; with follow up studies on any positive interactions. This work would further define the molecular events preventing EHEC uptake into bovine M cells.

Another key question is how important is this inhibitory activity to the colonisation of cattle; is colonisation of the terminal rectum predominately driven by the initial interaction of EHEC O157:H7 with M-cells. Testing of an EHEC O157 *espF* mutant should be carried out in cattle. This was to be part of my PhD but time, administrative (GMO permission) and financial restrictions have prevented this.

The next area to consider if EspF is shown to be critical is the value of its inclusion in vaccine preparations to limit excretion of EHEC O157 from cattle. An effective vaccine is still required for use in cattle and this would limit financial loses in the cattle industry and protect public health.

The second key part of my research was about antigen sampling M-cells; an important component of the intestinal mucosa immune system. The number of these cells has been shown to be increased when epithelial cells are challenged with number of micro-organisms including *S*. Typhimurium. However no one has studied the mechanisms underlying this differentiation leading to an increase in M cell numbers. So in this study I aimed to study bacterial factors that my promote such a phenomena. Specifically, I focused on pathways central to cell differentiation which I speculated to be modulated by *S*. Typhimurium. One of the salient findings was that *S*. Typhimurium effector protein SopB activates the Wnt pathway which leads to cytosolic stabilization of β-catenin. The stabilized β-catenin accumulates in the cytoplasm and translocates to the nucleus and acts as a co-factor for TCF/LEF transcription factor. Once β-catenin binds to TCF/LEF it activates the transcription of Wnt pathway proteins including Slug and Vimentin. Slug also acts as a transcription factor that induces transcription of vimentin and represses the transcription of E-Cadherin, a marker typical of epithelium. This leads to the transformation of the cells from epithelium to mesenchymal. It is known that Vimentin is a marker of M cells in rabbits and bovine species. Expression of vimentin is a co-relate of EMT as well as a marker for M cells across species including rabbits and bovine. Increased expression of vimentin following bacterial challenge, therefore, perhaps represents the cellular transformation of epithelial cells in to M cell phenotype in an EMT signalling dependent manner. Associated with this, I have shown that the cells expressing vimentin in bovine intestinal epithelial primaries have an uptake function similar to M cells.

It is know that RANKL (Receptor activator of nuclear factor kappa-B ligand) is a protein that can induce M cell 'transformation'. My results have shown that *S.* Typhimurium effector protein SopB increased the expression of RANKL at both the transcriptional and protein levels. RANKL induced the transformation of epithelial cells to the M cell phenotype in bovine terminal rectal primaries through the activation of the NF-kB pathway, in particular through the RelB transcription factor. This stimulates the transcription of the transcription factor Slug that stimulates the transcription of Vimentin, the M cell phenotype marker.

Taken together EspF is very important protein for *E. coli* O157 colonisation at the bovine terminal rectum that prevents its up takes by M cell. *Salmonella* effector protein SopB plays a crucial role in converting follicular associated epithelium to the antigen transporting M cell phenotype and thereby promoting bacterial translocation across gut. The future work will investigate if any of the EHEC effector proteins have a function identical to SopB and thereby could induce transformation of epithelial cells.

## 7.1 Concluding remarks

The $espF_{O157}$ allele was significantly less effective at inhibiting phagocytosis and also had reduced capacity to inhibit *E. coli* translocation through a human-derived in vitro M-cell co-culture system in comparison to $espF_{O127}$ and $espF_{O26}$. In contrast, espFO157 was the most effective allele at restricting bacterial uptake into and translocation through primary epithelial cells cultured from the bovine terminal rectum, the predominant colonisation site of EHEC O157 in cattle and a site containing M-like cells. Yeast-2-hybrid screening was used to identify additional host proteins that may interact with EspF. The anaphase promoting complex inhibitor, Mad2L2, and nuclear factor IC (NFIC) was identified from this screen.

The work focused on bovine rectal primary cell cultures interacting with *Salmonella enterica* serovar Typhimurium. The type III secreted protein, SopB, was required for Salmonella to: (i) activate parts of epithelial to mesenchymal transition (EMT) pathway; (ii) transform a subset of epithelial cells to a cell type that phenotypically and functionally resembles specialized antigen sampling M cells; (iii) induce RANKL and downstream RelB dependent NFκB signalling. The work suggests that Salmonella may induce this cellular transformation to promote its invasion and colonization of intestinal mucosa.

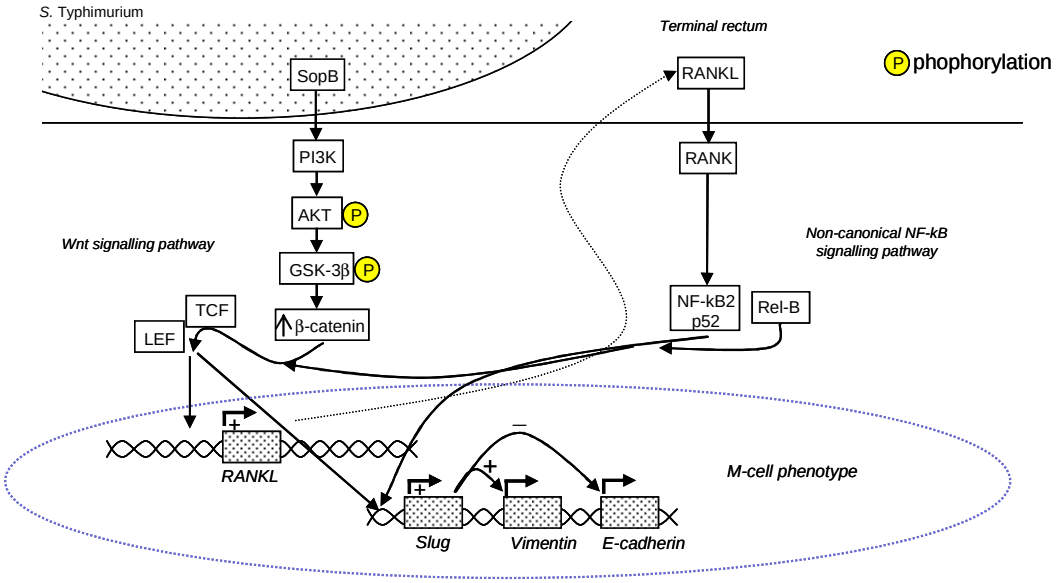


**Fig. 7.1 Schematic diagram to define role of *S.* Typhimurium dependent activation of Wnt/β-catenin pathway in induction of cellular differentiation.** *S.* Typhimurium in a SopB dependent manner activates Akt kinase (phosphorylation), that phosphorylates and inhibits GSK-3β, hence the increased cytosolic β-catenin. The free β-catenin binds to members of the T-cell factor (TCF) family of transcription factors (including LEF-1), then translocates to the cell nucleus and regulates transcription of RANKL, Slug, vimentin and E-cadherin. SopB dependent induction of Wnt/β-catenin pathway is critical to trans-differentiation of epithelial cells in to M cell phenotype in the bovine terminal rectal "Follicle Associated Crypt (FACs)" - epithelial cells. It works by induction of RANKL that in a RelB dependent manner triggers Slug and the downstream cellular trans-differentiation. In the FACs- epithelial cells the Wnt/β-catenin dependent changes in Slug expression and the enhanced RANKL expression acts in synergy to induce cellular transdifferentiation. In the bovine proximal rectal "Ordinary Crypt" epithelial cells in absence of RelB/ RANKL signalling the Wnt/b catenin mediated Slug activation of its own is not sufficient enough to induce the cellular trans-differentiation.

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