Investigations on how the enteropathogenic *Escherichia coli* (EPEC) EspF effector inhibits PI-3 kinase-dependent phagocytosis

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Abstract

Enteropathogenic *E. coli* inhibits phosphoinositide 3 (PI-3) kinase dependent phagocytosis via a Type Three Secretion System (T3SS) that delivers up to twenty effector proteins into target cells. The T3SS components are encoded on the Locus of Enterocyte Effacement (LEE) pathogenicity island alongside genes for a surface protein, Intimin, and seven effectors (Tir, Map, EspF, EspG, EspZ, EspH and EspB; latter also needed to deliver effectors). Inhibiting phagocytosis is linked to EspB, EspH, EspG and EspF activities with inhibitory mechanisms described for all except EspF. The aim of this study was to determine if EspF alone could inhibit phagocytosis and define the inhibitory mechanism.

Initial anti-phagocytosis studies, with J774A.1 macrophages, not only confirmed EspF and T3SS-dependent inhibition but suggested a T3SS-independent contribution. Moreover, studies with effectors-deficient EPEC and non-pathogenic *E. coli* carrying LEE on a plasmid argued for LEE sufficiency. Surprisingly, delivering EspF into macrophages without most (EPEC multi-mutant) or all (via T3SS of another pathogen, *Yersinia*) other EPEC effectors argued against EspF sufficiency. Interestingly, the data also argued against EspG driving the anti-phagocytosis process but suggested that EspF's contribution required Map, EspH, Tir, and/or the Intimin activities. Surprisingly, screening EspF/Map/EspH/Tir/Intimin single, double, triple, quadruple and quintuple mutants failed to confirm the critical roles for EspF or EspH that are linked to phenotypic instability and/or indirect contributions. Preliminary investigations on EspF's involvement revealed possible features and domains required for its efficient expression, secretion and/or inhibiting phagocytosis. Crucially, the screening data provided many hypotheses including Tir and Intimin being able to drive T3SS-dependent anti-phagocytosis, an idea supported by complementation studies. Collectively, this study provides important new insights on EPEC's ability to inhibit its uptake by J774A.1 macrophages and reveals unknown levels of complexity.

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Abbreviations

A/E	Attaching/Effacing
Arp2/3	Actin related protein 2/3
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BFP	Bundle Forming Pilus
Cb	Carbenicillin
Cm	Chloramphenicol
CR3	Complement receptor 3
DAPI	4’,6-diamidino-2-phenylindole
DMEM	Dulbecco’s minimal eagles medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EPEC	Enteropathogenic *Escherichia coli*
E2348/69	EPEC strain E2348/69
EAF	EPEC adherence factor
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EHEC	Enterohemorrhagic *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
Esc	EPEC secretion
Esp	EPEC secreted/signalling protein
FCS	Foetal Calf Serum
GEF	Guanine-nucleotide Exchange Factor
GFP	Green fluorescent protein
GTP	Guanosine triphosphate
h	Hour
ITAM	Immune receptor tyrosine based activation motifs

ITIM	Immune receptor tyrosine based inhibition motifs
Km	Kanamycin
LB	Luria-Bertani
LEE	Locus of enterocyte effacement
LPS	Lipopolysaccharide
M-cells	Microfold cells
Map	Mitochondrial associated protein
Min	Minute
MOI	Multiplicity of infection
NaCl	Sodium chloride
Nal	Nalidixic acid
NF-κB	Nuclear factor-kappa B
Nle	Non-LEE encoded
N-WASP	Neural Wiskott-Aldrich syndrome protein
OD600	Optical density 600 nm
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerised chain reaction
PFA	Paraformaldehyde
PH	Pleckstrin homology
PI	Phosphoinositide
PI-3K	Phosphoinositide 3-kinase
PI[4,5]P2	Phosphatidylinositol 4,5-bisphosphate
PI[3,4]P2	Phosphatidylinositol 3,4-bisphosphate
PI[3,4,5]P3	Phosphatidylinositol 3,4,5-trisphosphate
PMSF	Phenylmethane sulfonyl fluoride
RPEC	Rabbit pathogenic *E. coli*
RT	Room temperature

SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Ser	Serine
SHIP2	SH2 domain containing inositol 5-phosphatase
SH2	Src homology 2
SNX9	Sorting nexin 9
SOC	Super optimal broth with catabolite repression
Tet	Tetracycline
TJ	Tight junctions
Tir	Translocated intimin receptor
TLR	Toll-like receptor
Tyr	Tyrosine
T3SS	Type 3 secretion system
UPEC	Uropathogenic *Escherichia coli*
WT	Wild type
WAVE	WASP and verprolin homolog

Chapter 1. Introduction

1.1 Gastrointestinal tract

The gastrointestinal tract is a complex organ which has many important functions including the digestion and absorption of nutrients (Vereecke *et al.*, 2011). The gastrointestinal tract can be divided into two main regions: the small intestine, from which 95% of nutrients are absorbed, and the large intestine which is primarily responsible for the absorption of most of the remaining water in the gut lumen (Vereecke *et al.*, 2011). The gastrointestinal epithelium consists of a monolayer of cells composed of goblet (mucus-secreting), enteroendocrine (hormone-secreting), paneth (antimicrobial peptide-secreting) and microfold (M) cells (antigen-sampling) as well as enterocytes (absorptive cells) (Abreu, 2010; Vereecke *et al.*, 2011). Enterocytes are the dominant cell type (~80%) of the small intestinal cell lining (Abreu, 2010) that have two distinct surfaces- apical and basolateral (Figure 1) (Snoeck *et al.*, 2005; Abreu, 2010). The apical membrane is distinguished by the presence of microvilli (Snoeck *et al.*, 2005; Abreu, 2010). Microvilli increase the absorptive surface area and facilitate the uptake of nutrients, ions and fluid (Halbleib *et al.*, 2007). Microvilli are covered by large, negatively charged, mucin-like glycoproteins (glycocalyx) that are integrated in the membrane (Snoeck *et al.*, 2005; Abreu, 2010).

The glycocalyx contains adsorbed pancreatic enzymes thereby providing a highly degradative microenvironment that promotes nutrient absorption. It also hinders macro-particles (for example gut micro-organisms and antigens) from accessing the epithelia (Snoeck *et al.*, 2005). The basolateral membrane also has distinct features including specific proteins to mediate cell-substratum interactions or transport absorbed nutrients into the blood. The basolateral membrane is enriched in antigen-sensing receptors which trigger an immune response against foreign antigens that penetrate across the epithelia (Abreu, 2010). All types of gastrointestinal epithelial cells are connected by tight junctions (TJ) (Abreu, 2010; Vereecke *et al.*, 2011) (Figure1). These belt-like structures have two main functions, a dynamic barrier function that regulates paracellular movement of molecules between adjacent cells. In addition, TJ have a fence function to block intermixing of molecules between the apical and basolateral membranes, thus maintaining the polarised nature of the epithelium (Tsukita *et al.*, 2008; Zihni *et al.*, 2016). Given the key role of the tight junctions and microvilli in fluid, ion and nutrient uptake, disruption of these structures by pathogenic micro-organisms can lead to diarrheal diseases (Guttman and Finlay, 2009; Thanabalasuriar *et al.*, 2010; Singh and Aijaz, 2015). As the tightly-packed microvilli and the thick glycocalyx are effective barriers to protect enterocytes against micro-organisms, some pathogens target M-cells which have a reduced

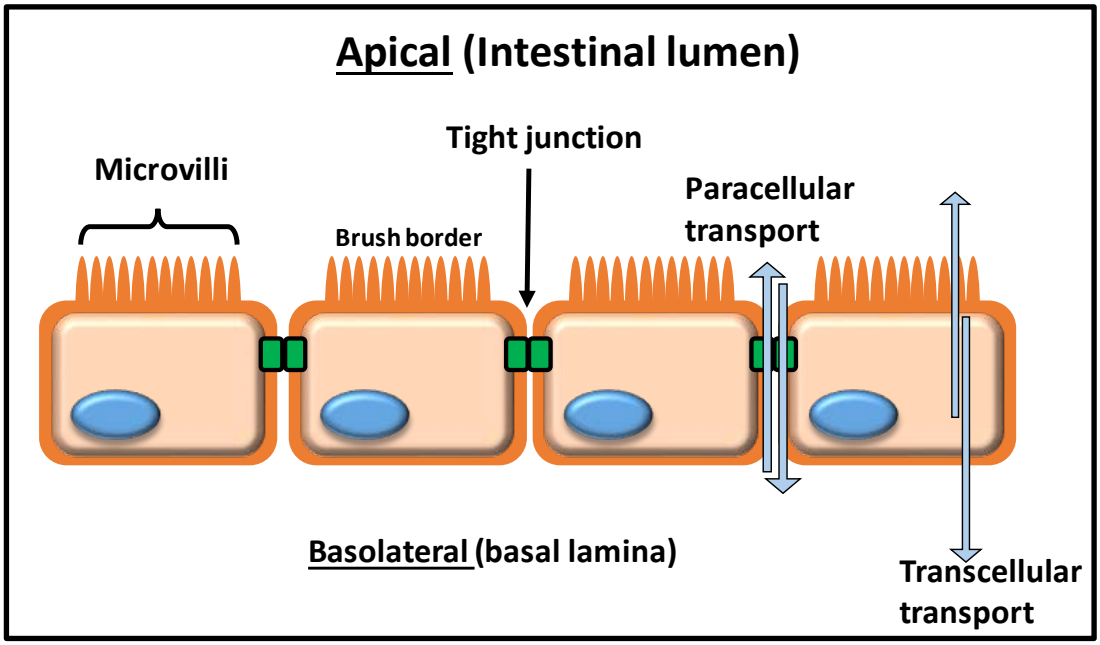


Figure 1. Schematic representation of an enterocyte. Enterocytes are composed of two discreet membrane regions: the apical membrane also known as brush-border, increases the absorption and secretory capacity of epithelial cells and protects against invading pathogens. The brush-border is made up of a number of finger like projections, named microvilli. The second membrane region is the basolateral region. Epithelial cells are connected by belt-like structures called tight junctions, which have two main function, a dynamic barrier function that regulates paracellular movement of molecules between adjacent cells and a fence function to block intermixing of molecules between the apical and basolateral membranes, thus maintaining the polarised nature of the epithelium.

number of microvilli (Mabbott *et al.*, 2013). Moreover, M-cells actively sample gut content and present it to immune cells located at the basolateral surface via transcellular vesicular trafficking. M-cells transport antigens without modifying their antigenic properties (Miller *et al.*, 2007; Mabbott *et al.*, 2013). M-cells are mostly clustered in Peyer's patches, dome-like structures associated with focal centres of immune cells, that display reduced glycocalyx covering to aid antigen sampling (Miller *et al.*, 2007; Mabbott *et al.*, 2013).

1.2 *Escherichia coli* (EPEC) in gut health and disease

E. coli is a gram negative, facultative anaerobic, commensal bacterium of the human and mammalian intestine. *E. coli* has been widely studied and utilized as a host for general molecular biology techniques in laboratory science as it can be easily grown and genetically manipulated. Although *E. coli* forms part of the normal gut flora, commensal strains can be transformed into human pathogens by acquiring genetically mobile material. These acquired virulence elements promote the adaptation to a new lifestyle and enable the bacteria to cause diseases such as gastrointestinal disease, urinary tract infection, septicaemia and meningitis (Kaper *et al.*, 2004; Greenwood *et al.*, 2012). Traditionally, *E. coli* has been divided into eight pathogenic groups which cause diarrhoeal disease using different mechanisms. These include six long-established pathotypes: enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC), as well as two more recently identified pathotypes: adherent invasive *E. coli* (AIEC) and Shiga toxin (Stx) producing enteroaggregative *E. coli* (STEAEC) (Table 1)(Croxen and Finlay, 2010; Croxen *et al.*, 2013).

1.3 Enteropathogenic *Escherichia coli*

EPEC is a non-invasive bacteria which was first linked to enteric disease in studies of hospitalised infants in the UK and USA during the 1940s and 1950s (Bray, 1945). However, enteric disease is now rare in developed countries but remains a major cause of infant morbidity in developing countries, causing profuse diarrhoea, often with fever and vomiting (Greenwood *et al.*, 2012). Comprehending the strategies which pathogenic *E. coli* strains such as EPEC use to cause disease is of increasing importance due to approximately 526 000 diarrhoea related deaths every year (Bhutta and Das, 2013; UNICEF, 2016). In spite of the significant advances in the understanding of EPEC pathogenesis, the precise molecular mechanism causing watery diarrhoea is not completely understood (Singh and Aijaz, 2015).

Pathogen name	Site of infection	Disease symptoms
Enteropathogenic *E. coli* (EPEC)	Small intestine	Watery diarrhoea
Enterotoxigenic *E. coli* (ETEC)		Watery diarrhoea
Diffusely adherent *E. coli* (DAEC)		Watery diarrhoea
Enteroaggregative *E. coli* (EAEC)	Small and large intestine	Watery diarrhoea, can be bloody
Enterohemorrhagic *E. coli* (EHEC)	Large intestine	Bloody diarrhoea, may lead to haemolytic uremic syndrome
Enteroinvasive *E. coli* (EIEC)		Bloody diarrhoea and bacillary dysentery
Uropathogenic *E. coli* (UPEC)	Urinary tract and kidneys	Urinary tract infections and acute pyelonephritis in the kidneys
Neonatal meningitis *E. coli* (NMEC)	Gastrointestinal tract and can cross blood-brain barrier	Meningitis

Table 1. List of the main pathogenic *E. coli*. This table illustrate the strains name and their site of infection and disease symptoms (Croxen and Finlay, 2010).

1.3.1 Attaching/Effacing phenotype and Locus of Enterocyte Effacement (LEE) region

EPEC is a member of the attaching and effacing (A/E) family of pathogens, which also includes the human pathogen enterohemorrhagic *E. coli* (EHEC), the mouse pathogen *Citrobacter rodentium* (CR) and rabbit pathogenic *E. coli* (RPEC). This family of A/E pathogens promotes the formation of characteristic histopathological lesions (Knutton *et al.*, 1989). These intestinal lesions are caused by intimate attachment of the microbes to the host plasma membrane followed by the dissolution of microvilli of the intestinal epithelial cells. The latter process is known as effacement (Moon *et al.*, 1983; Knutton *et al.*, 1989). Considerable cytoskeletal changes lead to the formation of pedestals consisting of polymerized actin beneath adherent bacteria (Figure 2) (Knutton *et al.*, 1989). Previous studies have suggested that the pedestal structure is critical for full virulence in both animal and human models and also enhances the colonization of bacteria, however, the precise role of pedestals remains incompletely understood (Donnenberg *et al.*, 1993a; Deng *et al.*, 2003; Ritchie *et al.*, 2003).

The formation of A/E lesions is dependent on the highly conserved 35.6 kb pathogenicity island named the Locus of Enterocyte Effacement (LEE; Figure 3) in the genome of pathogenic bacteria. Introducing the LEE region of the EPEC (E2348/69) strain into commensal *E. coli* K12 strain proved that the LEE region possess all the essential genes which are required to induce A/E lesions (McDaniel and Kaper, 1997). The LEE region of EPEC (E2348/69) includes 41 open reading frames (ORFs) organised in five polycistronic operons. The LEE region encodes the proteins that form the type 3 secretion system (T3SS) as well as gene regulators, chaperones, effector proteins and the bacterial outer membrane protein Intimin (McDaniel *et al.*, 1995; Elliott *et al.*, 1998) as discussed below.

1.3.2 Structure and function of T3SS

The T3SS is an important virulence factor for A/E pathogens, due to its role in delivering the effector proteins into host cells (Gaytan *et al.*, 2016). Similar to A/E pathogens, pathogenicity of several other Gram negative bacteria, including *Shigella flexneri* and *Salmonella enterica* is dependent on the delivery of many effector proteins into host cells through T3SS; subverting cellular systems to promote their colonisation (Coburn *et al.*, 2007). The T3SS is a syringe-like structure that transfers effector proteins from the bacterial cytosol directly into target host cells (Gaytan *et al.*, 2016). The proteins which form the T3SS of A/E pathogens are encoded by LEE, a total of around 20 proteins that form a needle-like complex which extends across both the inner and outer bacterial membranes (Figure 4)(Garmendia *et al.*, 2005; Coburn *et al.*, 2007). The last part of the T3SS is known as the translocon, which links the T3SS with the host cell. The translocon consists of three secreted

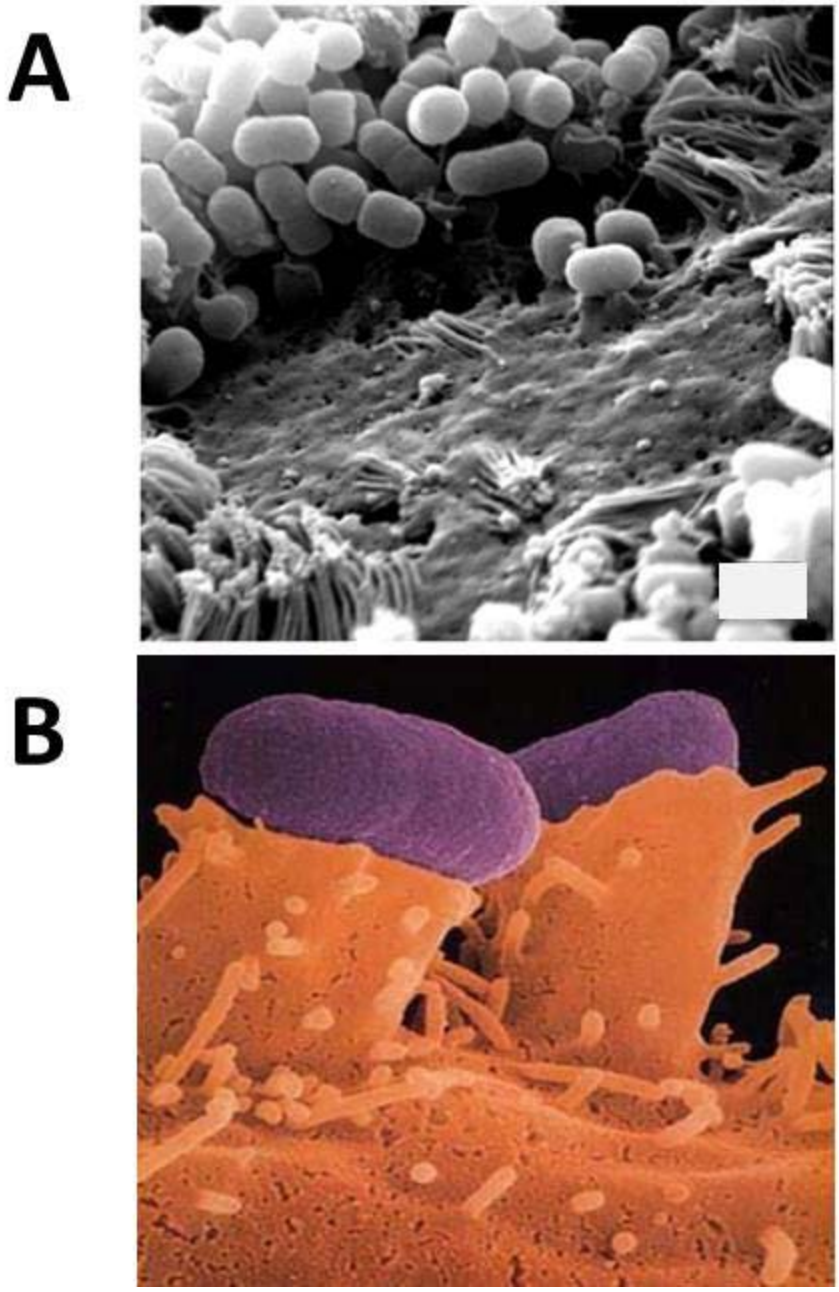


Figure 2. Intimate attachment of EPEC to the host plasma membrane induces effacing lesions and actin rich pedestals. Illustrative images by scanning electron microscopy (SEM) of EPEC induced infection characteristics. (A) The dissolution of microvilli of the intestinal epithelial cells (effacement) is shown after four hours of infection of a Caco-2 cell model with wild type EPEC. Figure taken from Dean *et al*., (Dean *et al*., 2006). (B) The formation of actin-rich pedestals beneath adherent bacteria revealed by SEM, using a HeLa cell model. Taken from Rosenshine *et al*., (Rosenshine *et al*., 1996b).

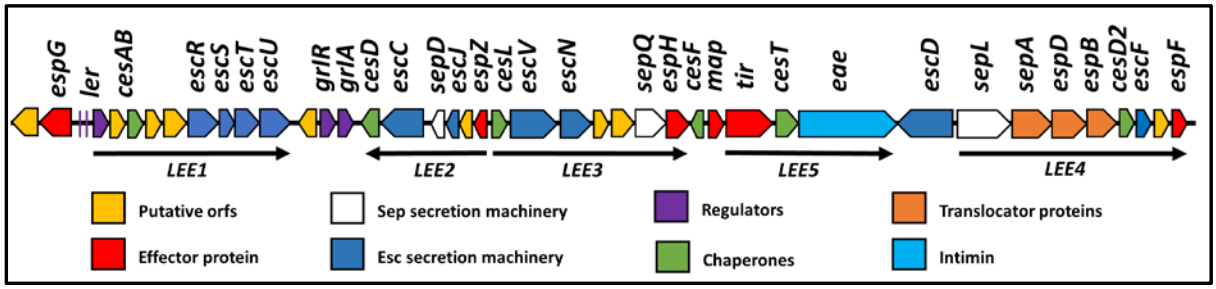


Figure 3. Diagram demonstrating the genetic organization of the locus of enterocyte effacement (LEE) region from EPEC E2348/69. The locus of enterocyte effacement (LEE) region is a highly conserved 35.6 kb pathogenicity island in the genome of pathogenic bacteria. The LEE region of EPEC (E2348/69) includes 41 open reading frames organised in five polycistronic operons indicated LEE1-LEE5. The LEE region encodes proteins which form the T3SS including Esc (Blue) and Sep (White), regulatory factors (Ler, GrlA, GrlR, and Mpc; Purple), chaperones (Green), translocator proteins (Orange), effector proteins (Esp; Red; that include Tir & Map), the bacterial outer membrane protein Intimin (*eae*; sky Blue) and other putative open reading frames (Orf; Yellow). Adapted from Wong *et al.*,(Wong *et al*., 2011).

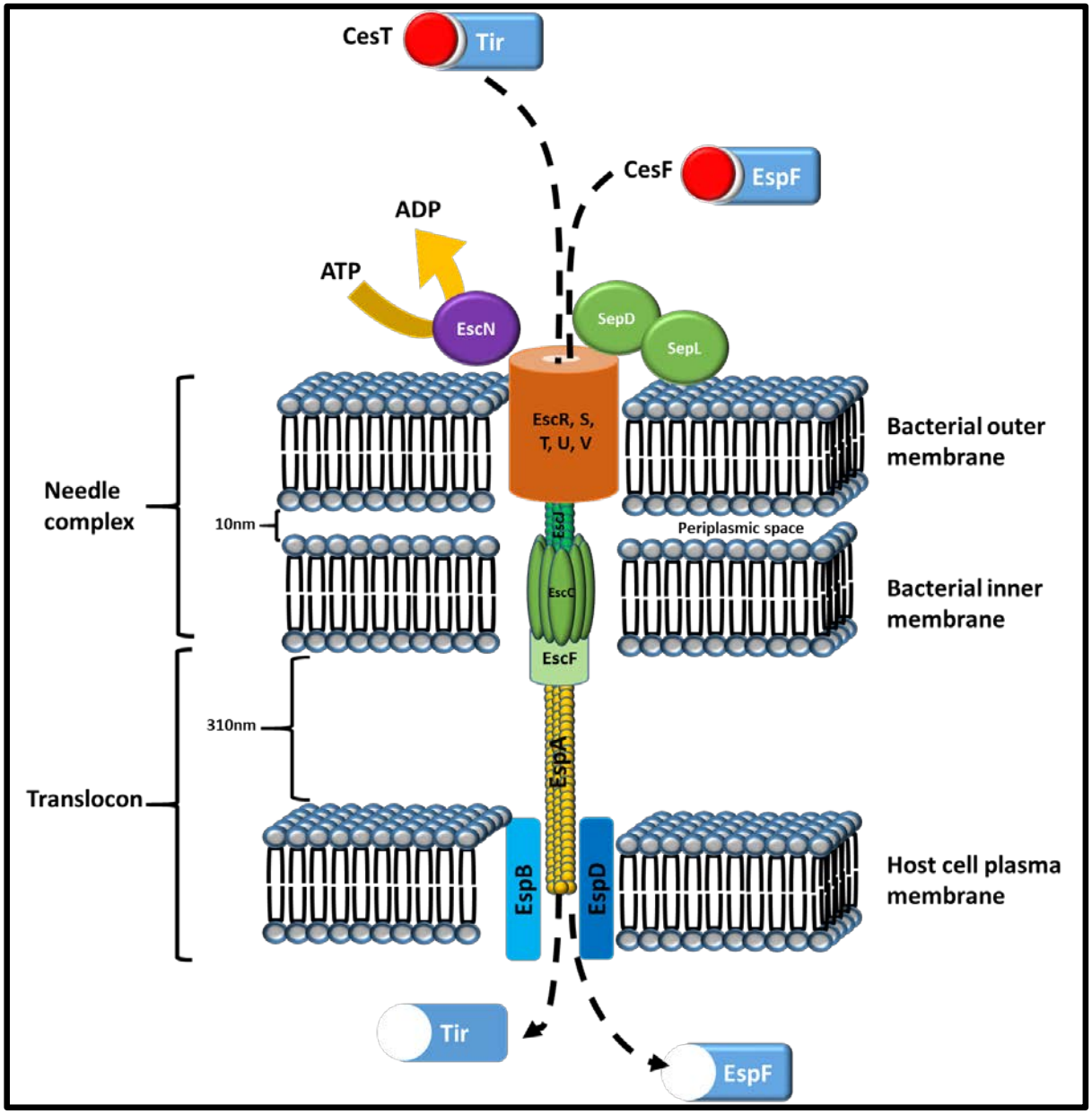


Figure 4. Schematic of the EPEC T3SS. The T3SS is a multi-complex apparatus, a total of around 20 proteins which form a needle-like complex. The proteins that forms T3SS are Esc and Sep. Esc traverses the inner membrane loop which is composed of spanning proteins EscR, EscS, EscT, EscU and EscV as well as EscJ, spanning the periplasmic space and the external layer ring which is formed from the subunit EscC. The secretion and polymerisation of the subunit EscF forms the external needle known as translocon which links the T3SS with the host cell. The translocon consists of three secreted proteins: EspA which forms a long filamentous structure, and membrane inserted proteins EspB and EspD which from a translocation pore within the host cell membrane to deliver of effector proteins (for example Tir and EspF) directly into the host cell cytosol. The energy to release these effector proteins is provided by the ATPase, EscN. The switch secretion of translocator proteins to the secretion of EPEC effectors is regulated by proteins SepD, SepL and SepQ via the T3SS. The efficient translocation is dependent on chaperone such as CesF and CesT, however, EspG, NleD and NleF are translocated independently of both chaperones. Adapted from Garmendia *et al.*, (Garmendia *et al.*, 2005).

proteins; EspA, EspB and EspD, that are also essential for EPEC virulence (Donnenberg *et al.*, 1993b; Kenny *et al.*, 1996; Frankel *et al.*, 1998; Deng *et al.*, 2004). The EspA protein polymerises at the end of the T3SS to form a filamentous extension that links the bacteria to the membrane of the host target where EspB and EspD insert to form a protein-delivery pore in the host membrane (Frankel *et al.*, 1998; Knutton *et al.*, 1998; Hartland *et al.*, 2000; Daniell *et al.*, 2001; Luo and Donnenberg, 2011). When the secretion component of the translocon is completed the bacteria can then begin to inject its effector proteins into target cells. Thus, the T3SS and translocator system form a continuous conduit that is critical for successful translocation and transferring a suite of 'effector' proteins into gut epithelia to subvert cellular processes in a manner that promotes the bacteria's lifecycle (Coburn *et al.*, 2007; Gaytan *et al.*, 2016).

1.3.3 EPEC effector proteins (LEE and Nle)

The LEE region encodes seven identified effector proteins, Tir, Map, EspF, EspG, EspZ, EspH and EspB as well as the outer membrane protein Intimin. In addition, the LEE region encodes three translocator proteins (EspA, EspB and EspD). The secreted proteins that form the translocon have already been discussed. The EspB translocator is also known to have effector functions as it is also found in the host cytoplasm and interacts with host proteins to subvert cellular processes (Taylor *et al.*, 1998; Iizumi *et al.*, 2007).

The first A/E effector to be delivered is named Tir, translocated Intimin receptor. Tir is inserted into the host membrane where it acts as a receptor for Intimin to mediate intimate host-pathogen interactions and to induce pedestal formation (Kenny *et al.*, 1997b). The insertion of Tir into the host membrane is linked to distinct shifts in apparent molecular mass leading to the T' and T'' forms, due to the addition of phosphate groups onto serine residues by host kinases, followed by additional phosphorylation (a tyrosine phosphorylation mediated by host tyrosine kinases) of T'' producing the T''pY form (Kenny and Warawa, 2001; Phillips *et al.*, 2004; Swimm *et al.*, 2004). Intimin interaction with T''pY triggers the recruitment of the Nck adapter protein, which in turn leads to sequestration of the neural Wiskott–Aldrich syndrome protein (N-WASP) and activation of the Arp2/3 actin-nucleating machinery to produce pedestals (Campellone *et al.*, 2002; Lai *et al.*, 2013). In contrast, Tir from EHEC O157:H7 does not employ this mechanism but uses a second effector, Tccp (Tir cytoskeleton-coupling protein), also known as EspFu, which directly activates N-WASP and the insulin receptor tyrosine kinase substrate (IRTKS) that triggers pedestal formation (Campellone *et al.*, 2004b; Vingadassalom *et al.*, 2009). Although Tir-Intimin interaction triggers downstream signalling events leading to

the formation of actin-rich pedestals beneath the adherent bacteria (Kenny *et al.*, 1997b) and causing microvilli effacement (Kenny *et al.*, 1997b; Dean *et al.*, 2006), Tir also has functions independent of Intimin. For example, Tir inhibits signalling pathways that induce anti-microbial and inflammatory responses (Ruchaud-Sparagano *et al.*, 2011). Moreover, Tir induces cell survival through supressing the ability of EspG/EspG2 to promote calpain activity whose function is to induce host cell detachment and cleavage of the host proteins (Dean *et al.*, 2010a).

A second LEE effector, mitochondrial-associated protein (Map) is named after the observation that it targets and alters the function of mitochondria via its N-terminal sequence (Kenny and Jepson, 2000; Kenny, 2001b; Papatheodorou *et al.*, 2006). Map is a guanine nucleotide exchange factor (GEF) that possess a WxxxE motif which activates host Rho GTPase Cdc42 at the cell membrane, leading to filopodia formation during the early stages of EPEC infection (Kenny *et al.*, 2002; Alto *et al.*, 2006; Huang *et al.*, 2009). Activation of Cdc42 leads to the recruitment of N-WASP which triggers Arp2/3-mediated actin nucleation and filopodia formation (Miki *et al.*, 1998; Svitkina *et al.*, 2003; Wong *et al.*, 2011). There is coordinated regulation between Map and other effectors; for example, Tir and Map regulate each other during infection (Kenny *et al.*, 2002). Tir downregulates Map through the downstream target N-WASP and Arp2/3-mediated actin nucleation (Kenny, 2002).

As a consequence, filopodia formation is transient due to a down-regulatory pathway dependent on the expression of Tir or Intimin (Dean *et al.*, 2005). On the other hand, there is cooperativity with other effectors in altering host cell activity illustrated by the coordinated action of Map, Tir, EspF and Intimin to disrupt TJs (Dean and Kenny, 2004). Indeed, Tir and Map work synergistically mediating the uptake of bacteria by HeLa cells (Jepson *et al.*, 2003).

The EspF effector can be detected in several cellular compartments including the cytoplasm, mitochondria, apical and lateral membranes, and it has the ability to interact with at least 12 host cell proteins (Dean and Kenny, 2009; Holmes *et al.*, 2010). EspF, like other LEE effectors, has multifunctional behaviour and induces various specific cellular responses (Dean and Kenny, 2009; Holmes *et al.*, 2010). For instance, targeting mitochondria causes permeabilisation of the mitochondrial membrane and the release of cytochrome *c* from mitochondria into the cytoplasm followed by activation of caspase to trigger apoptosis (Nougayrède and Donnenberg, 2004; Nagai *et al.*, 2005). Furthermore, EspF works synergistically with other effectors, for example EspF and Map decrease mitochondrial membrane potential and change the mitochondrial shape during EPEC infection (Kenny and Jepson, 2000; Nougayrède and Donnenberg, 2004). Moreover, it acts with Map and Intimin to disrupt intestinal barrier function (Dean and Kenny, 2004). In addition, EspF works

cooperatively with three effectors Tir, Map and Intimin (outer membrane protein) to trigger the effacement of absorptive microvilli (Dean *et al.*, 2006).

Like EspF, the EspG effector has multifunctional behaviour, with the major activities associated to actin cytoskeleton remodelling (Glotfelty and Hecht, 2012). EspG is a 398-amino-acid protein that is also delivered into host cells via T3SS (Elliott *et al.*, 2001). EspG displayes 21% amino acids identity with the *Shigella flexneri* effector VirA (Elliott *et al.*, 2001). The latter triggers the destabilization of host microtubule (Elliott *et al.*, 2001). The EspG effector protein has a Nle homologue EspG2 (discussed below) which works together with EspG to disrupt the barrier function (Dean *et al.*, 2010a). However, the presence of Tir suppresses this activity during infection (Dean *et al.*, 2010a). In addition, disintegration of microtubules into tubulin subunits is ascribed to both EspG and EspG2 (Matsuzawa *et al.*, 2004; Shaw *et al.*, 2005b).

Another LEE-encoded effector is EspH; our knowledge about this effector is largely based upon empirical studies that investigated how EspH modulated actin dynamics of the host. Deletion of EspH caused prolonged filopodia formation concomitant with delayed pedestal formation. However, over-expressing of EspH lead to actin polymerization and pedestal elongation (Tu *et al.*, 2003). EspH acts synergistically with Tir to modulate the actin pedestals through recruiting N-WASP activity and the Arp2/3 complex (explained below) (Wong *et al.*, 2012).

EspZ is the smallest LEE encoded effector (98 amino acid). Previous research has indicated that EspZ is involved in virulence during rabbit EPEC infection (Wilbur *et al.*, 2015). EspZ has also been shown to promote host cell survival by interaction with the transmembrane protein CD98, thus stimulating ß1-integrin/FAK mediated host survival signals (Shames *et al.*, 2010). EspZ plays an important role in limiting cell death through inhibition of the apoptotic pathway (Roxas *et al.*, 2012). It also interacts with the mitochondrial membrane translocase Tim17b, thus avoiding the release of cytochrome c and stabilising the mitochondrial membrane potential (Shames *et al.*, 2010; Roxas *et al.*, 2012). In order to regulate effector protein translocation into host cells by T3SS, EspZ interacts with EspD in the membrane pore and regulates the balance of pro- and anti-apoptotic effectors, consequently promoting host cell survival (Berger *et al.*, 2012).

EPEC also possesses additional effectors encoded outside the LEE region known as non-LEE (Nle) effectors, although some of these proteins retain the ‘Esp’ nomenclature. Up to seventeen known Nle effector proteins are encoded on prophages (PP) and integrative elements (IE) (Figure 5). Previous research has demonstrated that LEE and Nle effector proteins work

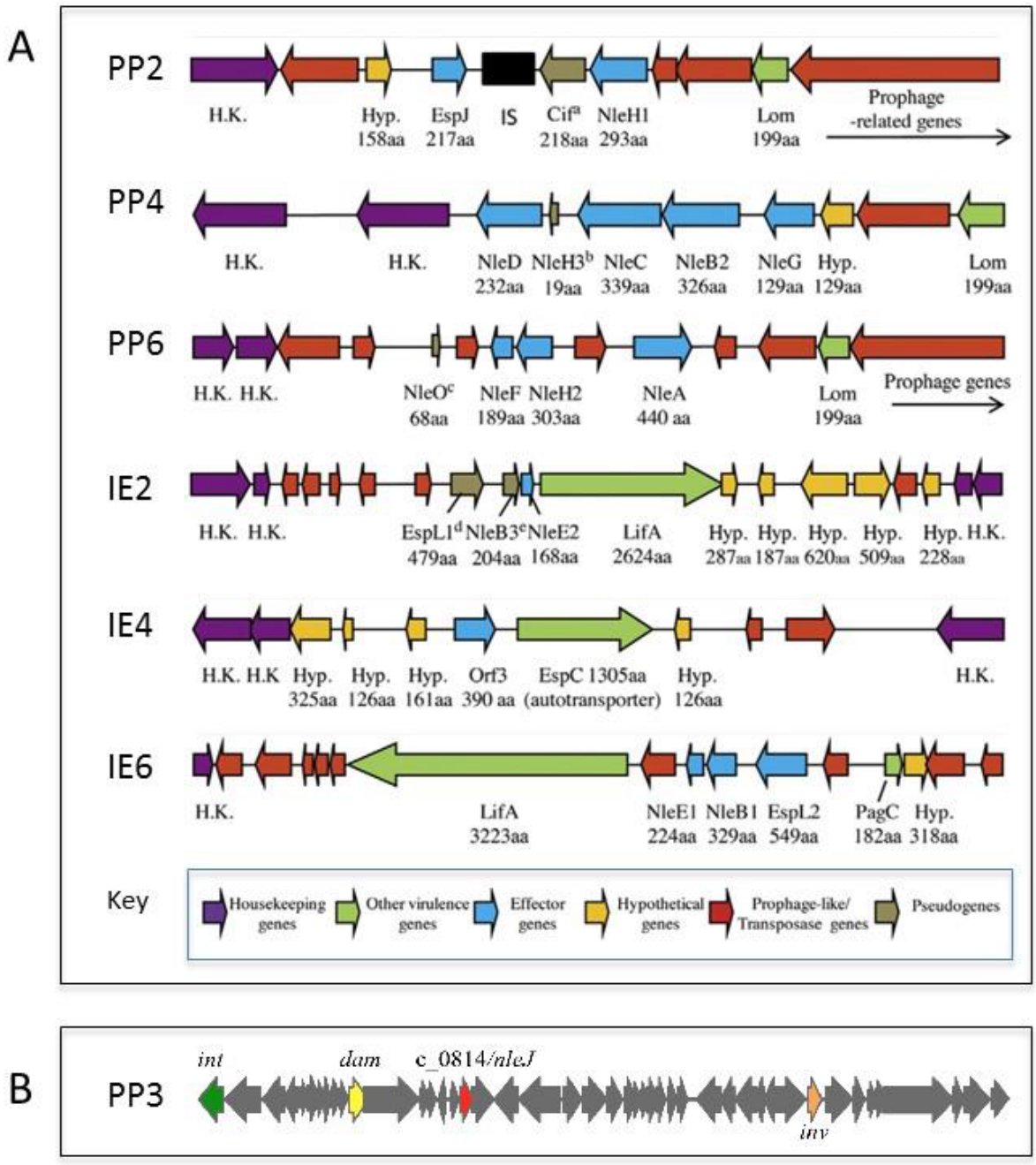


Figure 5. Schematic of the non-LEE effector encoding pathogenicity islands. EPEC possesses seventeen known effectors encoded outside the LEE region known as non-LEE (Nle), although some of these proteins retain the 'Esp' nomenclature. These effector proteins are encoded on three integrative elements (IE2, IE5 & IE6) and four prophages (PP2, PP3, PP4 & PP6). The direction of the strand and genes are indicated using distinct arrows. (A) Demonstrating the fourteen effector proteins (Blue) encoded by the pathogenicity islands IE3, IE5, IE6, PP2, PP4 and PP6 pathogenicity islands; moreover effectors LifA (IE6; Green) and EspC (IE4; Green). Taken from Dean and Kenny (Dean and Kenny, 2009). (B) Diagram demonstrating the prophage, PP3, encoding the NleJ effector (Red). Taken from Deng *et al.*, (Deng *et al.*, 2012).

antagonistically or synergistically to subvert several host cellular activities. For example LEE encoded Tir as well as NleB, NleC and NleE are associated with disrupting the inflammatory signalling pathway NF-κB (Nadler *et al.*, 2010; Newton *et al.*, 2010; Pearson *et al.*, 2011; Ruchaud-Sparagano *et al.*, 2011; Wong *et al.*, 2011). In addition, a number of Nle effectors including NleD delay host cell apoptosis by targeting c-Jun N-terminal kinase (JNK) and MAP kinase which subsequently inactivate the transcription factor AP-1, involved in inflammation, apoptosis and cell differentiation (Baruch *et al.*, 2011). Both NleH1 and NleH2 delay host cell apoptosis through targeting the anti-apoptosis protein Bax-inhibitor1 (Hemrajani *et al.*, 2010). NleF also has anti-apoptotic effects as it inhibits caspase activity (Blasche *et al.*, 2013). Additionally, the EPEC Nle effector protein EspJ was found to inhibit opsonic phagocytosis including both IgG and iC3b pathways. This is likely mediated through its capacity to inhibit Src family kinases (SFK), which block actin-driven opsono-phagocytosis (Marchès *et al.*, 2008; Young *et al.*, 2014).

1.1.4 Chaperones

The delivery of most LEE and Nle effectors through T3SS depends on chaperones encoded in the LEE region such as CesF and CesT (Elliott *et al.*, 1999; Elliott *et al.*, 2002; Thomas *et al.*, 2005). The main functions of the chaperones are (I) to stabilise the effector proteins within the bacterial cytosol, (II) to assist in trafficking/docking with the T3SS at the inner membrane and (III) to regulate gene expression. In addition, the chaperones are critical for efficient secretion of virulence proteins (Elliott *et al.*, 1999; Elliott *et al.*, 2002; Creasey *et al.*, 2003; Thomas *et al.*, 2005). It has been demonstrated that CesF is essential for only EspF intra-bacterial stability and T3SS delivery (Elliott *et al.*, 2002). In contrast, CesT is essential for the delivery of most LEE effectors (Tir, Map, EspH, EspZ). Indeed, Nle are either fully dependent (NleB2, NleG, EspJ, NleH1, NleH2) or only partially dependent (NleB1; NleC) on the chaperone CesT. However, some effectors such as EspG, NleD and NleF have no CesF/CesT dependence (Thomas *et al.*, 2005; Mills *et al.*, 2013).

1.3.5 EPEC pathogenicity

The transmission of EPEC mainly occurs via the fecal-oral contact with contaminated drink or foods (Julian, 2016). When bacteria reach the small digestive system, the pathogenicity of EPEC infection is characterised by four stages: The first stage of infection involves EPEC binding to epithelia via the Bundle Forming Pilus (BFP) (Giron *et al.*, 1991), which is encoded on the EPEC plasmid known as EPEC adherent factor (EAF) (Levine *et al.*, 1985; Jarvis *et al.*, 1995). It has been demonstrated that BFP retraction promotes EPEC aggregation to form microcolonies and promotes efficient translocation of bacterial effector proteins into host cells

(Giron *et al.*, 1991; Zahavi *et al.*, 2011). The formation of the BFP allows interaction with other EPEC bacteria, forming characteristic EPEC micro-colonies, which are termed 'localised adherence pattern' (Baldini *et al.*, 1983; Knutton *et al.*, 1987; Frankel *et al.*, 1998). Noticeably, the expression of BFP is coordinated by a collection of regulatory genes, encoded by EAF, known as plasmid encoded regulator genes A, B and C (*perA*, *perB* & *perC*). Plasmid encoded regulators have also been shown to influence other critical virulence factors including the T3SS via the LEE encoded regulator (LER) (Mellies *et al.*, 1999). Nevertheless, some EPEC strains known as atypical EPEC strains do not display a localised adherence pattern as they lack the EAF plasmid. Atypical EPEC strains still have the ability to form A/E lesions, though with significantly delayed lesion formation; consequently these strains are less likely to cause human diarrheal disease (Levine *et al.*, 1985; Chen and Frankel, 2005; Bueris *et al.*, 2015). The second stage of infection involves T3SS mediated delivery of effector proteins into host cells, which cause activation of cell signalling pathways, actin polymerisation and cytoskeletal rearrangement (Figure 6)(Hodges and Gill, 2010; Croxen *et al.*, 2013). The defining features of the last two stages of infection are the diarrhoea-associated events of losing absorptive microvilli and the formation of actin-rich pedestal-like structures beneath the adherent bacteria (Clarke *et al.*, 2003; Kaper *et al.*, 2004; Hodges and Gill, 2010). Additional diarrhoeal-associated events include altering the function of apical transporters before the effacement process and disrupting barrier function by altering tight junction associated cell-cell interactions (Hodges and Gill, 2010; Singh and Aijaz, 2015).

1.4. Phagocytosis

There are several strategies that cells utilize to internalise molecules: phagocytosis, micropinocytosis and endocytosis (Rosales, 2005; Bohdanowicz and Grinstein, 2013). Phagocytosis is an important biological process, a key mechanism of innate immunity, that involves ingestion and destruction of large particles (≥0.5-µm). The main characteristic of phagocytosis is phagosome formation that is independent of clathrin but dependent on actin polymerization as well as phosphoinositide 3-kinase (PI-3 kinase) activity (Aderem and Underhill, 1999; Bohdanowicz and Grinstein, 2013; Naik and Harrison, 2013). PI-3 kinase is crucial for phagocytosis but also plays key roles in regulating membrane trafficking and cell growth (Araki *et al.*, 1996; Bohdanowicz and Grinstein, 2013). PI-3 kinase in mammalian cells are composed of two subunits, a catalytic (p110) and a regulatory (p85) subunit (Araki *et al.*, 1996; Naik and Harrison, 2013). PI-3 kinase phosphorylates hydroxyl groups of inositol to

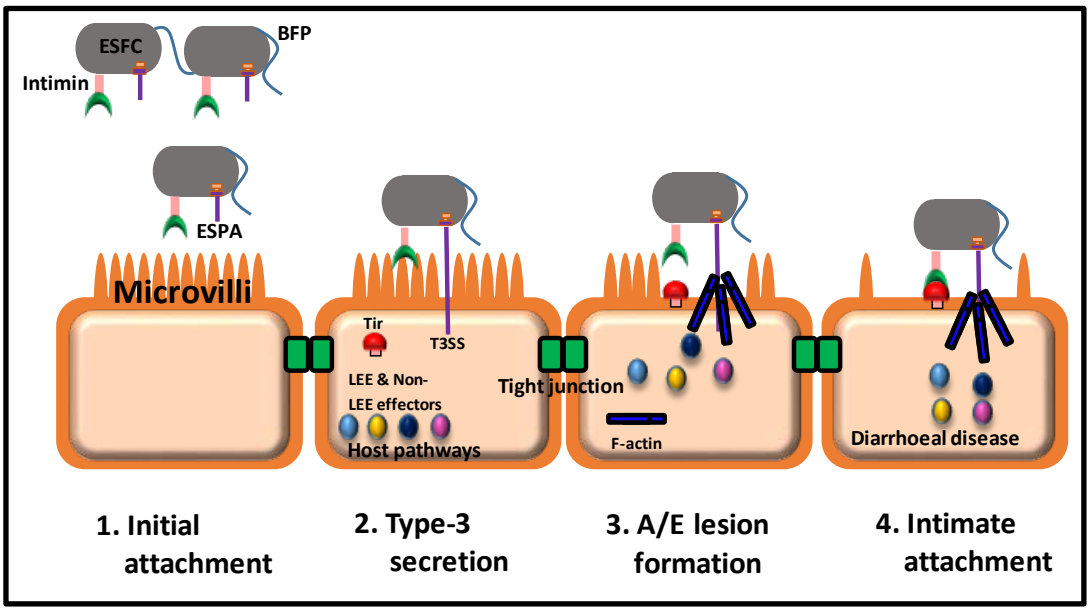


Figure 6. The four distinct stages of EPEC infection. The first stage of infection involves EPEC binding to the apical surface of enterocytes via the Bundle Forming Pilus (BFP), the formation of the BFP allows the interaction with other EPEC bacteria, forming characteristic EPEC micro-colonies which are termed 'localised adherence pattern'. The second stage involves T3SS mediated delivery of a plethora effector proteins into host cells, which cause activation of cell signalling pathways, actin polymerisation and cytoskeletal rearrangement. The disruption of the host cytoskeleton is caused by the cooperative action of LEE effectors which are encoded on the pathogenicity island termed the locus of enterocyte effacement (LEE). These include seven identified effector proteins of which one (EspB) is part of the translocon as well as Nle effector proteins with some also named Esp (e.g. EspG2, EspL and EspJ). As a consequence, the absorptive microvilli on the apical surface of enterocytes is lost extensively which is known as effacement. The defining features of the final stage is the formation of actin rich pedestals. Pedestals formation is a very active process which includes the binding of the T3SS delivered translocated intimin receptor (Tir) effector, which is inserted into the host cell membrane and the outer membrane protein Intimin.

produce phosphatidylinositol 3-phosphate, phosphatidylinositol (3,4)-bisphosphate, or phosphatidylinositol (3,4,5)-trisphosphate (De Camilli *et al.*, 1996; Naik and Harrison, 2013).

In contrast, endocytosis uses two ways to form endosomes; caveolar endocytosis depends on caveolin and cavins to form a coat (Figure 7; (Krauss and Haucke, 2011; Bohdanowicz and Grinstein, 2013). Clathrin-mediated endocytosis uses the GTPase motor protein dynamin and the coat protein clathrin (Krauss and Haucke, 2011; Bohdanowicz and Grinstein, 2013). PI-3 kinase also plays multiple roles in the regulation of endocytosis, for example in intracellular membrane trafficking that follows internalisation (Araki *et al.*, 1996; Krauss and Haucke, 2011). By inhibition of post endocytic trafficking it affects the rates of fluid-phase endocytosis versus receptor-mediated endocytosis (Krauss and Haucke, 2011; Elkin *et al.*, 2016).

The cells that are capable of phagocytosis include neutrophils, macrophages and dendritic cells (Flannagan *et al.*, 2012). Macrophages are derived from monocytes which differentiate into macrophages or dendritic cells after leaving the circulation (Murray and Wynn, 2011).

Monocytes can be considered as professional phagocytic cells since they express surface receptors which recognise the conserved microbial structures of bacteria and viruses (Murray and Wynn, 2011). The most abundant type (40 to 75%) of white blood cells are neutrophils, also known as neutrocytes. Although the neutrophils are short-lived, they are essential components of the innate immune system as they have the ability to enter any tissue and directly target and destroy microbes (Witko-Sarsat *et al.*, 2000). In contrast, dendritic cells (antigen-presenting cells) capture the antigen, process it and then present it to other immune cells (Hivroz *et al.*, 2012).

There are a number of features that are shared between different phagocytic mechanisms: Firstly, ligands on the surface of the microorganism interact with receptors on the surface of the phagocyte, thus triggering internalisation, which in turn depends on the polymerization of actin at the site of interaction (Araki *et al.*, 1996; Aderem and Underhill, 1999; Flannagan *et al.*, 2012). Following actin-mediated internalisation, actin is lost from the phagosome which then matures, through a series of vesicle fusion and fission events, to a microorganism-destructive phagolysosomal compartment (Figure 8) (Aderem and Underhill, 1999; Flannagan *et al.*, 2012).

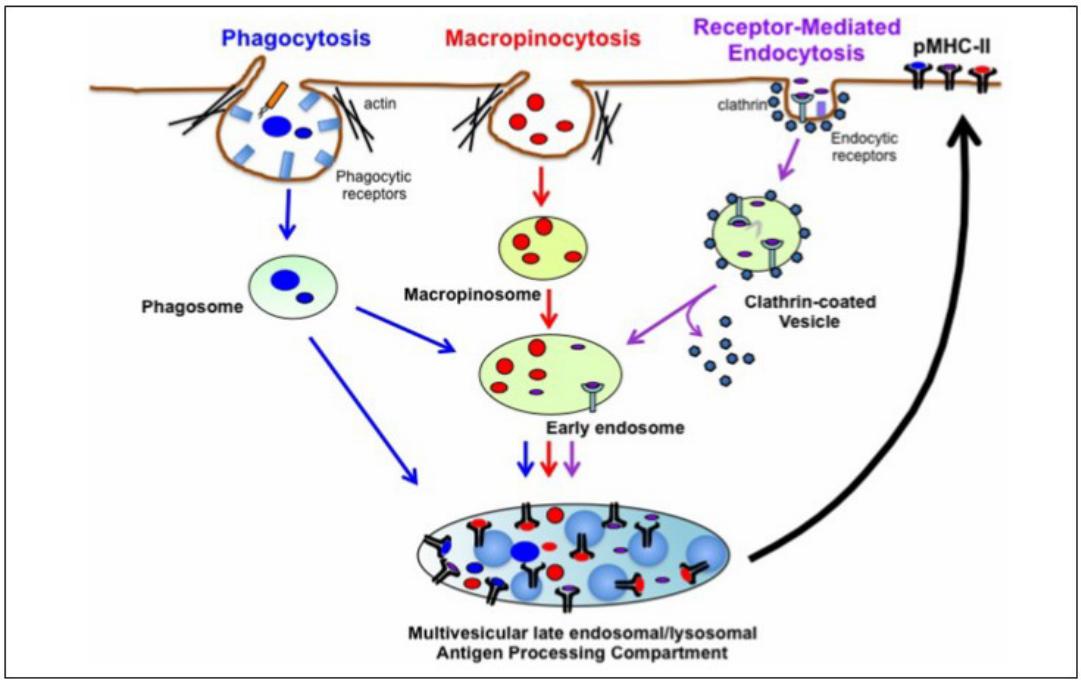


Figure 7. Pathways of entry into cells. There are several mechanisms of internalisation depending on the size of the particles. Large solid particles of >0.5 µm can be internalised by phagocytosis, while fluid is taken up by micropinocytosis. Both mechanisms are dependent on actin-mediated remodelling of the plasma membrane. The main characteristic of phagocytosis is phagosome formation that is independent of clathrin but dependent on actin polymerization as well as phosphoinositide 3-kinase (PI-3 kinase) activity. In contrast, endocytosis uses two ways to form endosomes; caveolar endocytosis depends on caveolin and cavins to form a coat. Clathrin-mediated endocytosis uses the GTPase motor protein dynamin and the coat protein clathrin. Whereas the caveolin-mediated endocytosis depends on intermediate compartments, such as the caveosome that is enriched early endosome. Taken from Liu and Roche, (Liu and Roche, 2015).

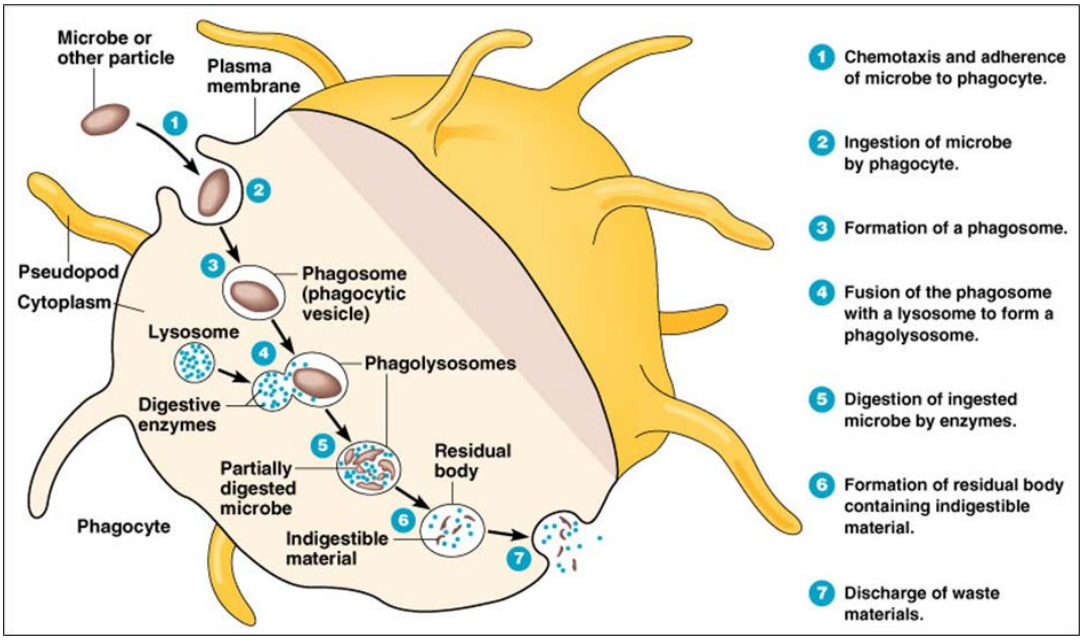


Figure 8. Schematic representation of phagocytosis stages. Taken from https://www.score95.com/blog/wp-content/uploads/2011/11/USMLE-step-2-ck2.jpg

1.4.1 Receptor mediated phagocytosis

One of the fundamental features of phagocytosis is the clearance the foreign bodies, for instance microbes which cause infection, a task that is mediated by a variety of specific surface receptors. Dependent on the type of receptors involved, phagocytosis can be divided into two main types: opsonized phagocytosis and non-opsonized (Figure 9).

1.4.1.1 Opsonic phagocytosis

The receptor-mediated opsonic phagocytosis can be categorised into two major groups according to the receptor involved: the immunoglobulin IgG protein and complement protein antibody. Both recognise effectively the foreign particles which circulate within the blood and interstitial fluids.

1.4.1.1.1 The immunoglobulin IgG protein

The IgG molecule consists of two portions, one known as Fab (variable) region that recognize and bind to the epitopes on foreign antigens, while the Fc domain of IgG attaches to Fcγ receptors (FcγRs; Figure 10) (Anderson *et al.*, 1990; Rosales and Uribe-Querol, 2017). The FcγRs in human macrophages are divided into two groups: The activating FcγRs such as FcγRI, FcγRIIA and FcγRIII receptors, activate effector functions and phagocytosis signalling to induce internalisation of the particles (Figure 11). The second type of FcγRs are the inhibitory receptors and include FcγRIIB that regulate effector functions negatively (Naik and Harrison, 2013; Rosales and Uribe-Querol, 2017) .

Opsonic phagocytosis includes three steps: (I) attachment of ligand-coated particles to receptors. (II) Assembly of the FcγR complex which activates a signalling cascade. (III) Uptake of molecules dependent on actin polymerisation that form membrane extensions known as pseudopods (Griffin et al., 1975; Flannagan et al., 2012; Naik and Harrison, 2013; Rosales and Uribe-Querol, 2017). Importantly, iterative activation of FcγR leads to 'zippering' the membrane around the foreign particle; this includes interaction between receptors and available ligands on the host membrane prior to the internalisation process (Griffin *et al.*, 1975; Naik and Harrison, 2013; Rosales and Uribe-Querol, 2017).

Despite similar extracellular domains in all types of FcγRs, the position of their immune-receptor tyrosine-based activation motif (ITAM) differs which is important for FcγRs clustering (Flannagan *et al.*, 2012; Naik and Harrison, 2013; Rosales and Uribe-Querol, 2017). The ITAM of FcγRIIA is located in the cytoplasmic domains (Muta *et al.*, 1994; Naik and Harrison, 2013). In contrast, the ITAM of FcγRI and FcγRIII is found in separate

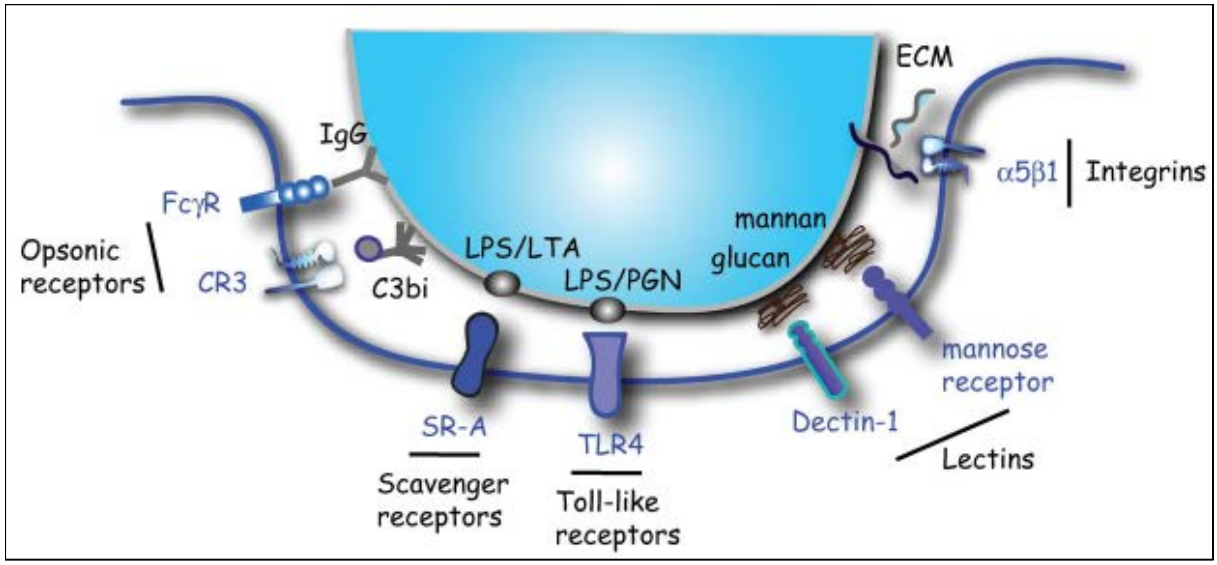


Figure 9. Schematic representation of the opsonic and non-opsonic receptors that initiate phagocytosis in macrophages. Macrophages possess both opsonic and non-opsonic receptors that interact with the microbes. They target coated particles "opsonized" with either C3bi or IgG to the FcγR or CR3 receptors, respectively. The scavenger receptors recognise bacterial ligands such as lipopolysaccharide (LPS) and lipoteichoic acid (LTA). In contrast, to the Toll-like receptors which recognise the bacterial lipopolysaccharide (LPS) and peptidoglycan (PGN), while, the integrines recognise with the bacterial extracellular matrix (ECM) including fibronectin and vitronectin. Taken from Naik and Harrison, 2013 (Naik and Harrison, 2013).

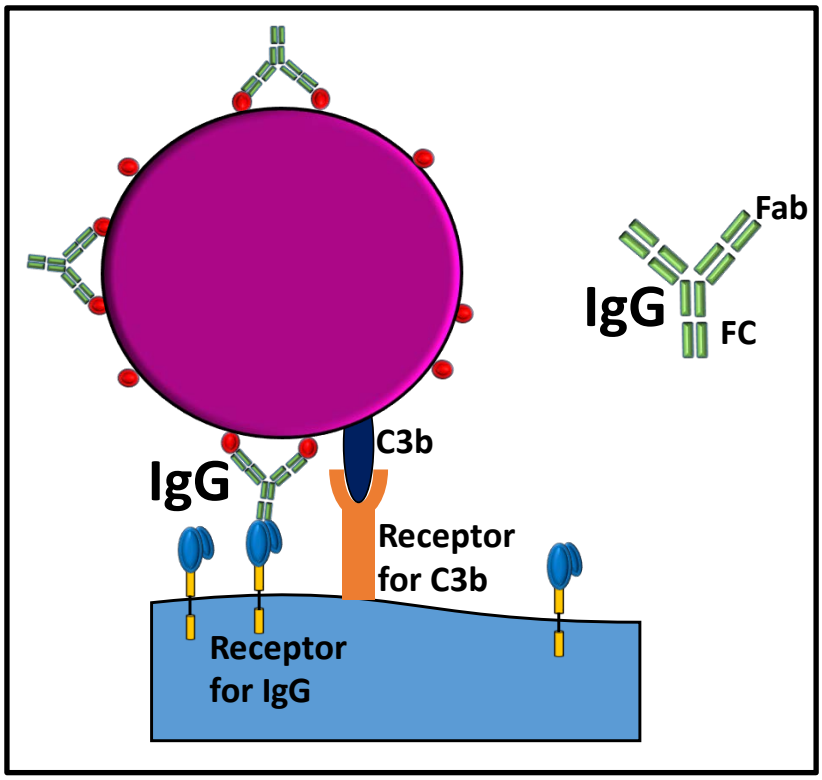


Figure 10. Simplified schematic of the IgG. The immunoglobulin G (IgG) is the most abundant type of antibody in blood. It contains two portions, one known as Fab (variable) region that recognize and attach to the epitopes on pathogens, while the Fc domain of IgG attaches to Fcγ receptors (FcγRs). The complement protein responsible for opsonising bacteria for phagocytosis is C3b that attach to its receptor on macrophages. Adapted from https://es.slideshare.net/munevarjuan/sistema-inmune-curso / picture 17.

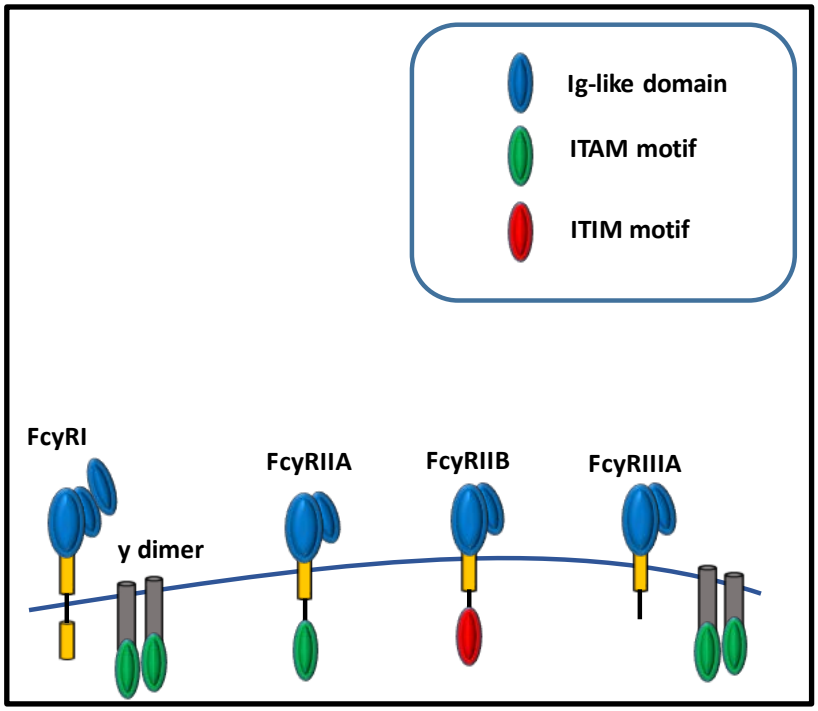


Figure 11. Different types of FcγRs receptors in phagocytic cells. The FcγRI in human macrophages possess Ig-like domains so that it has high affinity to bind with IgG. Activation of the FcγRI receptors is achieved by binding with their γ dimers which have a stimulatory ITAM motif within their cytosolic tails. While the ITAM motif is located in the cytoplasmic domains of the FcγRIIA. FcγRIIB possess an immune-receptor tyrosine-based inhibition motif (ITIM) within its transmembrane tail. Adapted from Naik and Harrison, 2013 (Naik and Harrison, 2013).

γ-dimers, which associate non-covalently with the ligand binding subunit of the receptors (Figure 11) (Hibbs *et al.*, 1989; Ernst *et al.*, 1993). Importantly, the ITAM has a conserved tyrosine residue in a YXXL sequence motif which is critical for competent phagocytic signalling through FcγRs, which is phosphorylated by tyrosine kinases of the Src family (Mitchell *et al.*, 1994; Naik and Harrison, 2013).

Src tyrosine kinases consist of five conserved components: The N-terminal domain (enables it to bind to the plasma membrane), SH2 domains, SH3 domains, the functional catalytic domain and the C- terminal with the tyrosine residue (Y527). Phosphorylation of Y527 lead to binds to the SH2 domain and induces a closed conformation that inactivates the enzyme.

Following the phosphorylation of the ITAM, spleen tyrosine kinase (Syk) is recruited by Src tyrosine kinase via binding to the SH2 domains of Syk kinase (Kimura *et al.*, 1996; Naik and Harrison, 2013; Rosales and Uribe-Querol, 2017). Activation of Syk kinase triggers different pathways which lead to cytoskeletal rearrangement as well as the release of inflammatory mediators (Latour *et al.*, 1996; Naik and Harrison, 2013; Rosales and Uribe-Querol, 2017). Previous research has found that Syc participates in the recruitment of downstream effectors such as PI-3 kinase via the phosphorylation of its p85 subunit (Crowley *et al.*, 1997). For pseudopod elongation PI-3 kinase catalyses the phosphorylation of (PI(4,5)P2) to (PI(3,4,5) P3) at the plasma membrane during FcγR-mediated phagocytosis (Crowley *et al.*, 1997; Cox and Greenberg, 2001). The production of the PI-3 kinase is essential for recruiting proteins which are important for F-actin remodelling in the phagocytic cup (Naik and Harrison, 2013; Rosales and Uribe-Querol, 2017).

Interestingly, FcγR activation stimulates F-actin polymerisation beneath the attached particles and consequently promotes membrane protrusions (pseudopods, Figure 12) (Griffin *et al.*, 1975). The key factors in the regulation of actin dynamics in eukaryotic cells are GTPases of the Rho family such as RhoA, Rac and Cdc42, that switch between the GTP-bound, active form and the GDP-bound inactive form (Hall, 1998). It has been shown that Src and Syc tyrosine kinases participate in F-actin regulation by activating Rac 1 and Cdc42 (Deckert *et al.*, 1996). Indeed, Rho family activated by nucleotide exchange, for instance the guanine nucleotide exchange factor (GEF), catalyses the exchange of GDP for GTP for activation. GTP-bound Rac 1 and Cdc42 regulate the F- actin binding protein to assist the formation of the membrane protrusions filopodia and lamellipodia (Hoppe and Swanson, 2004). Cdc42 GTPase is an important component in FcγR-mediated phagocytosis in macrophages as it plays a key role for activation and recruitment of the downstream effector Wiskott - Aldrich syndrome

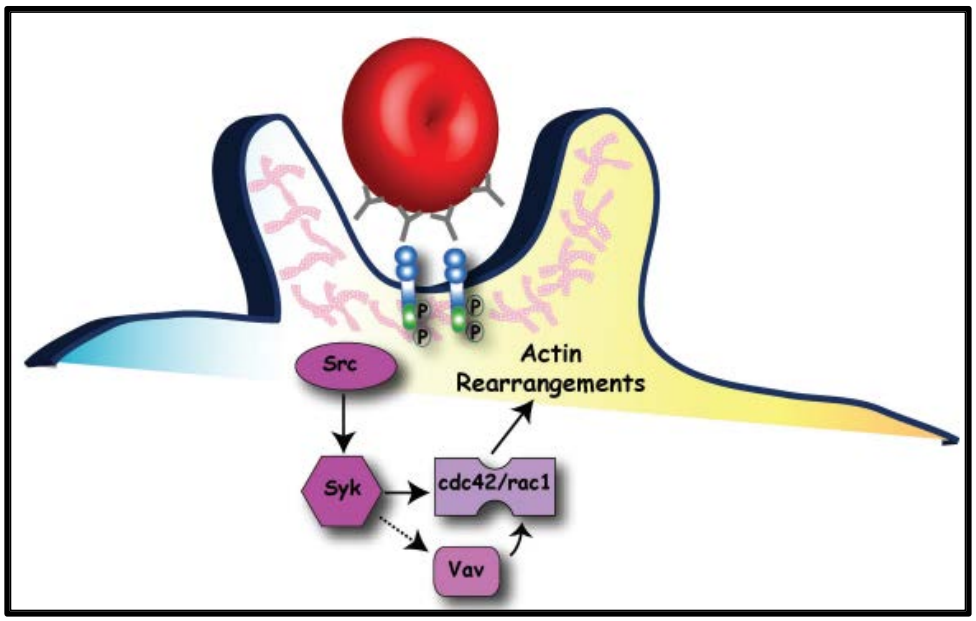


Figure 12. Activation of Fcγ receptor signalling leads to actin rearrangement and pseudopod formation. Following the activation of the Src and Syk, the small GTPases Cdc42 and Rac1 are recruited and activated by the guanine nucleotide exchange factor Vav. Activation of Rac1 and Cdc42 regulates F-actin binding proteins to facilitate F-actin cup formation and membrane pseudopod extension around the target particle. Taken from Naik and Harrison, 2013 (Naik and Harrison, 2013).

protein (N-WASP). N-WASP activates the seven-protein nucleator complex (Arp2/3), which stimulates the formation of actin filaments that drive the engulfment of particles (Machesky and Insall, 1998; May *et al.*, 2000). In addition, activation of Cdc42 by FcγR stimulates PI-3 kinase, the resulting increase of 3' PIs in cup membranes leads to inactivation of Cdc42 to permit actin recycling necessary for phagosome formation (Beemiller *et al.*, 2010).

However, not all FcγR stimulate phagocytosis: the FcγRIIB has an immune-receptor tyrosine-based inhibition motif (ITIM) which recruits the phosphatase Src homology 2 domain containing inositol 5-phosphatase (SHIP). SHIP inhibits phagocytosis through dephosphorylation of PI (3,4,5) P3 to PI(4,5)P2 which terminates the PI-3 kinase signalling (Cox *et al.*, 2001; Nakamura *et al.*, 2002; Rosales and Uribe-Querol, 2017).

1.4.1.1.2 Complement protein antibody

The complement protein responsible for opsonising bacteria for phagocytosis that is present in serum is C3b, also called Mac-1, CD11b/CD18 or integrin $\alpha_m\beta_2$. The latter refers to an integrin present in macrophages which consists of two conserved α and β chains (Ross *et al.*, 1992). There are two pathways that activate the complement system: the classical pathway includes indirect attachment to immunoglobulin molecules present on microbes, particularly IgM/IgG-opsonized pathogens (Hartung and Hadding, 1983; Naik and Harrison, 2013). The second and rapid response is the alternative pathway, in which the carbohydrate motifs on the surface of the pathogen are recognized directly by complement without relying on the production of antibodies (Carroll, 1998; Naik and Harrison, 2013).

Briefly, the signalling pathway of integrin includes two routes: inside out signalling which is important in activating the complement receptor CR3 and causing membrane ruffling in order to capture C3Bi- opsonised particles in macrophages (Patel and Harrison, 2008; Naik and Harrison, 2013; Rosales and Uribe-Querol, 2017). This process is followed by an outside-in signalling process that stimulates F-actin remodelling. Contrasting with the FcγR-mediated phagocytosis, the role of Syk tyrosine kinase is not well understood (Kiefer *et al.*, 1998; Naik and Harrison, 2013; Rosales and Uribe-Querol, 2017) but it appears that Syk is also phosphorylated during CR3-mediated phagocytosis (Shi *et al.*, 2006). While F-actin polymerisation is regulated by Cdc42 in FcγR-mediated phagocytosis, it is regulated by RhoA GTPase in CR3-mediated phagocytosis (Wiedemann *et al.*, 2006). The regulation of F-actin by RhoA GTPase involves two signalling pathways: In first pathway RhoA activates Rho kinase (ROCK) and phosphorylates myosin II which stimulates F-actin polymerisation through the Arp2/3 complex (Olazabal *et al.*, 2002). The second direct pathway includes the activation of mammalian diaphanous-related formin (mDia) by RhoA. mDia in turn stimulates F-actin

assembly which is recruited by a microtubule associated protein (Lewkowicz *et al.*, 2008; Naik and Harrison, 2013; Rosales and Uribe-Querol, 2017).

1.4.1.2 Non-opsonic phagocytosis

Microorganisms such as bacteria, fungi and parasites expose various types of conserved molecules which are never observed in the higher organisms, known as pathogens-associated molecular patterns (PAMPs). Non-opsonic phagocytosis, also known as cis-phagocytosis, is activated directly through the recognition of PAMPs by receptors of phagocytic cells (Janeway, 1992). For example, the polysaccharides mannan and β-glucan which are displayed on the surface of certain yeast cells are recognised by mannose- and dectin-1 receptors, respectively (Ezekowitz *et al.*, 1990; Brown and Gordon, 2001; Herre *et al.*, 2004; Naik and Harrison, 2013). Both belong to the lectin receptor family (Figure 9). The cytosolic domain of the dectin-1 receptor contains an immune-receptor tyrosine-based activation motif (ITAM) which recruits kinases via interaction with SH2 domains (Herre et al., 2004; Flannagan et al., 2012). Researchers have found that the macrophage dectin-1 mediated phagocytosis of zymosan, one of the components of the *Saccharomyces cerevisiae* cell wall, was less dependent on Syk, Src, PI-3 kinase and Rho GTPases (Herre et al., 2004).

Other receptors, such as Toll-like receptors (TLRs) and Scavenger receptors SR-A are expressed in macrophages and recognise lipopolysaccharides (LPS) on the surface of bacteria (Poltorak *et al.*, 1998; Rock *et al.*, 1998; Hoebe *et al.*, 2005; Flannagan *et al.*, 2012). In addition, they work co-ordinately to recognise peptidoglycans, lipoteichoic acid and flagellin molecules on the cell wall of gram-positive bacteria (Underhill *et al.*, 1999). Research into SR-A demonstrated that the receptor is critical for efficient binding and uptake of bacteria by non-opsonic phagocytosis. The experiments used inhibiters to block SR-A receptors and consequently decreased internalisation was observed. Moreover, SR-A was shown to recognise not only LPS but also lipoteichoic acid of bacteria (Poltorak *et al.*, 1998; Peiser *et al.*, 2000). The signalling pathways triggered by SR-A-mediated non-opsonic phagocytosis include a number of molecules such as protein kinase C, Src, MARKS as well as PI-3 kinase (Nikolic *et al.*, 2007; Sulahian *et al.*, 2008). In contrast, integrin receptors recognise and attach to extracellular matrix proteins including fibronectin and vitronectin (Figure 9) (Freeman and Grinstein, 2014).

1.4.2 Strategies used by pathogens to inhibit phagocytosis

There are several ways for bacterial pathogens to avoid both opsonic and non-opsonic phagocytosis. These include interfering with opsonin-binding which is considered to be the most common strategy against opsonic phagocytosis. One of the bacterial virulence factors is the capsule which consists of polysaccharides and is used by bacterial and fungal species such as EPEC, *Streptococcus pneumoniae*, *Neisseria meningitidis* and *Haemophilus influenzae* as a shield to prevent the deposition of opsonin (Flannagan *et al.*, 2012). Another way to avoid being internalised, is the synthesis of protein A and M-protein by *Staphylococcus aureus* and *Streptococcus pyogenes* respectively. Protein A binds to the Fc portion of IgG thereby preventing its interaction with Fc receptors (Foster, 2005). On the other hand, the M-protein recruits an inhibitor of complement activation called C2b-binding protein. Likewise the YadA of *Yersinia enterocolitica* prevents deposition of the complement component iC3b (China *et al.*, 1993).

To survive in the presence of non-opsonic phagocytosis, bacteria must be able to inhibit signalling pathways required for the effective uptake. Some microbes such as EPEC (see below) and *Pseudomonas aeruginosa* use their T3SS to inject effector proteins into phagocytic cells (Flannagan *et al*., 2012). Delivering these effectors inhibits Rho-family GTPases or antagonize tyrosine phosphorylation and PI-3-dependent signalling by imitating the function of inhibitory host proteins (Flannagan *et al*., 2012). For example, the *Yersinia* inhibitory mechanism has been linked to the subversive activity of YopH and YopE delivered by T3SS. YepE inhibits uptake of *Yersinia* by acting as a GTPase-activating protein (GAP), negatively regulates the switch by enhancing the intrinsic GTPase activity, for RhoA, Rac1 or Cdc42, which are responsible for regulating actin cytoskeleton dynamics (Black and Bliska, 2000; Pawel-Rammingen *et al.*, 2000). YopH inhibits bacterial uptake by inducing the de-phosphorylation of the focal adhesion kinase in both professional and non-professional phagocytes (Fallman *et al.*, 2002; de la Puerta *et al.*, 2009).

However, some pathogens such as HIV and *Haemophilus ducreyi* do not have an injection system, thus, they use a different mechanism to inhibit phagocytosis, named Trojan horse-like strategy. In this strategy, the microbes engulf normally and once they are internalised their effectors block the phagocytic capacity of the host cells preventing further uptake of additional microbes (Wood *et al*., 2001; Mazzolini *et al*., 2010).

1.4.2.1 EPEC anti-phagocytosis activity

EPEC has a T3SS-dependent capacity to inhibit its uptake by J774.A1 macrophages via phosphoinositide 3 (PI-3 kinase) dependent pathways (Goosney *et al.*, 1999; Celli *et al.*, 2001; Quitard *et al.*, 2006). However, many EPEC bacteria (~40%) are still internalised via PI-3 kinase independent pathways (Goosney *et al.*, 1999; Celli *et al.*, 2001; Quitard *et al.*, 2006). While EPEC uptake occurs through both pathways in macrophages, in the M-cell model system (microfold; antigen-sampling cells in gut) it is mainly through PI-3 kinase dependent pathways (Martinez-Argudo *et al.*, 2007). It is speculated that delaying EPEC exposure to immune cells provides an advantage to the bacteria's life cycle (Quitard *et al.*, 2006). The PI-3 kinase inhibitory mechanism has been linked to the subversive activity of EspB, EspH, EspG and EspF proteins (Quitard *et al.*, 2006; Iizumi *et al.*, 2007; Dong *et al.*, 2010; Humphreys *et al.*, 2016). Studies using M-cells have revealed that the inhibition of EPEC translocation is EspF dependent (Martinez-Argudo *et al.*, 2007). However, in bovine M-cells model, EspF-dependent inhibition of uptake is less effective than EHEC, in contrast with murine macrophages (Tahoun *et al.*, 2011). However, the mechanism of EspF inhibition remains undefined (Quitard *et al.*, 2006). In addition, studies in the macrophage model have ruled out roles for several other effectors (Tir, Map) and Intimin in the EspF-dependent inhibitory process (Celli *et al.*, 2001; Quitard *et al.*, 2006). Studies with EspF variants found that its mitochondrial targeting and SNX9 binding motifs were non-essential for its inhibitory activity (Quitard *et al.*, 2006; Weflen *et al.*, 2010; Young, 2013). However, a fusion protein in which the first 101 amino acids were replaced with those of Tir (provides a T3SS targeting signal) failed to complement the EspF mutant anti-phagocytosis defect (Quitard *et al.*, 2006). The latter indicates a key role for features within the N-terminal 101 amino acids, though the presence of the Tir domain may simply be hindering EspF's anti-phagocytic activity; for example by preventing EspF from adopting a functional conformation or targeting it away from a binding partner.

Additionally, EspB can also inhibit phagocytosis; as discussed earlier EspB is not only an effector protein but also one of the components of the EPEC translocon that links the T3SS to host cells (Iizumi *et al.*, 2007). EspB can disrupt myosin interaction with actin filaments through binding to the myosin motor domains of myosin family proteins (myosin-1c, -2, -5, -6 and -10). This interaction interrupts actin dynamics, consequently preventing pseudopod extension of the membrane around the invading bacterium and loss of myosin dependent closure of the phagocytic cup (Iizumi *et al.*, 2007).

In contrast, EspH inhibits uptake of EPEC by counteracting both opsonic and non-opsonic phagocytosis through inhibiting the activity of RhoGEFs (Dong *et al.*, 2010). EspH has the ability to target the DH-PH domain of RhoGEFs, which disrupts the interaction between the

GEF and their Rho GTPase target. Blocking this interaction inhibits host GTPase activation and downstream signalling, consequently, prevents actin remodelling and phagosome formation (Dong *et al.*, 2010).

Interestingly, EspG has recently been identified as an inhibitor of non-opsonic phagocytosis through inhibition of the WAVE regulatory complex (WRC; a five-subunit protein complex in the Wiskott-Aldrich syndrome protein (WASP)) which interferes with phagocytosis and enables EPEC to evade phagocytic cells (Humphreys *et al.*, 2016). Inhibition of WRC is achieved by binding to the small GTPase Arf1, thereby either preventing the signalling to ARNO (Arf1 activator) or blocking the interaction with the small GTPases-Rac1, which blocks WRC recruitment and activation (Humphreys *et al.*, 2016).

1.5 Summary and project aims

Bacterial pathogens such as EPEC employ a wide range of strategies to colonise, survive and cause diarrhoeal disease. Through T3SS, EPEC delivers many effector proteins directly into host cells and subverts a number of host cell functions and signalling pathways including inhibition of phosphoinositide PI-3 kinase dependent uptake by macrophages. EPEC's ability to inhibit PI-3 kinase dependent pathways has been linked to the subversive activity of the LEE-encoded EspB, EspH, EspG and EspF effectors. EspB inhibits bacterial uptake by targeting myosin motor proteins which promote pseudopod extension. In contrast, EspH inhibits the activity of RhoGEF which is responsible for regulating actin cytoskeleton dynamics. EspG inhibits the activity of the WAVE regulatory complex which is responsible for actin polymerisation through targeting the small GTPases Arf6 and Arf1. However, the mechanism by which EspF inhibits phagocytosis remains undefined. Understanding how EPEC inhibits its uptake by macrophages would provide greater understanding of the EPEC infection strategy and provide critical insights into the anti-phagocytic mechanism. Thus, the project aims are to examine if EspF alone could inhibit phagocytosis and investigate the mechanism by which EPEC inhibit its uptake by macrophages.

Chapter 2. Materials and Methods

2.1 Cell culture

2.1.1 Culturing of bacterial strains

Bacterial strains used in this study are listed in Table 2. Bacteria were streaked from frozen cultures (-80 °C) onto Luria-Bertani (LB) agar plates and incubated at 37 °C or 26 °C for *E. coli* and *Yersinia* strains, respectively. Agar plates were supplemented, when appropriate, with the antibiotic(s) Nalidixic acid [Nal], Carbencillin [Cb], Chloramphenicol [Cm] and Kanamycin [Km] at 50, 100, 25 and 25 µg/ml final concentration, respectively. LB broth containing appropriate antibiotics was inoculated with a single bacterial colony picked from an LB agar plate for overnight (16-18 h) incubation at 37 ˚C (or at 26 ˚C *Yersinia*) with, or for infection studies, without shaking at the required temperature. Bacteria were usually diluted 1:10 into serum free Dulbecco's minimal Eagles medium (DMEM; Sigma D5796), containing appropriate antibiotic(s), for 3 h to pre-induce T3SS expression (Rosenshine *et al.*, 1996a). When needed, overnight LB broth cultures (post-shaking) were used to make frozen stocks (-80 ˚C) by adding 10% glycerol (final concentration) prior to freezing.

2.1.2 Mammalian cell culture

Mammalian cell culturing was implemented under sterilised conditions in a Class 2 laminar flow hood (BioMat 2) with studies using HeLa (human cervix-derived epithelial-like; ATCC_CCL2) and J774.A1 (mouse macrophage-like; ATCC_TIB-67) cells. Mammalian cells were grown in Dulbecco's minimal Eagles medium (DMEM), supplemented with 10% foetal calf serum (FCS; GIBCO) in a 5% CO_2/37°C incubator (LEEC; Research CO_2 Incubator). Cells were routinely maintained in 25 cm^2, 75 cm^2 or 175 cm^2 (Corning; 430639, 430641, 431080; respectively) culture flasks at 37 ˚C in a 5% CO_2 atmosphere. Every 2-3 days the cell media was replaced with fresh media. When the cells reached 80-90% confluence they were washed twice with Phosphate Buffered Saline pH 7.4 (PBS; 137 mM sodium chloride [NaCl], 2.7 mM potassium chloride [KCl], 10 mM disodium phosphate and 1.8 mM monopotassium phosphate; pH 7.4) and detached, for macrophages, into DMEM using a scraper (TC Cell Scraper 25 cm; Sarstedt 83.183) and, for HeLa cells, by adding 1x trypsin solution (Trypsin-EDTA [ethylenediamine acetic acid]; Sigma; T4174) for 10-15 min prior to adding DMEM. A sample was removed to determine cell viability - percentage of cells that exclude trypan blue (Sigma; T8154) - and cell concentration, using a haemocytometer following the manufacturer's instructions (Hawksley; AC1000). The cells were seeded into a new flask [routinely 1:6 dilution] or into wells of a 24 well plate (NunclonTMΔ Surface, Nunc), 60mm dishes (SPL Life Sciences) or onto sterile 13 mm glass coverslips (VWR, 631-1578) within wells of a 24 well

plate to obtain 70-80 confluency for the day of the experiment. The cell passage number was recorded and cells replaced following 20 (J774.A1) and 30 (HeLa cells) passages. Frozen culture stocks were generated by placing cells (10^6/ml) in DMEM containing 10% DMSO (Sigma; D2650)/10% FCS into a -80 $^{\circ}$C freezer overnight inside a closed polystyrene box (after wrapping within paper towels) before transferring to liquid nitrogen storage.

2.2 Molecular biology

2.2.1 DNA Isolation

LB shaking cultures of strains carrying appropriate plasmids (Table 3), routinely 5 ml, were used to harvest the appropriate bacterial strain by centrifugation (13,000 xg, 5 min, room temperature [RT]) with plasmid DNA isolated using the AccuPrep plasmid miniprep kit (Bioneer; K-3030) according to manufacturer's instructions. Concentration and purity of isolated plasmids were determined using a NanoDrop 100 spectrophotometer (Thermo Scientific) following manufacturer's instructions.

2.2.2 Polymerase chain reaction (PCR) of EPEC genes and agarose gel electrophoresis

Genes were amplified using purified plasmids or DNA released from bacteria following lysis in dH_2O (100°C, 5 min) using oligonucleotide primers (Table 4) specific to the target gene. These primers were designed using the Serial Cloner 2.6.1 software (Scientific and Educational Software; Franck Perez, Serial basics) and PCR reactions undertaken using a Veriti 96 well thermocycler (Applied Biosystems). PCR reactions were routinely in a total volume of 25 µl containing DNA, 1x Taq reaction buffer (New England Biolabs; B9014S), dNTPs (200 µM final concentration; New England Biolabs; N0447S), Taq DNA Polymerase (1.25 units/50 µl final concentration; New England Biolabs M0273L) and each primer (0.2 µM final concentration) using conditions given in Table 5.

The PCR products were assessed by adding 10x ficoll loading dye (to 1x final concentration) and loading, alongside 2-log DNA ladder (New England Biolabs; N3200L), on agarose gels (0.7-1% w/v agarose in TAE [40 mM Tris-acetate, 1mM EDTA] buffer) containing the fluorescent nucleic acid stain GelRedTM (Biotium; BT4100 at 1:30,000 dilution). Samples were run at 100 volts for an appropriate time with DNA visualised using a UV transilluminator (Biorad) for image capture.

2.2.2.1 Sequence analysis

The target gene (or region thereof) was PCR amplified using Q5 high-fidelity DNA polymerase (New England Bio-labs), the product isolated using the GenElute™ PCR Clean-Up Kit (Sigma; NA1020) prior to sending for sequencing (Source Bioscience, Cambridge, UK). The resulting sequences were analysed using the Serial Cloner 2.6.1 software.

2.2.3 Preparation of electro-competent bacteria and transformation with DNA

LB shaking cultures, routinely 5 ml, were diluted 1:100 in 100 ml LB broth (supplemented with appropriate antibiotics) for growth at 37°C or, for *Yersinia* strains, 28°C with shaking (225-250 rpm) until reached optical density (OD_{600}; 1 OD = 10^9 bacteria) of 0.4-0.6 as assessed using a UV 1101 Biotech photometer (WPA). Bacterial cultures were rapidly cooled (on ice with shaking) and then pelleted (12,000 x g, 15 min, 4°C) and re-suspended in 50 ml ice-cold sterile H_2O. This washing step was repeated twice with the bacterial pellet re-suspended in 20 ml sterile, ice cold, 10% glycerol before pelleting as before. The resulting pellet was re-suspended in 500 µl ice-cold sterile 10% glycerol with 40 µl aliquots placed into ice cold 0.5 ml Eppendorf tubes (Sarstedt; 72.690.001) for immediate use or snap freezing (in liquid nitrogen) for long term storage (-80°C).

For electroporation, 25-50 ng/µl of plasmid DNA (see Table 3) was added to an aliquot of (thawed) cells and transferred into an ice-cold electroporation cuvette (2mm-gap; GenPulser II; Bio-Rad) on ice for 5-10 min prior to electroporation using setting 2.5 KV, 200 Ω, 25 mF, 4.5µs. The transformed cells were immediately resuspended in 1 ml pre-warmed, 37°C, super optimal broth with catabolite repression (SOC; 0.5% Yeast Extract, 2% Tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl2, 10 mM MgSO4 & 20 mM Glucose) media and incubated 1 h at 37°C (28°C for *Yersinia* strains) with 50-1000 µl plated onto LB agar plates containing appropriate antibiotic(s). Colonies evident after 16-20 incubation at 37°C (28°C for *Yersinia* strains) were screened for presence of electroporation-introduced gene by PCR and/or, see below, western blot analysis.

2.2.4 Disrupting EPEC mutants by allelic exchange

EPEC genes were inactivated using available suicide vectors (Table 6) introduced via conjugation (using SM10 *λpir* strain carrying the suicide vector) as previously described (Kenny *et al.*, 1997b; Philippe *et al.*, 2004). Briefly, the donor SM10 and recipient EPEC strains were grown separately in LB broth cultures (supplemented with appropriate antibiotics) at 37°C shaking, then, mixed together and plated onto LB agar plates at 37°C to allow conjugation between recipient and donor strains. Bacteria (from the resulting bacterial growth) were

inoculated into 3 ml of LB broth containing antibiotics to select for the trans-conjugant (EPEC with suicide vector inserted into chromosome via single cross-over event) and incubated with shaking for 4-6 h at 37°C. The next day, the resulting culture was serially diluted onto LB agar plates supplemented with appropriate antibiotic(s) and incubated at 37°C overnight. Following incubation, single colonies were streaked out on four LB agar plates at 37°C overnight (supplemented with Nal, Cb or Km antibiotics) to identify putative trans-conjugants. To enable the excision of inserted suicide plasmid, candidate trans-conjugant was grown, with shaking, in LB broth (supplemented with appropriate antibiotic(s) beyond plating on LB agar plates containing 5% sucrose with appropriate antibiotic(s) and incubated 24-42 h at 30°C. To test for suicide vector loss, single colonies were streaked out on four LB agar plates (supplement with Nal, Cb or Km antibiotic(s)) and incubated at 37°C overnight. Finally, the potential gene disrupted strains colonies were screened for loss of target gene by PCR analysis.

2.3 Mammalian cells infection

2.3.1. Standard phagocytosis assay

Studies used a previously described protocol (Goosney *et al.*, 1999) with minor modifications. Briefly, J774.A1 cells were seeded (~$1.1x10^5$) on glass coverslips 2 days prior to infection to obtain 60-70% confluence on the infection day. LB standing *E. coli* cultures (*Yersinia* strains grown with aeration at 28°C) were diluted (1:10) into DMEM (with antibiotic when appropriate) for 3 h (30 min for *Yersinia* strains) prior to determining the OD_{600} value and calculating the volume for a Multiplicity of Infection (MOI) of 100:1 (bacteria to J774.A1 macrophage). Thirty minutes prior to infection, the macrophages were washed (37°C PBS) and incubated in DMEM. Following an hour infection, the cells were washed (37°C PBS) and incubated in DMEM containing chloramphenicol (bacterial protein synthesis inhibitor; 25 µg/ml final concentration) for 1 h to promote uptake of adherent bacteria unable to inhibit phagocytosis. Finally, the cells were washed twice (ice cold PBS) and fixed by incubating 20 min with PBS containing 2.5% para-formaldehyde (PFA; Santa Cruz Biotechnology ; sc-281692) and storing (4°C; short term).

The percentage of bacterial internalisation was determined as previously described (Quitard *et al.*, 2006). Briefly, extracellular bacteria were labelled by incubating 30 min (at RT) with anti-EPEC 0127 rabbit or anti-*E. coli* all serotypes antibodies (Table 7) diluted in PBS. Following three washes (PBS), the cells were incubated 30 min with goat anti-rabbit Alexa-555 (fluoresces Red) conjugated secondary antibodies (Table 7). The cells were then washed again (3 x with PBS) with all cell-associated bacteria labelled by incubating with the same primary antibody (diluted in PBS containing 1% Triton X-100 [makes macrophage membrane

permeable to antibodies]) followed by goat anti-rabbit Alexa-488 (fluoresces Green) conjugated secondary antibodies (Figure 13; Table 7). The dye 4'6-diamidino-2-phenylinodole (DAPI - fluoresces blue) was routinely added in final antibody incubations to detect bacterial and host DNA. Coverslips were placed onto FluorSave reagent (Calbiochem; 345789) on glass slides (Thermo Scientific) for phase contrast/fluorescent microscopy examination (Zeiss Axioskop Epifluorescence microscope) (Figure 13). Fifty macrophages with 20-50 cell-associated bacteria (identified in phase/contrast or 'blue' channel) were randomly selected to count the number of extracellular (fluoresce Red) and total-cell associated (fluoresce Green) bacteria enabling the percentage internalisation to be calculated as: (number of Green bacteria minus number of Red bacteria) divided by number of Green bacteria) multiplied by 100. These studies were undertaken in a semi-blind manner i.e. samples were placed (usually by Prof Kenny) in a withheld order until all counting and the percentage of internalisation calculation were made. A range of multiplicity of infections (MOIs) were examined with 100:1 (bacteria: macrophages) found to be the best to obtain 70-80% of the macrophages (each strain) having 20-50 cell-associated bacteria. Higher MOIs (200:1) lead to 70-80% of the macrophages having more than 50 cell-associated bacteria which were too many to reliably count. Lower MOIs (50:1) lead to 70-80% of the macrophages having less than 20 cell-associated bacteria which was considered too few to reliably detect differences between strains.

2.3.2 Alternative (two-wave infection) phagocytosis assay

J774.A1 macrophages were prepared and infected for 1 h as described (2.3.1) prior to washing (37°C 1 x PBS) and incubating 1 h in DMEM containing gentamicin (bactericidal antibiotic; 100 µg/ml final concentration). The macrophages were then washed (37°C PBS) and incubated in DMEM supplemented with 10% FCS for 1 h before replacing with serum free DMEM (30 min) and infecting (MOI 100:1) with the EPEC T3SS mutant (carries Green fluorescent protein-expressing plasmid) for 1 h. The cells were then washed three times (37°C PBS), incubated 1 h in DMEM containing chloramphenicol (25 µg/ml final concentration) and processes as described earlier (2.3.1.1). As the total number of cell-associated second-wave bacteria are revealed by their expression of GFP (fluoresces Green), the antibody labelling procedure was only needed to identify the extracellular bacteria (fluoresce Red). The percentage internalisation of second-wave infecting bacteria was calculated using data of extracellular (fluoresce Green & Red) and total cell-associated (only fluoresce Green) bacteria as described above.

2.3.3. Infection for immunoblot analysis

J774.A1 cells or HeLa cells were seeded (~$5x10^5$) into 6 well plates 2 days prior to infection to

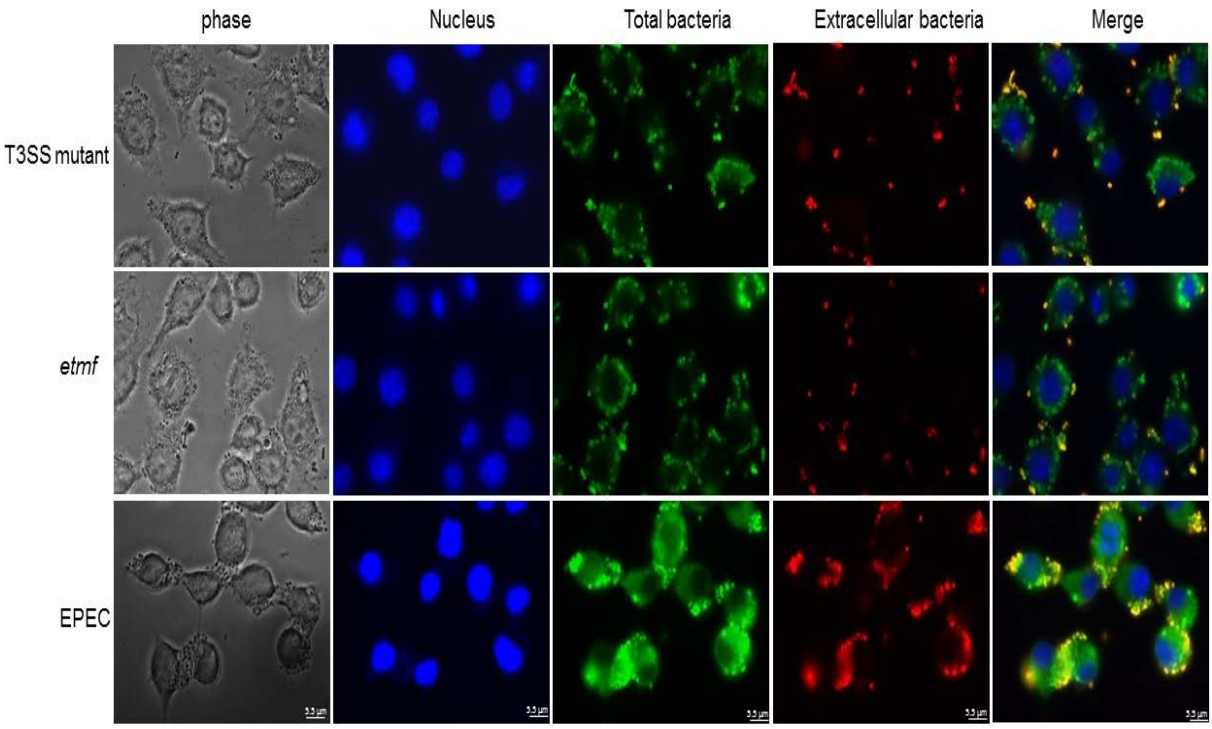


Figure 13. Standard phagocytic assay. J774.A1 macrophages were infected for 1 h (MOI 100:1) with the indicated strains (pre-grown in DMEM for 3 h) prior to determining the percentage of internalised bacteria using differential antibody staining to label extracellular bacteria (Red) and total cell-associated bacteria (Green). Fifty macrophages with 20-50 cell-associated bacteria were randomly chosen to count the number of 'Red' and 'Green' bacteria. Strains used were EPEC and strains lacking a functional T3SS (T3SS) or four LEE proteins: Intimin, Tir, Map and EspF (*etmf*). Images are representative of at least three independent experiments taken by epifluorescence microscope with an 100x oil objective. Scale bar 5.5 µm.

obtain 80-90% confluence on the infection day. Following infection - using previously described conditions/MOI - the cells were washed twice (ice cold PBS) and incubated 5 min in 100 µl ice cold PBS (Sigma)/Triton X-100 lysis buffer (1% Triton, 1mM phenylmethylsulphonyl fluoride [PMSF; Sigma P7626], 0.5 mM sodium fluoride [NaF], 1mM sodium orthvanadate [NaVO4]; protease inhibitor cocktail (Sigma; P8340 [1/100 dilution]). The cells were detached using a cell scraper, and transferred to 1.5 ml centrifuge tubes (Sarstedt; 72.690.001) for centrifugation (13,000 xg, 4 min, 4°C). The resulting 'soluble' fraction (contains host cytoplasmic and membrane proteins plus T3SS-delivered effectors) was transferred to a new tube where 5x Laemmli sample buffer (Laemmli, 1970) was added to a final 1x concentration. The remaining Triton X-100 'insoluble' pellet (contains host nuclei and cytoskeleton plus adherent bacteria) was washed with cold PBS and re-suspended in 1x Laemmli sample buffer. The samples were heated (100°C, 5 min), vortexed and used immediately (for western blot analysis; see below) or stored (-20°C).

2.4 Immunoblot analysis

Protein samples derived from $5x10^5$ mammalian cells were loaded into separating wells for SDS gel electrophoresis as described (Laemmli, 1970). Protein samples were separated using a 15% and 12% polyacrylamide resolving gel and 5% polyacrylamide stacking gel (0.125 M Tris-HCl pH 6.8, 0.1% w/v SDS, 0.1% w/v ammonium persulfate & 0.1% v/v TEMED); all gels were prepared in dH_2O (Laemmli, 1970). Polyacrylamide gels were loaded into a vertical electrophoresis cell (Mini Trans-Blot Cell (Bio-Rad)) system, with electrophoresis performed using 1X SDS running buffer (25 mM Tris-HCl, 192 mM glycine and 0.1 % w/v SDS; pH 8.3) at 200 volts for 1 h. Proteins were then transferred onto polyvinylidene fluoride (PVDF) membrane (GE Healthcare) in 1X transfer buffer (25 mM Tris & 192 mM glycine; pH 8.3) using the Bio-Rad transfer system at 110 volts for 60 min (Kenny and Finlay, 1995). Protein transfer efficacy was assessed by Ponsceau Red staining (Sigma bP7170; prior to washing away with PBS). The membranes were blocked overnight at 4 °C with gentle agitation in 5% skimmed milk powder (Marvel) i) PBS solution if probing for alkaline phosphatase (AP) or ii) Tris-buffered saline with Tween-20 (TBS-T, 150 mM NaCl and 10 mM Tris-Hcl; VWR Scientific, 0.1% Tween-20 pH 7.5; Sigma-Aldrich) if probing for horseradish peroxidase (HRP). The next day, the membrane was washed 5 min with PBS (x 3) and then incubated 1 h in PBS containing anti -Tir, anti -EspF or anti -EspB antibodies (Table 7). Post-incubation and washing (5 min with PBS x 3 for AP or TBSTx3 for HRP) the membrane was incubated with appropriate AP-conjugated antibodies or HRP-conjugated antibody (Table 7). Blots were developed using NBT/BCIP reagent diluted in developing buffer (Promega; 2 M Tris, 5 M

NaCl, 1 M $MgCl_2$, pH 9.5) for AP. For HRP, PVDF membranes were exposed to ECL chemiluminesence western blotting substrate (Thermo Fisher Scientific, 1:1 ratio) for 5 min. The membrane then was exposed to Hyperfilm ECL (Amersham Biosciences) and developed.

2.5 Immunofluorescent microscopy analysis

Studies used a previously described protocol (Nougayrède and Donnenberg, 2004; Dean *et al.*, 2010b). Briefly, cells were seeded (~$1.1x10^5$ per well) onto glass coverslips 2 days prior to infection for 70-90% confluency on the infection day. The cells were washed (37°C PBS) and DMEM - containing antibiotics when appropriate - added prior to a 3 h infection (MOI 100:1) with bacteria pre-grown in DMEM. The cells were washed three times (ice cold PBS), fixed (2.5% PFA, 20 min) and, post PBS washing, incubated (30 min; RT) with anti-EspF antibody (Table 7) in PBS alone or PBS containing 1% Triton X-100. Following three washes (PBS), the cells were incubated 30 min with goat anti-rabbit Alexa-488 (fluoresces green) conjugated secondary antibodies and DAPI (stains mammalian/bacterial DNA; fluoresces blue). Coverslips were mounted in FluorSave reagent on glass slides for analysis (Zeiss Axioskop Epifluorescence microscope with an 100x oil objective and/or Leica TCS SP2 UV confocal laser-scanning microscope [Leica Microsystems GMBH Heidelberg]) with an 63x 1.32 NA oil-immersion objective; housed within the Bio-Imaging Unit, Newcastle University. The captured images were processed using Image-J software.

2.6 Detecting T3S secreted proteins

Studies used a previously described protocol (Kenny and Finlay, 1995). Following infection in DMEM- using previously described conditions/MOI (see above 2.5), the media was collected and centrifuged for 10 min to remove the bacteria. Trichloroacetic acid (TCA; 10% v/v final concentration) was added to the resulting supernatant which contains secreted proteins then left for 1 h at 4°C before pelleting the precipitated proteins and re-suspended in Laemmli sample buffer before resolution by SDS/PAGE 12%.

2.7 Statistical analysis

Statistical analysis was performed using GraphPad Prism V6.0 (GraphPad software) to analyse data sets using a one-way (ANOVA) analysis of variance with additional Dunnett's test (*) compared with control positive (EPEC) or (*) compared with control negative (T3SS) or Tukey's test for multiple comparisons between strains. P-values of $p<0.05$ were considered significant.

EPEC Strain	Description	Selection	Reference
EPEC E2348/69	Prototypical (O127:H6) strain.	Nal^R	(Levine *et al.*, 1985)
T3SS (*cfm*-14)	Lacks functional type 3 secretion system.	Nal^R, Km^R	(Jarvis *et al.*, 1995)
map	Lacks Map effector.	Nal^R	(Kenny and Jepson, 2000)
tir	Lacks residues 50-320 of Tir effector.	Nal^R	(Kenny *et al.*, 1997b)
tir_{full}	Lacks all Tir residues.	Nal^R	This study
eae	Lacks Intimin outer membrane protein.	Nal^R	(Donnenberg and Kaper, 1991)
espH::Km	Lacks EspH effector.	Km^R	(Tu *et al.*, 2003)
espH	Lacks EspH effector.	Nal^R, Km^R	Unpublished, Kenny lab
$espH_{full}$	Lacks EspH effector.	Nal^R	(Dong *et al.*, 2010)
espF	Lacks EspF effector.	Nal^R	(Warawa *et al.*, 1999)
espB (eaeB)	Lacks EspB translocator protein.	Nal^R	(Foubister *et al.*, 1994)
espZ (81)	Lacks EspZ effector.	Nal^R	Unpublished, Kenny lab
*espGorf3core**	Lacks EspG, EspG2/Orf3, EspH, Tir Map, Intimin and 2 chaperones (CesT, CesF).	Nal^R	(Ruchaud-Sparagano *et al.*, 2007)
espGorf3coreΔespZ(81)	As above but also lacks EspZ.	Nal^R	This study
me	Lacks Map & Intimin.	Nal^R	Unpublished, Kenny lab
tm	Lacks Tir & Map.	Nal^R	(Jepson *et al.*, 2003)
et	Lacks Intimin & Tir.	Nal^R	(Ruchaud-Sparagano *et al.*, 2011)
et_{AA}	Lacks Intimin & Tir.	Nal^R	This study
fh	Lacks EspF & EspH.	Km^R	Unpublished, Kenny lab
fh_{AA}	Lacks EspF & EspH.	Km^R	This study
fe	Lacks EspF & Intimin.	Nal^R	Unpublished, Kenny lab
fe_{AA}	Lacks EspF & Intimin.	Nal^R	This study
ft	Lacks EspF & Tir.	Nal^R	Unpublished, Kenny lab
ft_{AA}	Lacks EspF & Tir.	Nal^R	This study
th	Lacks Tir & EspH.	Km^R	Unpublished, Kenny lab
mh	Lacks Map & EspH.	Km^R	Unpublished, Kenny lab
fm	Lacks EspF & Map.	Nal^R	(Dean and Kenny, 2004)
fm_{AA}	Lacks EspF & Map.	Nal^R	This study
eh	Lacks Intimin & EspH.	Km^R	Unpublished, Kenny lab
feh	Lacks EspF, Intimin & EspH.	Km^R	Unpublished, Kenny lab
fth	Lacks EspF, Tir & EspH.	Km^R	Unpublished, Kenny lab
tmf	Lacks Tir, Map & EspF.	Nal^R	Unpublished, Kenny lab
fet	Lacks EspF, Intimin & Tir.	Nal^R	Unpublished, Kenny lab
fme	Lacks EspF, Map & Intimin.	Nal^R	Unpublished, Kenny lab
meh	Lacks Map, Intimin & EspH.	Km^R	Unpublished, Kenny lab
fmh	Lacks EspF, Map & EspH.	Km^R	This study
tmh	Lacks Tir, Map & EspH.	Km^R	This study
eth	Lacks Intimin, Tir & EspH.	Km^R	Unpublished, Kenny lab
etm	Lacks Intimin, Tir & Map.	Nal^R	Unpublished, Kenny lab
etmh	Lacks Intimin, Tir, Map & EspH.	Km^R	Unpublished, Kenny lab
tmfh	Lacks Tir, Map, EspF & EspH.	Km^R	Unpublished, Kenny lab

fmeh	Lacks EspF, Map, Intimin & EspH.	Km^R	Unpublished, Kenny lab
feth	Lacks EspF, Intimin, Tir & EspH.	Km^R	Unpublished, Kenny lab
etmf	Lacks Intimin, Tir, Map & EspF.	Nal^R	(Quitard *et al.*, 2006)
etmfh (quin)	Lacks Intimin, Tir, Map EspF & EspH.	Km^R	This study
TOEA7	Lacks 14 effectors (on PP2, IE2, PP4, IE6, PP6, IE5 gene regions) & EspG.		(Yen *et al.*, 2010)
TOEA7/pTet	As above but carries pACYC184 plasmid (lacks gene encoding Cm^R).	Tet^R	Unpublished, Kenny lab
TOEA7Δcore/pTet	As above but also lacks EspH, CesF, Map, Tir, CesT & Intimin.	Tet^R	Unpublished, Kenny lab

Bacterial Strain	Description	Selection	Reference
DH10B	Non-pathogenic K12 *E. coli* strain.		(Grant *et al.*, 1990)
TOB01 (*E. coli* K12)	K12 *E. coli* strain carrying EPEC B171-8 *bfp* and *perABC* operons on pTOK-01 plasmid. Also has pTOK-02 (no cloned inserts).	Km^R, Cm^R	(Yen *et al.*, 2010)
TOB02 (*E. coli* K12)	As per TOBO1 but also LEE region (from EPEC B171-8) on pTOK-02.	Km^R, Cm^R	(Yen *et al.*, 2010)
Yersinia enterocolitica MRS40 (pIML421)	Lacks main *Yersinia* T3SS Yop effectors.	Nal^R	(Iriarte and Cornelis, 1998)

Table 2. List of EPEC and non-EPEC strains used in this study providing a brief description of key characteristics, antibiotic used for selection (Nal^R, Km^R, Cb^R, Cm^R, Tet^R indicates resistance to Nalidixic acid, Kanamycin, Carbenicillin, Chloramphenicol and/or Tetracycline) and source of strain. "Km" and "full" indicates that associated genes was disrupted by introduction of a gene encoding Kanamycin resistance or removal of sequence between start and stop codons, respectively. Note, gene name indicates order of gene disruption, for example, *ft* reveals that the *tir* gene was disrupted from an *espF* mutant background though order of *espG*/*espG2* gene disruption in indicated (*) strain not known.

Plasmid	Encodes	Selection	Reference
pSK-*cesF*::HA	CesF as a CesF::HA fusion protein.	Cb^R	Provided by Brendan Kenny
pSK-*map*	Map.	Cb^R	(Kenny and Jepson, 2000)
pCVD438-*eae*	Intimin.	Cm^R	(Donnenberg and Kaper, 1991)
pACYC-*espH*::HA	EspH as an EspH::HA fusion protein.	Cm^R	Provided by Brendan Kenny
pGFP	Green Fluorescent Protein.	Cb^R	Provided by Brendan Kenny
pBR322-*espF*	EspF.	Cb^R	(Nagai *et al.*, 2005)
pBR322-*espF*(A3)	EspF with leucine 104, 151 and 197 substituted for alanine.	Cb^R	(Dean *et al.*, 2013)
pSK-Δ101*espF*	EspF lacking residues 1-101 (replaced with those from Tir).	Cb^R	(Quitard *et al.*, 2006)
pACYC-T7-*espF*	EspF as T7::EspF fusion protein.	Cm^R	(Dean and Kenny, 2004)

pBR322-*espF* (L16E)	EspF cannot target mitochondria.	Cb^R	(Nagai *et al.*, 2005)
pBR322-*espF* (L16E; Δ100-183)	As above but also lacks residues 100-183.	Cb^R	(Holmes *et al.*, 2010)
pBR322-*espF* (Δ21-183)	EspF lacking residues 21-183.	Cb^R	Provided by Brendan Kenny
pBR322-*espF* (L16E; Δ51-168)	As *espF* (L16E) but also lacks residues 51-168.	Cb^R	Provided by Brendan Kenny
pBR322-*espF* (L16E; Δ5)	As *espF* (L16E) but also lacks five base pairs (499-503).	Cb^R	Provided by Brendan Kenny
pBR322-*espF* (L16E; Δ51-73)	As *espF* (L16E) but also lacks residues 51-73.	Cb^R	Provided by Brendan Kenny
pBR322-*espF* (L16E; P74A)	As *espF* (L16E) but with 4 proline residues, 79-82, changed to alanine.	Cb^R	Provided by Brendan Kenny
pBR322-*espF* (L16E; Δ62-73)	As *espF* (L16E) but also lacks residues 62-73.	Cb^R	Provided by Brendan Kenny
pBR322-*espF* (L16E; Δ23-52)	As *espF* (L16E) but also lacks residues 23-52.	Cb^R	Provided by Brendan Kenny
pBR322-espF (Δ21-73)	EspF lacking residues 21-73.	Cb^R	(Holmes *et al.*, 2010)
pBR322-*espF* (L16E; Δ51-73 and Δ117-162)	As *espF* (L16E) but also lacks residues 51-73 & 117-162.	Cb^R	Provided by Brendan Kenny
pBR322-*espF* (L16E; S47A)	As *espF* (L16E) but also with serine 47 changed to alanine.	Cb^R	Provided by Brendan Kenny
pACYC-*tir_cesT*	Tir and CesT.	Cm^R	(Kenny *et al.*, 2002)
pACYC-*tir* R521_*cesT*	CesT and Tir with arginine 521 substituted for alanine.	Cm^R	(Kenny *et al.*, 2002)
pACYC-*tir* S463A_*cesT*	CesT and Tir unable to be phosphorylated on S463.	Cm^R	Provided by Brendan Kenny
pACYC-*tir* Y474F_*cesT*	CesT and Tir unable to be phosphorylated on Y474.	Cm^R	Provided by Brendan Kenny
pACYC-*tir* Y474-454F S434A-463A_*cesT*	CesT and Tir unable to be phosphorylated	Cm^R	Provided by Brendan Kenny
pACYC-*tir* polyproline_*cesT*	CesT and Tir cannot form pedestal.	Cm^R	Provided by Brendan Kenny
pACYC-*tir* S434A_*cesT*	CesT and Tir unable to be phosphorylated on S434.	Cm^R	Provided by Brendan Kenny
pACYC-*tir* S434/463A_*cesT*	CesT and Tir unable to be phosphorylated on S434 and S463.	Cm^R	Provided by Brendan Kenny

Table 3. List of plasmids used in this study providing a brief description of key encoded factor(s), antibiotic used for selection (Cb^R, Cm^R indicates resistance to Carbenicillin and Chloramphenicol respectively) and source of plasmid.

Name	Oligonucleotide sequence 5'-3'
eae Forward Primer	CATTCTAACTCATTGTGGTGG
eae Reverse Primer	CTAGCTAGAGACTTGATTACC
map Forward Primer	GTGCTGGAGGAAAAGTTCTG
map Reverse Primer	CAGCGCAGTAAGTTTCC
espH Forward Primer	CCCTTTGGCAACCGTAAAGC
espH Reverse Primer	AAATATCGTCCCCAGAACAG
espF Forward Primer	ATGGAATTAGTAACGCTGCTTCTACAC
espF Reverse Primer	TTGGTTACCCTTTCTTCGATTGCTCATAG
tir Forward Primer	CCGCCACTACCTTCACAAAC
tir Reverse Primer	GCGTTGGTGCGGCATTTACAG
nleF Forward Primer	AAGGGGGTTTTGATATGTTACCAACAAGTG
nleF Reverse Primer	CCACGAGGCATTTCATTGCTCGTAG
nleE1 Forward Primer	CCAGTATGTATACCAGCAGTTCATGGTAAG
*nleE1*Reverse Primer	ATTGGGCGTTTTCCGGATATAACTG

Table 4. Oligonucleotides used in this study to PCR determine the presence of intact or disrupted EPEC genes.

Stage	Temperature	Time	Cycles
Initial denaturation	95°C	30 sec	1
Denaturation	95°C	30 sec	30
Annealing	56-68°C	60 sec	
Extension	68°C	60 sec	
Final Extension	68°C	5 min	1

Table 5. Polymerase chain reaction (PCR) Thermocycling conditions used to amplify EPEC's genes with Taq DNA polymerase.

Suicide Plasmid	To disrupt the	Antibiotic Selection	Reference
pCVD422-Δ*tir*$_{full}$	*tir* gene.	Cb^R	Provided by Brendan Kenny
pCVD422-Δ*tir*	*tir* gene	Cb^R	(Kenny *et al.*, 1997b)
pCVD422-Δ*espZ* (81)	*espZ* gene.	Cb^R	Provided by Brendan Kenny
pCVD422-Δ*espF*	*espF* gene.	Cb^R	(Warawa *et al.*, 1999)
pCVD422-Δ*map*	*map* gene.	Cb^R	(Kenny and Jepson, 2000)
pCVD422-Δ*eae*	Intimin-encoding gene.	Cb^R	(Donnenberg and Kaper, 1991)
pCVD422-Δ*espH*::Km	*espH* gene	Cb^R	Provided by Brendan Kenny
pCVD422-Δ*espH*$_{full}$	*espH* gene.	Cb^R	(Dong *et al.*, 2010)

Table 6. List of suicide vectors used in this study indicating which gene can disrupt, antibiotic used for selection (Km^R, Cb^R indicates resistance to Kanamycin and Carbenicillin respectively) and source of plasmid "::Km" indicates that associated gene was disrupted by introduction of a gene encoding Kanamycin resistance while "81" reveals absence of sequence encoding 81 of 98 EspZ residues.

Antibody	Type	Dilution (IF)	Dilution (WB)	Supplier & Catalogue #
Primary Antibody				
Anti-EPEC 0127	Rabbit (monospecific *E. coli* OK) antiserum	1/100	NA	Statens Serum Institut; #44305
Anti-*E. coli*	Rabbit (polyclonal)	1/100	NA	AMS Biotech; Cat # B65001R
Anti-Tir	Rabbit (polyclonal)	NA	1/5000	Kenny Lab
Anti-EspF	Rabbit (polyclonal)	1/100	1/2000	Kenny Lab
Anti-EspB	Rabbit (polyclonal)	NA	1/2000	Kenny Lab
Anti-HA	Mouse (monoclonal)	NA	1/1000	Sigma; # H3663
Secondary Antibody				
Anti Rabbit IgG-HRP	Goat	NA	1/5000	Jackson Immuno Research; #111-035-003
Anti Mouse IgG-HRP	Goat	NA	1/5000	Jackson Immuno Research; #115-035-003
Anti-Rabbit IgG-AP	Goat	NA	1/5000	Jackson Immuno Research; #111-055-144
Anti-Mouse IgG-AP	Goat	NA	1/5000	Jackson Immuno Research; #115-055-146
Anti Rabbit Alexa Fluor 555	Goat	1/100	NA	Molecular Probes; #A11008
Anti Rabbit Alexa Fluor 488	Goat	1/100	NA	Molecular Probes; #A21428
DAPI (DNA staining)	NA	1/1000	NA	Invitrogen; #D1306

Table 7. List of antibodies used for immunofluorescence (IF) and/or western blot (WB) analyses providing information on type, dilution and source.

Chapter 3. EspF is not sufficient to inhibit the uptake of EPEC by J774.A1 macrophages

3.1 Introduction

The T3SS dependent delivery of EspF into host cells has been linked to many subversive events such as: microvilli effacement, apoptosis, mitochondrial dysfunction, nucleolus disruption, ion transport inactivation, TJ barrier disruption and anti-phagocytosis (Celli *et al.*, 2001; Crane *et al.*, 2001; McNamara *et al.*, 2001; Dean and Kenny, 2004; Nougayrède and Donnenberg, 2004; Nagai *et al.*, 2005; Shaw *et al.*, 2005a; Dean *et al.*, 2006; Guttman *et al.*, 2006; Quitard *et al.*, 2006; Alto *et al.*, 2007; Peralta-Ramirez *et al.*, 2008; Dean *et al.*, 2010b). EspF's capacity to do all these subversive events is linked to the presence of EspF in several cellular compartments i.e. host cytoplasm, mitochondria, plasma membrane (apical and lateral surfaces) and nucleus (Dean and Kenny, 2009; Holmes *et al.*, 2010).

To carry out all the subversive activity mentioned above, EspF need to be translocated efficiently into the host cells. Translocation of the effector EspF into host cells is dependent on the LEE encoded chaperone CesF chaperone, which interacts specifically with EspF but not with other EPEC effectors (Elliott *et al.*, 2002). CesF is essential for stability and efficient translocation of EspF into host cells; however it is not essential for EspF secretion (Elliott *et al.*, 2002).

It has been well established that EspF plays a key role in inhibiting EPEC uptake by phagocytic cells such as macrophage and M-cell models *in vitro* and *in vivo* (Quitard *et al*., 2006; Martinez-Argudo *et al*., 2007; Marchès *et al*., 2008; Tahoun *et al*., 2011). However, the EspF inhibitory mechanism remains undefined.

To address whether the EspF effector protein, in the absence of other EPEC factors, encodes sufficient information to enable EPEC to inhibit its uptake by macrophages, different approaches could be used to introduce EspF into mammalian cells. For example, i) introducing EspF protein by micro-injection and ii) delivering EspF protein into macrophages via T3SS of different pathogen, as used previously to deliver the EPEC Tir effector into host cells (Kenny and Warawa, 2001) iii) expressing EspF within the cells by transfection, as used previously to transfect HeLa cells and Caco-2 cells (Crane *et al.*, 2001; Dean *et al.*, 2010b; Dean *et al.*, 2013). However, it is very difficult to transfect phagocytic cells such as macrophages with the limitation of transfection including cytotoxicity and low transfection efficiency (Arkusz *et al.*, 2006).

The aim of this chapter was to test whether the EspF effector protein encodes sufficient information to enable EPEC to inhibit its uptake by macrophages. This was achieved through i) delivering EspF protein into macrophages via T3SS of *Yersinia* to avoid some of the

limitations identified in J774A.1 macrophages transfection ii) testing the anti-phagocytosis capacity of an EPEC strains unable to deliver most known effectors except EspF.

3.2 Results

3.2.1 EPEC requires a functional type three secretion system (T3SS) and EspF effector protein to inhibit its uptake by J774.A1 macrophages

EPEC has a T3SS-dependent capacity to inhibit its uptake by J774.A1 macrophages via phosphoinositide 3 (PI 3)-kinase dependent pathways (Celli *et al.*, 2001; Quitard *et al.*, 2006). However, many EPEC bacteria (~40%) are still internalised via PI 3-kinase independent pathways (Celli *et al.*, 2001; Quitard *et al.*, 2006). Prior to investigate whether EspF was sufficient to inhibit EPEC uptake by macrophages, it was necessary to re-establish the anti-phagocytosis assay. To determine the percentage of internalised bacteria a published standard anti-phagocytosis protocol (Quitard *et al.*, 2006) was followed with minor changes designed to aid re-establishment of a robust assay. Accordingly, J774.A1 macrophages (60-80% confluent) were infected for 1 h with pre-induced (to activate the T3SS) bacterial strains that carry a Green Fluorescent Protein (GFP)-expressing plasmid (see Materials and Methods). Moreover, after infection the cells were washed (to remove non-adherent bacteria) and incubated for 1 h with DMEM supplemented with chloramphenicol (stops bacterial protein synthesis) to promote uptake of adherent bacteria. An antibody based approach was used to label the extracellular bacteria (Red) as described (Goosney *et al.*, 1999; Celli *et al.*, 2001; Quitard *et al.*, 2006) (see Materials and Methods). The percentage of phagocytosed bacteria was calculated as: (Total cell-associated bacteria [Green] minus extracellular bacteria [Red]/Total cell-associated bacteria [Green]) x 100. A range of multiplicity of infections (MOIs) were examined with 100:1 (bacteria: macrophages) found to be the best and most reproducible to recapitulate previously published results (Quitard *et al.*, 2006). Initial infections involved EPEC, T3SS mutant, EspF deficient (*espF*) mutant and DH10B (non-pathogenic *E. coli*) strains carrying a GFP-expressing plasmid (see Materials and Methods). PCR analysis was used to confirm the presence of an intact *espF* gene in only EPEC and T3SS mutant strains (Figure 14A).

Prior to carrying out the phagocytosis assay, the nature of the T3SS mutant (unable to translocate effectors) was interrogated by infecting J774A.1 macrophages (see Materials and Methods) which were fractionated into Triton X-100 soluble fractions (contain host cytoplasmic and membrane proteins plus T3SS-delivered effectors) and insoluble (contains host nuclei and cytoskeleton proteins plus adherent bacteria) fractions for western blot analysis. As previously described (Kenny *et al.*, 1997b; Kenny and Finlay, 1997) Tir shifting in the soluble fraction was used as a marker of a functional effector delivery system, as it is the first and abundant EPEC protein delivered into target cells (Mills *et al.*, 2008) linked to shifts i

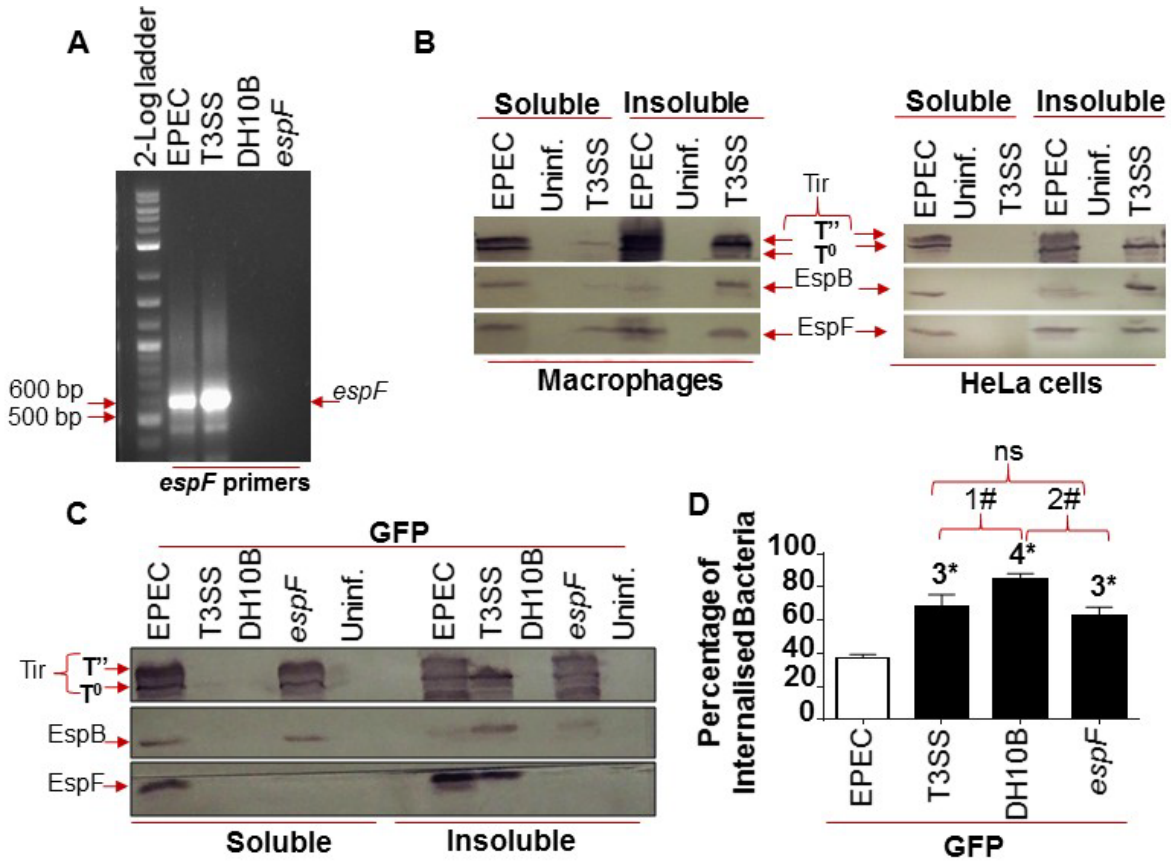


Figure 14. EPEC requires a functional type 3 secretion system (T3SS) and EspF effector protein to inhibit its uptake by J774.A1 macrophages. Indicated strains were used for (A) PCR analysis or to infect (B&D) J774.A1 macrophages or (B&C) HeLa cells. (A) Oligonucleotides specific to *espF* were used in PCR analyses to determine the presence of an intact (618bp) or deleted (~180bp) *espF* gene with PCR products visualised, alongside molecular mass markers 2-log (1000Kb ladder), on a 1% agarose gel. (B&D) Macrophages and HeLa (C&D) cells were left uninfected (Uninfect.) or infected MOI of 100:1 with indicated strains for 3 h before isolating Triton X-100 soluble (contains host cytoplasmic and membrane proteins plus T3SS-delivered effectors) and insoluble (contains host nuclei and cytoskeleton plus adherent bacteria) fractions. Samples were examined by western blot analysis (12% SDS-PAGE) probing with anti-EspF, anti-Tir and anti-EspB antibodies. Arrows indicate the position of Tir (unmodified T^0 and host kinase modified T'' forms), EspF and EspB proteins. (D) J774.A1 macrophages were infected for 1 h (MOI 100:1) with indicated strains (pre-grown in DMEM for 3 h) prior to determining the percentage of internalised bacteria using antibodies to label extracellular bacteria (Red) with total cell-associated bacteria detected by plasmid expression of the Green Fluorescent Protein (Green). Fifty macrophages with 20-50 cell-associated bacteria were randomly chosen to count the number of extracellular and total cell-associated bacteria (Green) enabling the percentage internalisation to be calculated. (D) Quantification studies were carried out in a semi-blind manner with the percentage of internalised bacteria displayed as the mean value (± standard deviation) from at least three independent experiments. * indicates statistically significant differences (Dunnett's post-test) compared to EPEC data (3* $p< 0.001$ 4* $p< 0.0001$; one-way ANOVA*)* or by Tukey's test for multiple comparisons between strains (1#$p<0.05$, 2#$p<0.01$ and ns not significant). Strains used were EPEC, T3SS-deficient (T3SS) and EspF-deficient (*espF*) mutants as well as non-pathogenic K12 (DH10B) *E. coli*, which all carried a GFP-expressing plasmid.

apparent molecular mass from its unmodified form (T^0) to modified form ($T^{''}$) due to the addition of phosphate groups on serine residues by host kinases (Warawa and Kenny, 2001). As expected (Kenny *et al.*, 1997b; Kenny and Finlay, 1997), EPEC infection led to the detection of the T'' form in the soluble and, due to its interaction with the EPEC surface protein Intimin, insoluble fractions (Figure 14B). Moreover, the EspB and EspF effector proteins were also evident in the soluble and insoluble fractions. Unexpectedly, the T3SS mutant infection was linked to low levels of Tir (only unmodified form), EspF and EspB proteins in the soluble fraction (Figure 14B and data not shown) suggestive of non-specific contamination as Tir would be modified to T'' form if in host cytoplasm. To interrogate whether this background issue related to the fractionation procedure or cell type (phagocytic), the infection was repeated using non-phagocytic HeLa cells routinely deployed for fractionation studies in the Kenny laboratory. As reported (Kenny *et al.*, 1997b; Kenny and Finlay, 1997) both forms of Tir (T^0 and $T^{''}$) were detected in the soluble and insoluble fractions of EPEC-infected cells. By contrast, Tir was only evident in the insoluble, not soluble, fraction of T3SS mutant-infected HeLa cells (Figure 14B) and only in the unmodified form thereby illustrating T3SS-dependent delivery. These results suggest that the standard fractionation protocol with macrophages cannot be used to demonstrate T3SS dependent delivery of effectors, possibly due to the internalisation of bacteria and their degradation in lysosomes. Therefore, fractionation studies were carried out with HeLa cells to test T3SS functionality prior to testing the anti-phagocytic capacity of strains. Thus, HeLa cells were infected with EPEC, T3SS mutant, EspF deficient (*espF*) and DH10B (non-pathogenic *E. coli*) strains. As expected, Tir, EspF or EspB proteins were not evident in the soluble fraction of uninfected, T3SS mutant or DH10B infected cells while EspF, EspB and Tir (T^0 and $T^{''}$ forms) were detected in the soluble and insoluble fractions of EPEC-infected cells (Figure 14C). No band for EspF protein was detected in the soluble and insoluble fractions of *espF* -infected cells which confirm the nature of this mutant strain (Figure 14C).

Having supported the correct nature of these GFP plasmid-carrying strains, they were used to infect macrophages to determine their capacity to inhibit phagocytosis, as outlined above. Infection with the T3SS mutant resulted in most (68.5 ± 5.7%) bacteria being internalised in contrast to only a minority (37.1 ± 1.7%) of cell-associated EPEC bacteria (Figure 14D), consistent with previous studies (Celli *et al.*, 2001; Quitard *et al.*, 2006). Noticeably, EPEC's ability to block PI 3-kinase-dependent phagocytic pathways was confirmed by infecting macrophages, which were treated with LY294002 inhibitor (to block the PI-3 kinase phagocytic pathways), with T3SS mutant that reduce the internalisation to levels indistinguishable from wild type EPEC (data not shown). The internalisation process

responsible for the uptake of ~37% of EPEC presumably relates to PI-3 kinase independent pathways (Celli *et al.*, 2001; Quitard *et al.*, 2006). Moreover, as reported, the *espF* mutant behaved like the T3SS mutant (no statistical difference) while, interestingly, the K12 strains displayed a greater ($p<0.01$) defect (Figure 14D). The latter finding suggests that EPEC also possesses T3SS-independent mechanisms that contribute to EPEC capacity to inhibit its uptake. This work confirms that EPEC inhibits its uptake by J774A.1 macrophages in a manner dependent on a functional T3SS and the EspF effector protein and also suggests that there may be a T3SS-independent inhibitory contribution.

3.2.2 Critical role for LEE, but not 14 Nle effectors in EPEC's T3SS-dependent anti-phagocytc activity

Having confirmed EspF's critical role in the anti-phagocytosis process, studies were undertaken to examine EspF sufficiency. EPEC subversion of host cell functions and signalling pathways depends, mostly, on the activity of LEE and/or non-LEE-encoded (Nle) effector proteins (Dean and Kenny, 2009). Thus, to explore whether EspF requires functions of non-LEE effectors to inhibit PI3-kinase dependent phagocytosis, studies examined phagocytosis of a strain, TOE-A7, which lacks 14 non-LEE encoded effectors and LEE EspG (Yen *et al.*, 2010). Strain genotype was supported by PCR analyses (Amin, 2016). Importantly, western blot analysis of soluble fractions obtained after infection of HeLa cells revealed delivery of EspF and Tir (both T^0 and T'' forms; not shown; but see Figure 23A). The latter promoted infection studies with J774.A1 macrophages to determine their anti-phagocytic capacity compared to a positive (EPEC) and negative (*etmf*) control strains. The latter strain doesn't express three LEE effectors (Tir, Map, EspF) or Intimin surface protein and, like the *espF* mutant, is unable to inhibit PI-3 kinase dependent uptake pathways (Quitard *et al.*, 2006). As these strains do not carry a GFP-expressing plasmid, the percentage internalisation was determined by antibody labelling extracellular bacteria (Red) followed by total cell associated bacteria (Green)(Figure 13) (Heesemann and Laufs, 1985; Quitard *et al.*, 2006) (see Materials and Methods).

As reported, the *etmf* mutant strain was readily internalised by macrophages with the majority (67.7 ± 0.32%) inside the cells (Figure 15). In contrast, only 28.2 ± 3.9% of EPEC bacteria were phagocytosed, significantly less than the negative control (*etmf* mutant; $p<0.0001$; Figure 15). Infection of cells with the TOE-A7 strain resulted in 31.9 ± 3.9% being internalised which was statistically indistinguishable from the EPEC data (Figure 15) ruling out critical roles, individual or cooperative, for 15 (14 non-LEE plus EspG) effectors in EPEC's T3SS dependent ability to inhibit its uptake by J774.A1 macrophages.

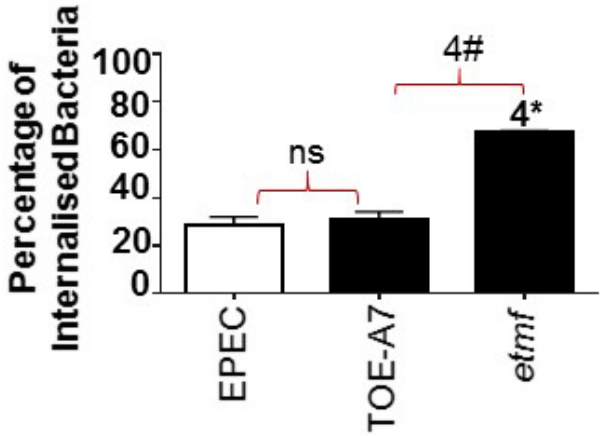


Figure 15. Ruling out critical roles for 14 non-LEE effectors and LEE EspG in EPEC's T3SS dependent ability to inhibit phagocytosis. J774.A1 macrophages were infected (MOI 100:1) with indicated strains (pre-grown in DMEM for 3 h) for 1 h prior to determining the percentage of bacterial internalisation following differential antibody stains labelling extracellular (Red) and total cell-associated (Green) bacteria. Fifty macrophages with 20-50 cell-associated bacteria were randomly chosen to count the number of extracellular and total cell-associated bacteria enabling the percentage internalisation to be calculated. Quantification studies were carried out in a semi-blind manner with the percentage of internalised bacteria displayed as the mean value (± standard deviation) from at least three independent experiments. * indicates statistically significant differences (Dunnett's post-test) compared to EPEC data (4* $p < 0.0001$ and ns not significant; one-way ANOVA*)* or by Tukey's test for multiple comparisons between strains (4# $p<0.0001$). Strains used were EPEC, *etmf* (lacks LEE-encoded Intimin [*eae* gene product] plus Tir, Map and EspF effectors) and TOE-A7 (lacks 14 non-LEE-encoded effectors plus LEE EspG).

As EPEC encodes at least three additional non-LEE-encoded effectors -EspC (Guignot *et al.*, 2015), NleJ and Lif A (Deng *et al.*, 2012) and probably other cryptic effectors- studies interrogated whether the LEE region encodes sufficient information to enable *E. coli* to inhibit its uptake. These studied used available (Yen *et al.*, 2010) non-pathogenic *E. coli* strains that carry two plasmids; one encodes EPEC's major adhesin (the bundle forming BFP) and positive regulator of LEE region expression (Per) (Giron *et al.*, 1991; Mellies *et al.*, 1999; Mellies *et al.*, 2007) with the second plasmid either encoding no new functions (TOB01 strain) or EPEC LEE region (TOB02 strains). PCR analysis supported strain genotype by confirming the presence of several *nle* genes in EPEC but not TOB01 or TOB02 strains (Figure 16A). Additionally, the EspF and Tir (T^0 and $T^{''}$ forms) proteins were detected in the soluble fraction of EPEC and TOB02, but not *etmf* or TOB01, -infected HeLa cells (Figure 16B). Infection of J774A.1 macrophages to determine strain anti-phagocytosis activity revealed TOB02 (25.9 ± 3.3% internalised) to behave like EPEC (30.02 ± 4.1 internalised) while TOB01 (81.1 ± 5.3% internalised) behaved like the negative, *etmf* mutant, control (74.8 ± 1.2 internalised; Figure 16C). Thus, these results reveal that the EPEC BFP and Per gene operons do not bestow onto *E. coli* the capacity to inhibit phagocytosis, unless the LEE region is also introduced thereby illustrating that the LEE region encodes sufficient information to enable *E. coli* (carrying BPF/Per operons) to inhibit its uptake by J774.A1 macrophages to a similar extent as EPEC presumably by providing the capacity to block PI 3-kinase dependent uptake pathways.

3.2.3. Yersinia can delivers EspF into host cells with efficient delivery dependent on co-expression of its chaperone, CesF

Having ruled out roles for 14 Nle effectors, demonstrated LEE sufficiency and confirmed that EspF is essential for *E. coli* to inhibit their uptake by J774.A1 macrophages, studies were undertaken to determine if EspF alone could inhibit phagocytosis. *Yersinia pseudotuberculosis* has been shown to deliver, via its T3SS, the EPEC Tir effector into host cells with efficient delivery dependent on co-expressing the Tir chaperone, CesT (Warawa and Kenny, 2001). These studies employed a mutant lacking the main *Yersinia* encoded (Yop) effectors (Warawa and Kenny, 2001). Thus, studies examined if *Yersinia* could also delivery EspF into host cells in a manner dependent, or not, on its chaperone (CesF) and, if so, the impact of this delivered effector on PI3K-dependent phagocytosis.

As the previously used *Yersinia* strain is resistant to many antibiotics – introduced when engineering *yop* gene loss – this hindered the introduction of antibiotic selectable plasmids,

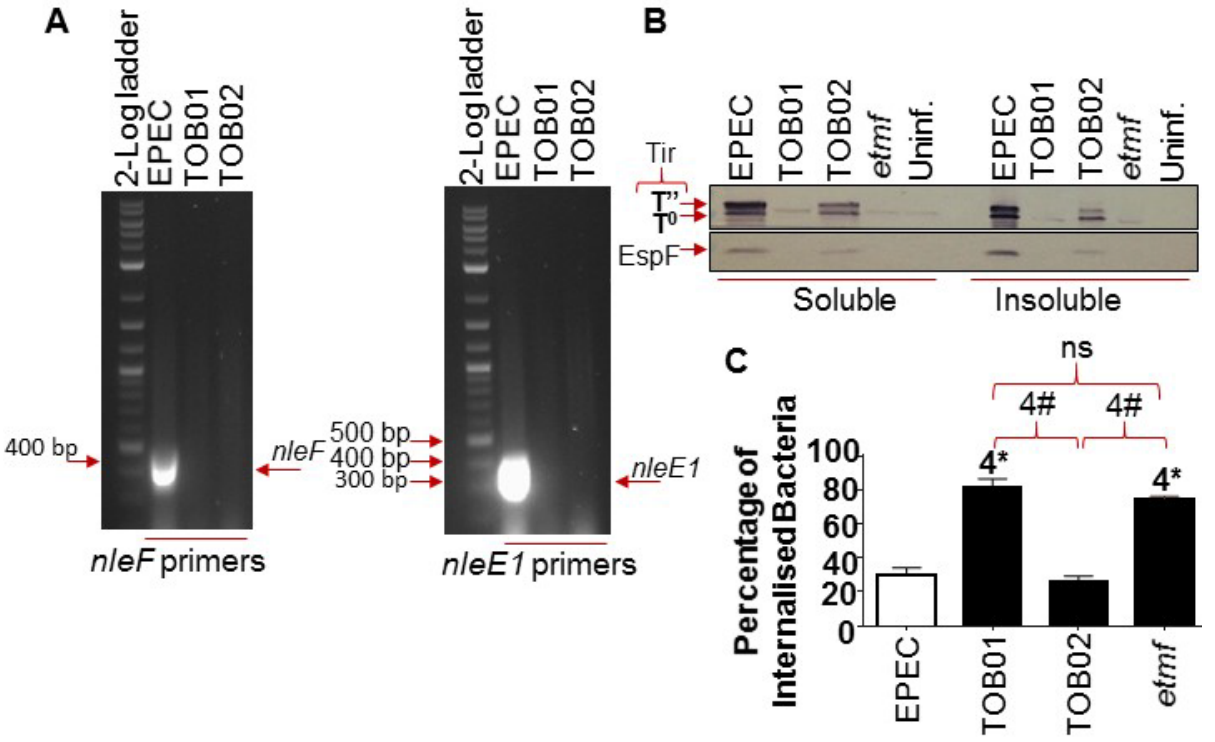


Figure 16. The EPEC LEE region encodes sufficient functions to enable non-pathogenic *E. coli* (carrying EPEC BFP and Per operons) to inhibit its uptake by J774.A1 macrophages. PCR probing for *nleF* and *nleE1* genes was used to support strain genotype (A) before infecting HeLa cells for western blot analysis (B) and J774A.1 macrophages for anti-phagocytosis assay (C). (A) PCR products were visualised, alongside molecular mass markers 2-log (1000Kb ladder), on a 1% agarose gel to determine the presence of intact *nleF* (390 bp) or *nleE1* (403 bp) genes. (B) HeLa cells were left uninfected or infected for 3 h (MOI 100:1) with indicated strains before isolating Triton X-100 soluble (contains host cytoplasmic and membrane proteins plus T3SS-delivered effectors) and insoluble (contains host nuclei and cytoskeleton plus adherent bacteria) fractions for western blot analysis (12% SDS-PAGE) probing for EspF and Tir proteins. Arrows indicate the position of Tir (unmodified T^0 and host kinase modified T'' forms) and EspF proteins. (C) J774.A1 macrophages were infected for 1 h (MOI 100:1) with the indicated strains (pre-grown in DMEM for 3 h) prior to determining the percentage of internalised bacteria using differential antibody staining to label extracellular (Red) and then total cell-associated (Green) bacteria. Fifty macrophages with 20-50 cell-associated bacteria were randomly chosen to count the number of extracellular and total cell-associated bacteria enabling the percentage internalisation to be calculated. Quantification studies were carried out in a semi-blind manner with the percentage internalised bacteria displayed as the mean value (± standard deviation) from at least three independent experiments. * indicates statistically significant differences (Dunnett's post-test) compared to EPEC data (4* $p < 0.0001$; one-way ANOVA*)* or by Tukey's test for multiple comparisons between strains (4# $p < 0.0001$; ns not significant). Strains used were EPEC, *etmf* (lacks LEE-encoded Intimin [*eae* gene product] plus Tir, Map and EspF effectors) and non-pathogenic *E. coli* strains TOB01 (carries plasmid encoding EPEC BFP adhesion and Per regulator plus 'empty' plasmid) and TOB02 (as TOB01 but with EPEC LEE region on an 'empty' plasmid).

and thus studies examined the utility of an antibiotic-sensitive Yop-deficient *Yersinia enterocolitica* strain (Iriarte and Cornelis, 1998). As the available *cesF* carrying plasmid encodes CesF as a CesF::HA fusion (Kenny; unpublished) shared the same antibiotic resistance (Chloramphenicol) as available EspF-expressing plasmids, studies used a carbenicillin-selectable plasmid that encodes EspF as a T7-EspF fusion protein (Dean and Kenny, 2004). Thus, initial studies were undertaken to determine if the N-terminal tag interfered with EspF's anti-phagocytic activity following introduction of the T7-EspF encoding plasmid into the EspF-deficient *etmf* quadruple mutant. First, infections were carried out on HeLa cells for western blot comparison of EspF and T7-EspF delivery levels. This analysis revealed similar EspF and, as evidenced by the slower migration (due to T7 tag), T7-EspF protein expression and delivery levels (Figure 17A). Importantly, these EspF and T7-EspF complemented strains were just as capable as EPEC, unlike the *etmf* control strain, at inhibiting their uptake by J774.A1 macrophages (Figure 17B) illustrating that the N-terminal T7 epitope tag does not affect EspF's ability to inhibit phagocytosis as reported (Quitard *et al.*, 2006).

Having confirmed that T7-EspF can be used as a substitute for EspF, studies examined the ability of *Yersinia* to express and deliver EspF into HeLa cells in a CesF-dependent or -independent manner. Thus, the T7-EspF plasmid was introduced into *Yersinia,* with or without the CesF-encoding plasmid, for a 3 h infection of HeLa cells at standard (100:1) MOI but with an additional centrifugation step to promote *Yersinia*-host cell interaction (Ruchaud-Sparagano *et al.*, 2011). Western blot examination of isolated soluble and insoluble fractions revealed EspF in the insoluble fractions of EPEC-infected cells, with T7-EspF evident in the insoluble fractions of cells infected with *Yersinia* strains carrying the pT7-*espF* plasmids in the presence or absence of p*cesF* plasmids (Figure 18). Linkage of the CesF-expressing plasmid with increased EspF signal is consistent with its published role in promoting EspF stability (Elliott *et al.*, 2002). Significantly, EspF related proteins were only detected in the soluble fractions of cells infected with EPEC or *Yersinia* carrying both, not single, *pcesF* and p*T7-espF* plasmids (Figure 18). CesF::HA and/or T7-EspF protein expression in the appropriate *Yersinia* strains was confirmed by probing with anti-T7 and anti-HA antibodies (not shown). In summary, these results demonstrate that *Yersinia* can express and deliver the EPEC EspF effector into HeLa cells in a manner promoted by co-expressing its chaperone, CesF.

3.2.4. Yersinia- delivered EspF does not inhibit the capacity of J774A.1 macrophages to internalise EPEC

Having demonstrated that *Yersinia* can deliver the EspF effector into cells in a manner

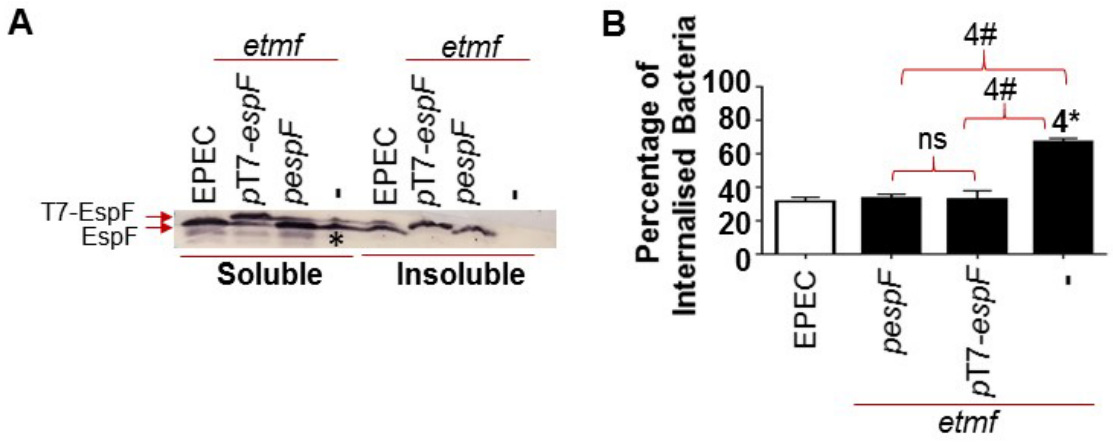


Figure 17. EspF and T7-EspF variants rescue the *etmf* mutant anti-phagocytosis defect. Indicated strains were used to infect (A) HeLa cells and (B) J774.A1 macrophages. (A) HeLa cells were infected for 3 h (MOI 100:1) with the indicated strains before isolating Triton X-100 soluble (contains host cytoplasmic and membrane proteins plus T3SS-delivered effectors) and insoluble (contains host nuclei and cytoskeleton plus adherent bacteria) fractions for western blot analysis (12% SDS-PAGE) probing for EspF. Arrows indicate the position of EspF and T7-EspF proteins. (A) * indicates cross-contamination of well. (B) J774.A1 macrophages were infected (MOI 100:1) with indicated strains (pre-grown in DMEM for 3 h) for 1 h prior to determining the percentage of internalised bacteria using differential antibody stains to label extracellular bacteria (Red) and then total cell-associated bacteria (Green). Fifty macrophages with 20-50 cell-associated bacteria were randomly chosen to count the number of extracellular and total cell-associated bacteria enabling the percentage internalisation to be calculated. (B) Quantification studies were carried out in a semi-blind manner with the percentage internalised bacteria displayed as the mean value (± standard deviation) from at least three independent experiments. * indicates statistically significant differences (Dunnett's post-test) compared to EPEC data (4* $p<0.0001$; one-way ANOVA*)* or by Tukey's test for multiple comparisons between strains (4# $p<0.0001$ and ns not significant). Strains used were EPEC, *etmf* (lacks LEE-encoded Intimin [*eae* gene product] plus Tir, Map and EspF effectors) and *etmf* carrying plasmids expressing EspF (*pespF*) or T7-EspF (p*T7-espF*).

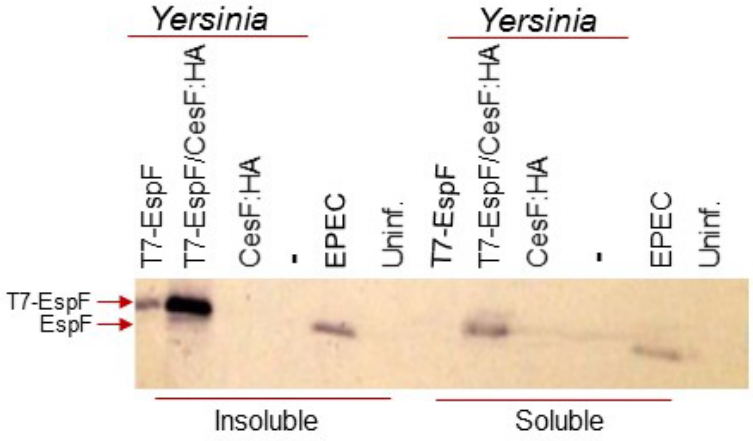


Figure 18. *Yersinia* delivers EspF into HeLa cells in a manner promoted by co-expressing its chaperone CesF. HeLa cells were left uninfected or infected (MOI 100:1) with indicated strains for 3 h before isolating Triton X-100 soluble (contains host cytoplasmic and membrane proteins plus T3SS-delivered effectors) and insoluble (contains host nuclei and cytoskeleton plus adherent bacteria) fractions for western blot analysis (12% SDS-PAGE) probing for EspF. Arrows indicate the position of EspF and T7-EspF proteins. The blot is representative of those obtained from three independent experiments. Strains used were EPEC, *Yersinia* carrying no plasmid (-) or plasmids encoding CesF (as a CesF::fusion protein) and/or T7-EspF proteins.

dependent on co-expressing its chaperone CesF, studies were undertaken to determine the best MOI to deliver T7-EspF to a similar extent as EPEC. Thus, HeLa cells were infected with *Yersinia* over a range of MOIs prior to isolating samples for western blot analysis which revealed an MOI of 80:1 to give T7-EspF signals comparable to the EspF signal of EPEC-infected cells (Figure 19). *Yersinia* uptake by macrophages is through PI-3 kinase independent pathways (Celli *et al.*, 2001), and thus assessing whether EspF delivery by this strain could inhibit PI-3 kinase independent pathways required the development of a two-wave infection protocol. For example determine if cells pre-infected with *Yersinia* would inhibit uptake of an EPEC T3SS (GFP-expressing) mutant. Thus, J774A.1 macrophages were infected with EPEC (MOI 100:1) and *Yersinia* (range of MOI's) strains for 1 h. The infections were stopped by adding fresh DMEM supplemented with antibiotic to kill extracellular bacteria for 1 h, followed by an hour recovery period (in DMEM +FCS). Post recovery, macrophages were infected for an hour with GFP-expressing EPEC T3SS mutant (second wave infection; MOI 100:1). Of note, the non-adherent bacteria were collected after the infection and retained for western blot analysis to support strains genotype (Figure 20A). Western blot analysis of the non-adherent bacterial samples revealed EspF and Tir expression in EPEC, but not *Yersinia* strains, with T7-EspF only evident in *Yersinia* strains carrying the pT7-*espF* and *pcesF* plasmids (Figure 20A) with signal strength increasing with MOI.

Following the second-wave infection, antibody staining was used to label the extracellular T3SS mutant bacteria (Red) with total cell-associated T3SS mutant bacteria revealed by their GFP-positive nature (see Materials and Methods). Calculating the percentage uptake of second-wave T3SS mutant bacteria revealed that only cells pre-infected with EPEC, not *Yersinia* or *Yersinia* carrying pT7-*espF* and *pcesF* plasmids, inhibited T3SS mutant uptake (~20% internalised compared to ~80% for *Yersinia* pre-infected cells; Figure 20B). The data is supported by additional experiments where the uptake was assessed, in a semi-blind manner, whether or not inhibit without quantification (not shown). These findings suggest that delivery of EspF into macrophages is not sufficient to inhibit their ability to internalise EPEC. However, an alternative unlikely possibility is that *Yersinia* can deliver EspF into HeLa but not J774A.1 macrophages. Therefore, imaging studies were undertaken to probe for *Yersinia* delivery of T7-EspF into macrophages.

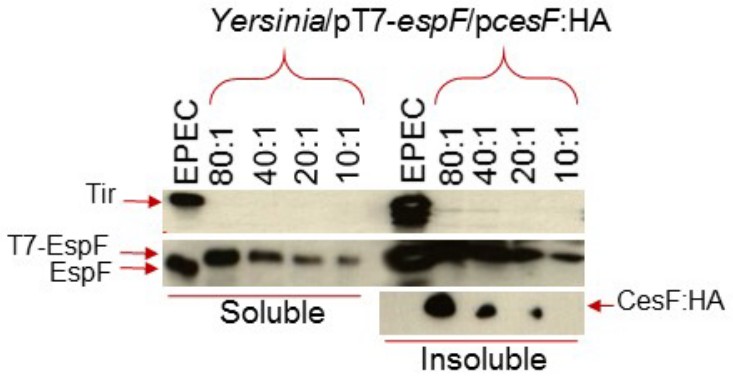


Figure 19. An MOI of 80:1 is best for *Yersinia* to deliver T7-EspF into HeLa cells to a similar extent as EPEC. HeLa cells were infected with EPEC (MOI of 100:1) and *Yersinia* (indicated MOI; using gentle centrifugation [500 xg, 5 minutes] to initiate macrophage-bacteria interaction) carrying plasmids encoding the EspF-specific chaperone CesF (as a CesF::Ha fusion protein) and/or T7-EspF protein. Following a 3 h infection the Triton X-100 soluble (contains host cytoplasmic and membrane proteins plus T3SS-delivered effectors) and insoluble (contains host nuclei and cytoskeleton plus adherent bacteria) fractions were isolated for western blot analysis (12% SDS-PAGE) to probe for EspF, Tir and HA-tagged proteins. Arrows indicate position of Tir, EspF, T7-EspF and CesF:HA proteins. The blot is representative of those obtained from three independent experiments.

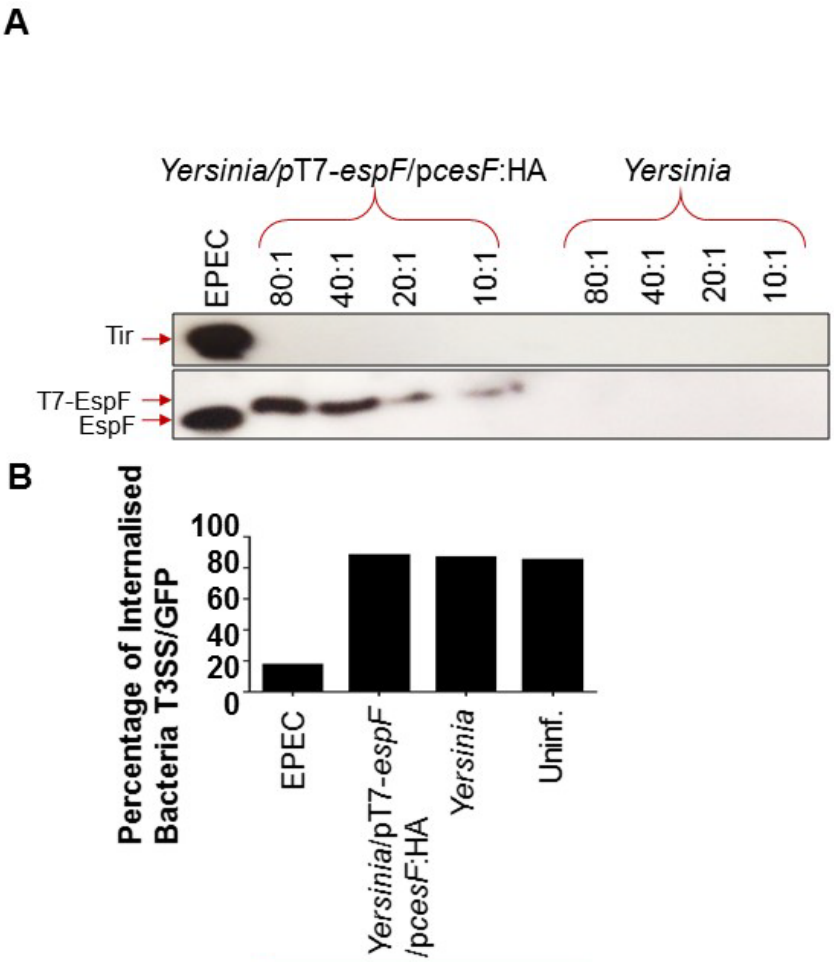


Figure 20. EspF is not sufficient to inhibit the uptake of EPEC by macrophages. J774.A1 macrophages were infected with indicated strains (pre-grown in DMEM for 3 h) using gentle centrifugation - 500 xg, 5 minutes- for 1 h prior to (A) isolating non-adherent bacteria for western blot analysis and (B) antibiotic killing extracellular bacteria prior to a 1 h infection with the T3SS mutant carrying a GFP-expressing plasmid (second wave of infection). *Yersinia* was added at the indicated MOI with EPEC and the T3SS mutant at 100:1. The extracellular bacteria were labelled 'Red' with total T3SS mutant evident by GFP 'Green' expression. Fifty macrophages with 20-50 cell-associated bacteria were randomly chosen to count the number of extracellular and total cell-associated bacteria. A two-wave infected T3SS/GFP bacteria were only counted which expresses both 'Green' (GFP) and 'Red', but not Red only (EPEC; first wave infection) (see Materials and Methods). Quantification studies were carried out in a semi-blind manner with the percentage of internalised bacteria revealed no difference between the different MOIs and, thus, only the highest was quantified. The data shown is representative of three independent experiments revealed similar results for each, thus, only one experiment was quantified. (A) Arrows indicate the position of Tir, EspF and T7-EspF proteins. Strains used were EPEC, T3SS/GFP, *Yersinia* carrying no plasmid or plasmids expressing T7-EspF and the EspF specific chaperone CesF::HA.

3.2.5. Immuno-detection of EspF, but not T7-EspF delivery into macrophages

EspF targets mitochondria where its accumulation can be detected by immunofluorescence microscopy (Nougayrède and Donnenberg, 2004; Dean *et al.*, 2010b). As previous studies used non-phagocytic cells, initial work involved HeLa cells infected for 3 h (MOI 100:1) with EPEC strains that can or cannot express or deliver EspF into cells. Post-infection, cells were washed and fixed before adding anti-EspF antibody in the presence and absence of detergent (1% Triton; enables antibodies to cross host membranes) for detection by secondary Alexa 488 conjugated (Green) antibodies. However, immunofluorescent imaging analysis revealed a high anti-EspF background signal with both uninfected and EPEC-infected cells when no detergent was present. Punctate aggregate signals in EPEC infected, not uninfected, cells when detergent was added (not shown) characteristic of EspF accumulation in mitochondria (Nougayrède and Donnenberg, 2004; Dean *et al.*, 2010b).

Repeat studies with J774.A1 macrophages provided similar results with high anti-EspF background for all cells that had not been exposed to detergent but also detergent-treated cells which were uninfected or infected with the effector delivery defective (*espB*) and EspF-deficient (*espF*) strains (Figure 21). Although more difficult to detect in macrophages, there was an obvious punctate EspF signal in EPEC infected cells that had been detergent-treated (Figure 21) that presumably reflects EspF accumulation in mitochondria. By contrast, no signal difference was evident for cells infected with strains (*etmf* mutant or *Yersinia*) expressing the T7-EspF variant when examined following addition or absence of detergent (data not shown). The latter data suggests that either T7-EspF is not delivered into macrophages or, more likely, that the N-terminal T7 tag prevents EspF targeting to mitochondria with accumulation in the cytoplasm obscured by the high background signal. To test the latter possibility, macrophages were infected with the EPEC and the *etmf*/pT7-EspF mutant strains to deliver EspF and pT7-EspF respectively with antibody probing revealing accumulation of only EspF in mitochondria (data not shown) supporting the idea that the T7 tag compromises the functionality of EspF's mitochondrial targeting sequence. Several approaches were undertaken to decrease the high anti-EspF background, for example by adding blocking agents, but none were successful.

Thus, another strategy was undertaken to negate the need to use the T7-EspF variant i.e. clone *espF* onto pACYC184 (chloramphenicol resistant) for introduction into *Yersinia* carrying p*cesF*::HA (carbenicillin resistant). Unexpectedly, introduction of pACYC-*espF* into this strain did not lead to high levels of EspF expression (sequencing confirmed the absence of unwanted PCR-introduced mutations) and/or EspF delivery into infected cells suggestive of plasmid incompatibility issues.

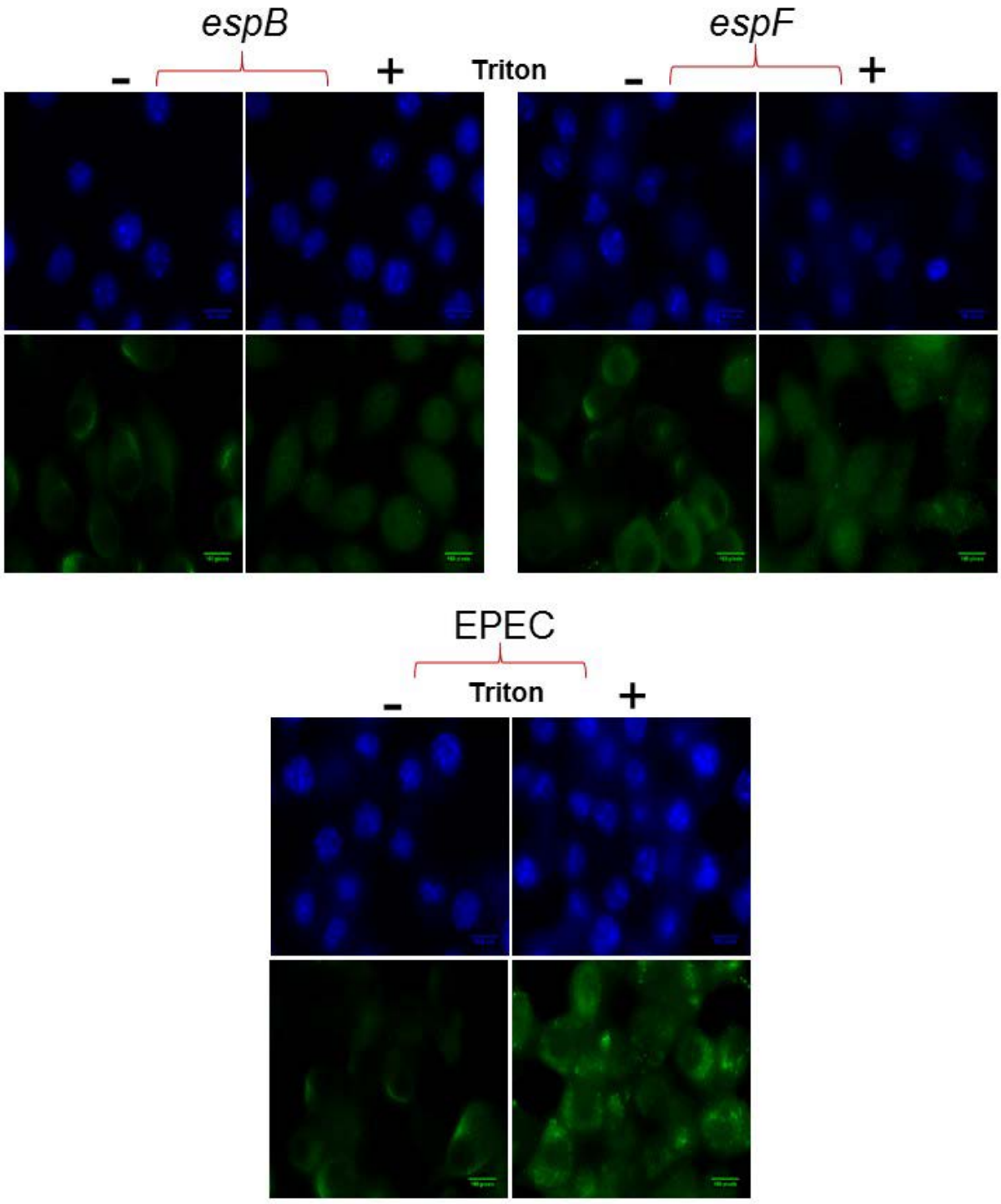


Figure 21. Immuno-detecting T3SS-dependent delivery of EspF. J774.A1 macrophages were infected for 3 h (MOI 100:1) with indicated strains prior to fixing cells and incubating with anti-EspF antibodies in PBS alone (-) or PBS containing 1% Triton (+) to make the host membrane permeable to the antibodies. The bound EspF antibodies were labelled with Alexa 488-conjugated (Green) secondary antibodies for immunofluorescent detection. DAPI was also added to detect DNA (host and bacterial; blue channel) see Materials and Methods. Strains used were EPEC, EspF-deficient (*espF*) and effector-delivery deficient (*espB*) mutants. The data shown is representative of three independent experiments. Images are representative of at least three independent experiments taken by epifluorescence microscope with an 100x oil objective. Scale bar 5.5 μm.

Despite the failure to demonstrate T7-EspF delivery into macrophages by *Yersinia*, the data collectively support the idea that EspF does not encode sufficient information to inhibit uptake of EPEC (T3SS mutant). To support this conclusion, another strategy was undertaken.

3.2.6. EspF requires the function of other effectors to inhibit EPEC's uptake by J774.A1 macrophages

To further explore EspF sufficiency to inhibit EPEC uptake by macrophages, studies interrogated the dependence of the subversive process on other effectors. Initial work focused on the available *espGorf3core* mutant strain which lack genes for Intimin, four LEE effectors (EspG, EspH, Tir, Map), a Nle (EspG2/Orf3) effector and the two effector-specific chaperones, CesF/CesT. While CesF is essential for EspF intra-bacterial stability and T3SS delivery (Elliott *et al.*, 2002), CesT is essential for the delivery of most LEE effectors (Tir, Map, EspH, EspZ). Indeed, Nle are either fully dependent (NleB2; NleG; EspJ; NleH1; NleH2) or only partially dependent (NleB1; NleC) on the chaperone CesT but, some effectors (eg EspG; NleD; NleF) have no CesF/CesT dependence (Thomas *et al.*, 2005; Mills *et al.*, 2013). Thus, this strain is, theoretically, unable to express and/or deliver many Nle and most LEE (including EspF) effectors. Thus, the p*cesF*::HA plasmid was introduced, generating *espGorf3core/pcesF*::HA, to restore EspF delivery prior to examining if the anti-phagocytosis mechanism depends on the activity of effectors not expressed or delivered by this strain.

Prior to investigating the strain's anti-phagocytosis capacity, HeLa cells were infected for western blot confirmation of restored EspF delivery. Examining the insoluble fractions illustrated that only one strain, *espGorf3core/*pcesF::HA, expressed a HA-tagged protein and that this was linked to restoring the EspF signal to the level found in the insoluble fraction of EPEC-infected cells (Figure 22A). As predicted, introducing p*cesF*::HA also restored the level of EspF found in the soluble fraction to that found in EPEC-infected cells (Figure 22A).

Having confirmed strain genotype, macrophages were infected to determine their ability to inhibit phagocytosis. As expected, the positive control, EPEC, inhibited uptake (only 30.1 ± 6.4% internalised) in contrast to the negative, *etmf* mutant, control (71.6 ± 7.6% internalised; Figure 22B). Crucially, the *espGorf3core* mutant behaved like the negative control whether it carried the p*cesF*::HA plasmid or not (Figure 22B) indicating roles for other effectors. Similar results (Figure 23A & 23B) were obtained with a more complex '*core*' mutant, TOE-A7Δ*core* (Table 2; Materials & Methods), which differs from *espGorf3core* by the additional absence of

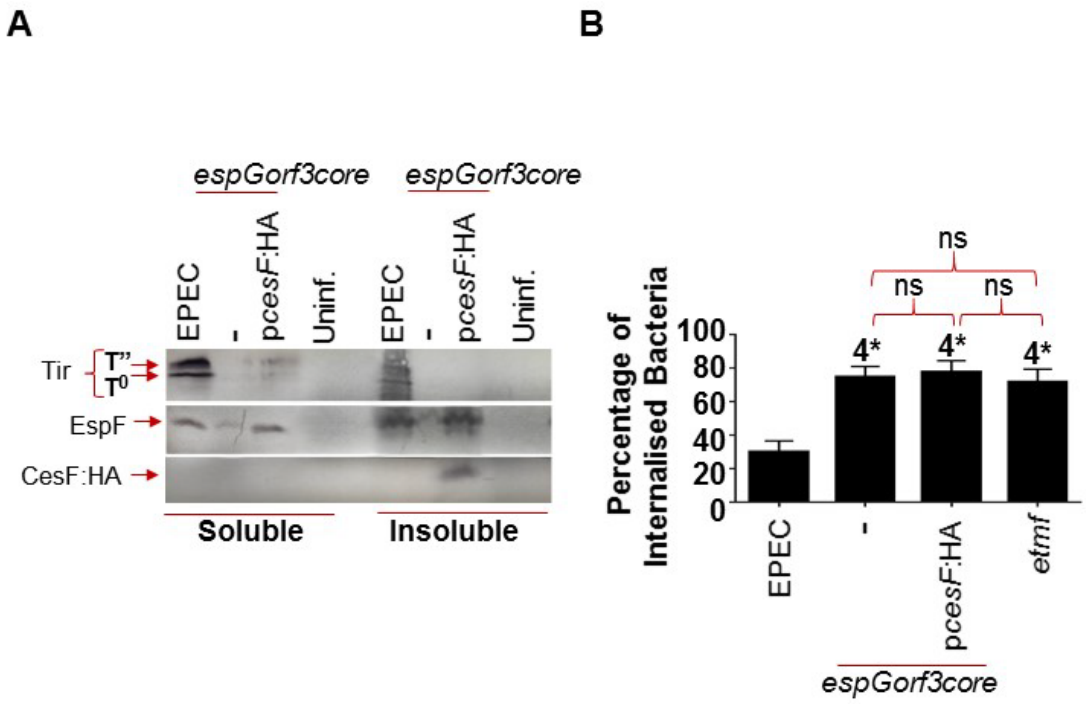


Figure 22. EspF requires the activity of other effectors to inhibit EPEC uptake by macrophages. Indicated strains were used to infect (A) HeLa cells for western blot analysis and (B) J774.A1 macrophages for the phagocytosis assay. (A) HeLa cells were left uninfected or infected for 3 h (MOI of 100:1) with indicated strains before isolating Triton X-100 soluble (contains host cytoplasmic and membrane proteins plus T3SS-delivered effectors) and insoluble (contains host nuclei and cytoskeleton plus adherent bacteria) fractions for western blot analysis (12% SDS-PAGE) probing for EspF, Tir and HA-tagged proteins. Arrows indicate the position of Tir (unmodified T^0 and host kinase modified T'' form), EspF and CesF::HA proteins. The blot is representative of those obtained from three independent experiments. (B) J774.A1 macrophages were infected 1 h (MOI 100:1) with indicated strains (pre-grown in DMEM 3 h) prior to determining the percentage bacterial internalisation using differential antibody stains distinguish extracellular bacteria (Red) from total cell-associated (Green) bacteria. Fifty macrophages, with 20-50 cell-associated bacteria, were randomly chosen to count the number of extracellular and total cell-associated bacteria enabling the percentage internalisation to be calculated. (B) Quantification studies were carried out in a semi-blind manner with percentage internalisation displayed as the mean value (± standard deviation) from at least three independent experiments. * indicates statistically significant differences (Dunnett's post-test) compared to EPEC data (4* $p < 0.0001$; one-way ANOVA) or by Tukey's test for multiple comparisons between strains (ns not significant). Strains used were EPEC, *etmf* (lacks LEE-encoded Intimin [*eae* gene product] plus Tir, Map and EspF effectors), *espGorf3Δcore* strain (lacks *espG*, *orf3*, *espH*, *tir*, *map*, *eae*, *cesF* and *cesT* genes) carrying no plasmid (-) or a plasmid encoding the EspF-specific chaperone CesF [as a CesF::HA fusion protein].

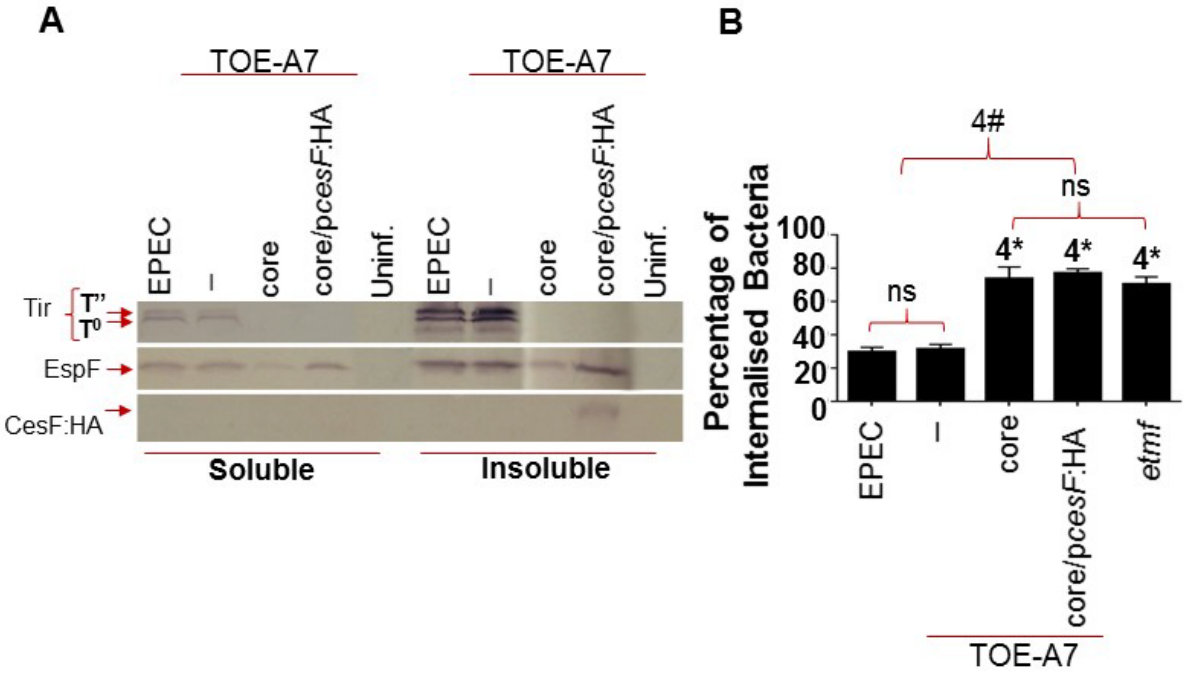


Figure 23. EspF requires the activity other effectors to inhibit EPEC uptake by macrophages. Indicated strains were used to infect (A) HeLa cells for western blot analysis and (B) J774.A1 macrophages for the phagocytosis assay. (A) HeLa cells were left uninfected or infected for 3 h (MOI of 100:1) with indicated strains before isolating Triton X-100 soluble (contains host cytoplasmic and membrane proteins plus T3SS-delivered effectors) and insoluble (contains host nuclei and cytoskeleton plus adherent bacteria) fractions for western blot analysis (12% SDS-PAGE) probing for EspF, Tir and HA-tagged proteins. Arrows indicate the position of Tir (unmodified T^0 and host kinase modified T'' form), EspF and CesF::HA proteins. The blot is representative of that obtained from three independent experiments. (B) J774.A1 macrophages were infected 1 h (MOI 100:1) with indicated strains (pre-grown in DMEM 3 h) prior to determining the percentage bacterial internalisation using differential antibody stains distinguish extracellular bacteria (Red) from total cell-associated (Green) bacteria. Fifty macrophages, with 20-50 cell-associated bacteria, were randomly chosen to count the number of extracellular and total cell-associated bacteria enabling the percentage internalisation to be calculated. (B) Quantification studies were carried out in a semi-blind manner with percentage internalisation displayed as the mean value (± standard deviation) from at least three independent experiments. * indicates statistically significant differences (Dunnett's post-test) compared to EPEC data (4* $p < 0.0001$; one-way ANOVA) or by Tukey's test for multiple comparisons between strains (4# $p<0.0001$ and ns not significant). Strains used were EPEC, *etmf* (lacks LEE-encoded Intimin [*eae* gene product] plus Tir, Map and EspF effectors), TOE-A7 (lacks 14 non-LEE encoded plus LEE EspG effectors) and TOE-A7Δ*core* strain (as TOE-A7 but also lacks *espH*, *tir*, *map*, *eae*, *cesF* and *cesT* genes) carrying no plasmid or a plasmid encoding the EspF-specific chaperone CesF [as a CesF::HA fusion protein].

thirteen Nle effector-encoding genes. Of note, similar western blot and phagocytosis data was obtained for EPEC and TOE-A7 (lacks 14 *nle* genes) strains reaffirming no detectable roles for 14 Nle effectors in delivering EspF and Tir as well as the anti-phagocytosis mechanism. By contrast, the '*core*' variants, with reduced (TOE-A7*core*) or EPEC-like (TOE-A7*core*/pCesF::HA) EspF expression/delivery levels (Figure 23A) could not inhibit phagocytosis (Figure 23B) supporting a requirement for other effectors in the anti-phagocytosis process.

3.2.7. Confirmation of T3SS/CesF-dependent EspF delivery into macrophages

The implied dependence of EPEC's EspF-driven anti-phagocytic activity on other effectors rests on the, western blot supported, assumption that introducing the p*cesF*::HA plasmid rescued the EspF delivery defect of *espGorf3core* and TOE-A7Δ*core* mutants. To provide further support for EspF delivery, J774A.1 macrophages were infected and fixed before antibody probing for EspF accumulation in mitochondria as before (see Materials and Methods). Immunofluorescent microscopy analysis revealed that adding antibodies in the absence of detergent led to, as before, a background EspF signal with detergent addition having no impact on the signal for *espB* (can't deliver effectors) infected cells (Figure 24). Interestingly, there was evidence of some EspF delivery (punctate staining) in *espGorf3core* and TOE-A7Δcore-infected cells consistent with the observed low level of EspF expression/delivery (Figure 22 and 23). By contrast, a much more dramatic punctate staining pattern was evident in detergent-treated cells that had been infected with EPEC, the *espGorf3core*/*p*cesF::HA or TOE-A7Δcore/p*cesF*::HA mutants (Figure 24). This work, together with the western blot data, illustrates that the EspF delivery-defect of CesF-deficient (*core* mutant) strains was completely rescued by introducing the CesF::HA encoding plasmid. Moreover, the failure of the CesF-complemented *espGorf3core* and TOE-A7Δ*core* strains to inhibit their uptake by J774A.1 macrophages supports the idea that EspF does not encode sufficient information to inhibit phagocytic pathways but depends on the activity of co-delivered effectors. As our studies also ruled out a role for the 14 Nle effectors and *core*-deleted LEE effectors, the findings implicate roles for other (CesT-dependent) effectors.

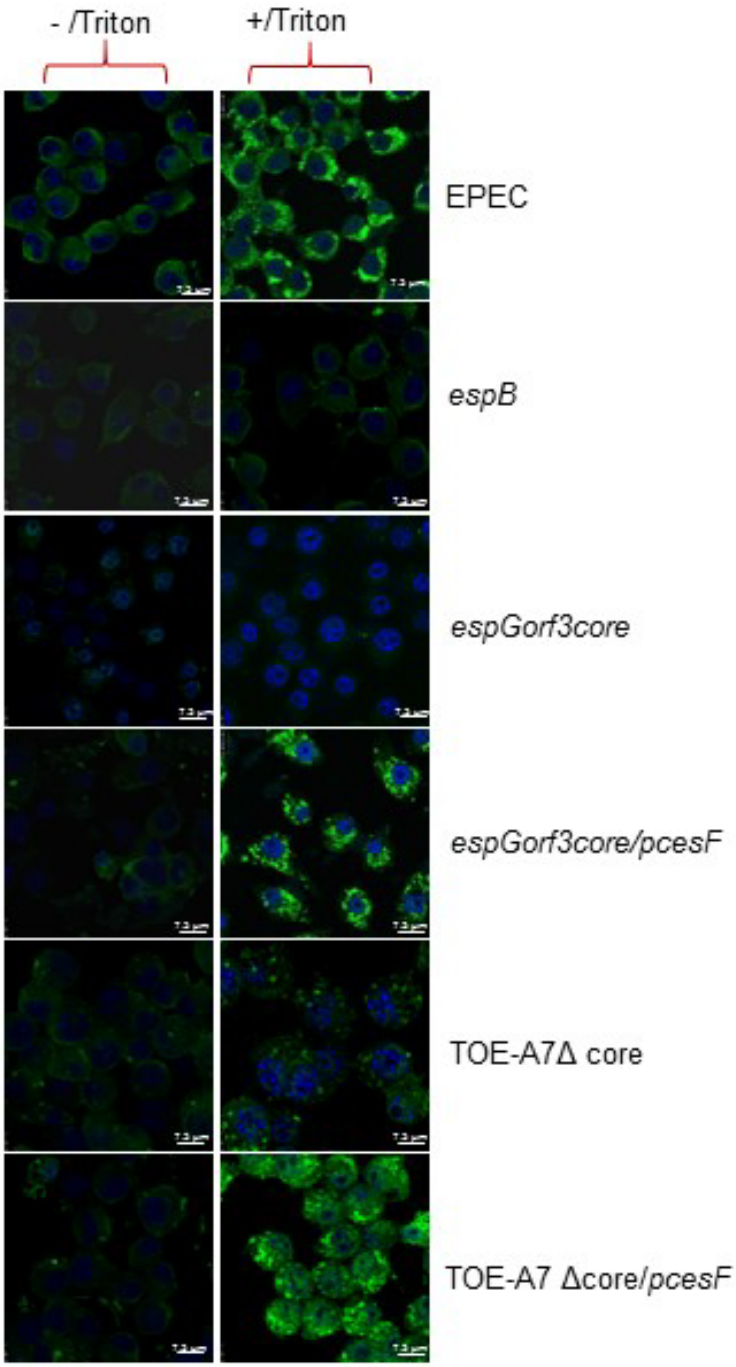


Figure 24. Rescuing EspF delivery defect of *core* mutants by introducing CesF::HA encoding plasmid. J774.A1 cells were infected for 3 h (MOI 100:1) with indicated strains prior to fixing cells and adding anti-EspF antibodies in PBS alone (-) or PBS containing 1% Triton (+) to enable antibody to cross host membranes. Bound antibodies were detected using Alexa 488-conjugated antibodies (green channel). Cells were also incubated with DAPI to stain DNA (host and bacterial; blue channel) see Materials and Methods. Strains used were EPEC, *espB* (can secrete EspF but cannot deliver EspF into cells), *espGorf3core* (lacks *espG*, *orf3*, *espH*, *tir*, *map*, *eae*, *cesF* and *cesT* genes) and TOE-A7Δ*core* (lacks 14 *nle* genes plus *espG*, *espH*, *tir*, *map*, *eae*, *cesF* and *cesT* genes). Indicated strains carried a plasmid (p*cesF*) encoding the EspF-specific chaperone CesF as a CesF::HA fusion protein. Images are representative of three independent experiments taken by confocal laser-scanning microscope at 63x 1.32 NA oil-immersion objective. Scale bar 7.5 μm.

3.3 Discussion

The T3SS-delivery of the EPEC EspF effector protein into host cells is essential for EPEC to inhibit their uptake through PI-3 kinase dependent pathways (Quitard *et al.*, 2006; Martinez-Argudo *et al.*, 2007; Marchès *et al.*, 2008; Tahoun *et al.*, 2011). The aim of this chapter was to test whether the EspF effector protein encodes sufficient information to enable EPEC to inhibit its uptake by macrophages. Here we clearly confirmed that EPEC has the ability to inhibit its uptake by J774A.1 macrophages in a manner dependent on a functional T3SS and EspF effector protein, and also suggest a T3SS-independent inhibitory contribution. Furthermore, we show that the LEE region encodes sufficient information to enable *E. coli* to inhibit its uptake by J774.A1 macrophages and, crucially, demonstrate that EspF is not sufficient to inhibit EPEC uptake.

The anti-phagocytic data generated in this study is consistent with others (Goosney *et al.*, 1999; Celli *et al.*, 2001; Quitard *et al.*, 2006; Marchès *et al.*, 2008) and confirms that EPEC has a T3SS-dependent capacity to inhibit its uptake by J774.A1 macrophages. Interestingly, studies with non-pathogenic K12 *E. coli* strains (DH10B and TOB01) suggested a T3SS-independent inhibitory contribution, which may implicate roles for other EPEC-specific T3SS-independent factors such as EspC protein (autotransporter link to cell cytotoxicity), T2SS-dependent substrate(s) required for EPEC virulence, surface components such as pili, membrane proteins or capsule (Giron *et al.*, 1991; Stein *et al.*, 1996; Whitfield, 2006; Baldi *et al.*, 2012; Guignot *et al.*, 2015). Further work is required to define the EPEC factors and mechanisms mediating this T3SS-independent inhibitory mechanism.

While anti-phagocytosis depends on LEE functions (Quitard *et al.*, 2006; Iizumi *et al.*, 2007; Dong *et al.*, 2010), it was unclear whether the LEE region encodes sufficient information to enable *E. coli* K12 to inhibit its uptake by macrophages. Here we show that the LEE region encodes sufficient information to enable *E. coli* K12 to inhibit its uptake to a similar level as EPEC. This finding was supported by subsequent work of another PhD student (Amin, 2016). The *E. coli* K12 strain also carries plasmids encoding an EPEC adhesin (BFP, facilitates the initial attachment of the microbe to its host cell surface) and Per (promotes LEE gene expression) but these factors are not responsible as present in the negative control strain which did not inhibit phagocytosis. However, these factors may be required (or promote) the LEE-mediated inhibitory event but previous studies discounted a role for BFP (Goosney *et al.*, 1999). Thus, it is likely that anti-phagocytosis is due to LEE-encoded effectors previously linked to this event i.e. EspF (Quitard *et al.*, 2006), EspB (Iizumi *et al.*, 2007), EspH (Dong *et al.*, 2010) and/or EspG (Humphreys *et al.*, 2016).

It is important to note that the EPEC factors in the *E. coli* K12 experiments came from EPEC B171-8; another prototypic typical EPEC strain (Yen *et al.*, 2010) with significant differences between LEE-encoded effectors, for example, only 66%, 66% and 77% identity between EspB, Tir and EspF protein respectively (McDaniel and Kaper, 1997; Simonovic *et al.*, 2000; Yen *et al.*, 2010). Nevertheless, we predict that the EPEC E2348/69 LEE region also encodes all the factors required to enable K12 *E. coli* (carrying *per* and/or *bfp* operons) to inhibit phagocytosis. Support from this suggestion comes from the finding that an EPEC E2348/69 strain lacking most (14 of 17) proven non-LEE-encoded effectors inhibited its uptake similar to the parental EPEC strain. However, as this strain lacks EspG (and its Nle homologue EspG2) our finding contrasts to a recent publication which suggested that the EPEC EspG effector inhibits phagocytosis (Humphreys *et al.*, 2016). A possible reason for the conflicting results may be use the different cell models; RAW264.7 (ATCC-TIB71) versus, in this study, J774A.1 (ATCC TIB-67) mouse macrophage-like cells, protocols and/or reagents.

The critical role of EspF has been supported by previous studies using macrophage models (Quitard *et al.*, 2006; Marchès *et al.*, 2008) M-cell, *in vitro* and *in vivo*, model (Martinez-Argudo *et al.*, 2007; Tahoun *et al.*, 2011), however, the EspF inhibitory mechanism remains undefined. One question arising from earlier studies is whether EspF encodes sufficient information to inhibit EPEC uptake by macrophages, or whether it requires additional EPEC factors in the anti-phagocytosis process. To address this, the EspF effector was delivered, via T3SSs, into host cells in the absence of all or most known EPEC effectors. The former involved a *Yersinia* strain -lacking genes encoding the key *Yersinia* encoded (Yop) effectors, as this strategy was used to deliver the EPEC Tir effector into HeLa cells in a manner greatly promoted by co-expressing the Tir chaperone CesT (Kenny and Warawa, 2001). Our studies revealed for the first time, that *Yersinia* can deliver EspF into HeLa cells in a manner dependent on co-expressing its chaperone CesF. While EspF delivery into HeLa cells was evident by western blot analysis of isolated (Triton X-100 soluble and insoluble) fractions, similar studies with macrophages were not able to illustrate T3SS-dependent effector delivery. The latter is probably due to the phagocytic nature of macrophages leading to non-specific internalisation (endocytosis) or T3SS-secreted proteins, perhaps, released during degradation of internalised bacteria within phago-lysosomes. Thus, a well-established protocol, using HeLa cells, was used in this study to support the T3SS dependent delivery of effectors (Kenny and Warawa, 2001; Grosdent *et al.*, 2002). It should be noted that these studies used a plasmid encoding a CesF::HA tagged protein which illustrates that adding a short C-terminal extension does not interfere with two of its chaperone functions: promoting intracellular stability of EspF and T3SS delivery.

The inability to detect T3SS-delivery into macrophages led to the development of a fluorescent microscopy approach (Nougayrède and Donnenberg, 2004; Dean *et al.*, 2010b), which could clearly illustrate T3SS-dependent delivery of EspF into HeLa cells and J774A.1 macrophages. However, this approach did not work with the T7-EspF varaint as the T7 tag, apparently, stops EspF from targeting mitochondria, the basis of the assay, with the cytoplasmic accumulation not evident due to high background signals. It should be noted that we confirmed that, as reported (Quitard *et al.*, 2006), T7 EspF could substitute for EspF to rescue the anti-phagocytosis defect of EspF deficient *etmf* mutant. As attempts to reduce the high background antibody signal were unsuccessful, attempts were made to generate a plasmid expressing EspF (un-tagged version) compatible with a plasmid expressing its chaperone CesF. However, unfortunately, introducing the new generated EspF into *pcesF*- carrying *Yersinia* did not increase EspF delivery level. As the sequencing analysis confirmed the absence of PCR-introduced mutations, possible explanations for this finding include incompatibility issues. In support of this hypothesis another PhD student (A. Madkour) has recently found that *Yersinia*'s ability to deliver Tir was abolished by the presence of a second plasmid CesT. Nevertheless, our data are consistent with *Yersinia* being able to deliver EspF (T7-EspF), in a CesF dependent manner, into HeLa cells, and presumably J774.A1 macrophages, to a similar level as EPEC but this fail to interfere with the phagocytic activity of J774.A1 macrophages towards EPEC. It should be noted that *Yersinia*'s ability to inhibit phagocytosis was assessed through a two-wave approach i.e. first-wave EPEC or *Yersinia*/pT7-*espF/pcesF*::HA (to pre-deliver EspF) infection before infecting with an EPEC T3SS mutant (GFP expressing) and determining the percentage of internalisation of the second wave T3SS/GPF mutant bacteria. Collectively, while this work failed to illustrate *Yersinia* delivery of T7-EspF into macrophages, unlike HeLa cells, the finding make it highly likely that it was delivered but failed to inhibit phagocytosis implicating crucial role(s) for other EPEC encoded factor(s).

Support for the above hypothesis came from a second strategy i.e studies with an EPEC strains unable to deliver most known effector except EspF. Firstly, we confirmed the expectation that an *espGorf3core* (and later TOE-A7Δcore) mutant, which lacks CesF, delivered little EspF into HeLa cells or (via fluorescent microscopy studies) macrophages and could not inhibit phagocytosis. Importantly, plasmid introducing CesF (as a CesF-HA fusion protein) rescued the EspF delivery, but not anti-phagocytosis, defect supporting the idea that EspF does not encode sufficient information to inhibit EPEC uptake by macrophages. Moreover, the finding implicates key role for factors encoded within the core region i.e. Intimin three effectors (EspH, Map, Tir).

In summary, this study not only confirms a dependence of EPEC's anti-phagocytosis activity on a functional T3SS and EspF effector protein, but discounted a key role of fifteen (fourteen Nle and EspG) effectors and argues that all required factors are encoded on the LEE region. Furthermore, this work demonstrates that delivering EspF into macrophages is not sufficient to inhibit EPEC uptake and implicates a critical role for one or more of the gene products encoded on the LEE core region i.e. Intimin, EspH, Map and/or Tir.

Chapter 4. Investigating roles for LEE *core* region proteins in EPEC's capacity to inhibit its uptake by J774.A1 macrophages

4.1 Introduction

In the previous chapter we have demonstrated that EspF requires the function of other effector proteins to inhibit EPEC's uptake, probably one or more of the LEE *core* region proteins EspH, Map, Tir and Intimin.

Tir (translocated Intimin receptor) is the first LEE effector delivered into the host cells by the T3SS. Tir is inserted into the host membrane where it acts as a receptor for Intimin to mediate intimate host-pathogen interactions (Kenny *et al.*, 1997a; Campellone *et al.*, 2004a). As previously discussed, phosphorylation of two serine residues, S434 and S463, is linked to shifts in apparent molecular mass and membrane insertion as well as efficient pedestal formation (Warawa and Kenny, 2001). Phosphorylation on two tyrosine residues, Y454 and Y474, is linked to actin nucleation by inefficient and efficient mechanisms respectively. Most is known about the latter process, as phosphorylation of residue Y474 recruits an adaptor protein, Nck, which in turn recruits N-WASP to sequester the Arp2/3 actin nucleating machinery inducing pedestal formation (Kenny, 1999; Gruenheid *et al.*, 2001; Campellone *et al.*, 2002). Previous research has examined the anti-phagocytosis capacity of *tir* and *eae* mutant strains revealed an intermediate anti-phagocytosis activity compared to EPEC (Goosney *et al.*, 1999). However, other studies could not confirm a role in the phagocytosis inhibition for either of them (Celli *et al.*, 2001; Quitard *et al.*, 2006) .

The other two LEE effector molecules, Map (mitochondrial-associated protein) and EspF, target mitochondria and decrease mitochondrial membrane potential during EPEC infection leading to cell death (Kenny and Jepson, 2000; Nougayrède and Donnenberg, 2004; Nagai *et al.*, 2005). Importantly, it has been shown that EPEC has the ability to inhibit its uptake which is dependent on a functional T3SS and the EspF effector protein (Quitard *et al.*, 2006; Martinez-Argudo *et al.*, 2007; Marchès *et al.*, 2008; Tahoun *et al.*, 2011), but no essential role for Map was revealed (Quitard *et al.*, 2006). However, the mechanism of EspF inhibition remains undefined (Quitard *et al.*, 2006).

Current knowledge of the effector EspH showed that its loss leads to prolonged filopodia formation concomitant with delayed pedestal formation. However, the over-expression of EspH led to actin polymerization and pedestal elongation (Tu *et al.*, 2003). Indeed, EspH inhibits uptake of EPEC by inhibiting the activity of RhoGEF which is responsible for regulating actin cytoskeleton dynamics (Dong *et al.*, 2010).

The core region also includes two chaperones, CesF and CesT, which are encoded on the LEE region. The main chaperone's functions are regulating gene expression, supporting the effectors

trafficking with the T3SS at the inner membrane and stabilisation of the effector proteins within the bacterial cytosol (Cornelis *et al.*, 1998; Elliott *et al.*, 1999; Creasey *et al.*, 2003). It has been demonstrated that CesF binds only to the EspF effector protein (Elliott *et al.*, 2002). In contrast, CesT binds and regulates the efficient translocation of at least twelve effector proteins, either fully (Tir, EspZ, EspH, Map, EspJ, NleG, NleH1 & NleH2) or partially (NleB1, NleB2, NleC & NleA) (Thomas *et al.*, 2005; Mills *et al.*, 2013).

Previous research has demonstrated that LEE effectors have overlapping functions (redundancy) and can work cooperatively and/or antagonistically in subverting host cell activities (Dean and Kenny, 2009; Schmidt, 2010; Santos and Finlay, 2015). For example, the three effectors (Tir, Map and EspF) and the outer membrane protein Intimin act cooperatively to efface absorptive microvilli of enterocytes (Dean *et al.*, 2006). This not only reflects the complexity of host pathogen interactions but also reveals the difficulty in determining the contribution of specific stimuli to subvert host cellular pathways such as inhibiting phagocytosis.

The aim of this chapter was to examine the role of the *core*-encoded factors in inhibiting EPEC uptake by macrophages through screening a bank of available mutants in which implicated core region genes (encode Map, EspH, Tir, Intimin), plus the EspF-encoding gene, were inactivated in all combinations.

4.2 Results

4.2.1 Unexpected role for Tir but not EspF or EspH in EPEC's anti-phagocytic activity in the J774.A1 macrophage model

The data presented in chapter 3 suggested that the LEE region encodes sufficient information to enable EPEC to inhibit its uptake by J774.A1 macrophages and discounted major roles for 14 Nle effectors in phagocytosis inhibition. By contrast, studies with the *espGorf3core*/*pcesF* mutant implied that EspF capacity to inhibit phagocytosis depended on one or more factors encoded on the *core* LEE region i.e. three effectors (EspH, Map, Tir), Intimin or CesT*;* required for delivery of most LEE and many Nle effectors (Thomas *et al.*, 2005; Mills *et al.*, 2013). As CesT is required for the T3SS-dependent delivery of EspH, Map and Tir, studies were re-focused to study the roles of the *core*-encoded effectors and Intimin protein by examining a bank of available mutants in which the genes encoding these proteins, as well as the EspF effector, were inactivated in all combinations (See Table 2; Materials and Methods).

The usual anti-phagocytosis assay was carried out on each strain in at least three independent experiments, with many strains included in additional experiments while the control (EPEC and T3SS mutant) strains were assessed in over 20 experiments. The percentage internalisation for each experiment was recorded in a single file with the resulting mean (± SD) used to generated individual graphs for the single, doubles, triples and quadruple/quintuplet mutants alongside EPEC and T3SS mutant data. Examining the data from the single mutants-genotypes supported by PCR analysis (Figure 25A)- reveals, that as before, most T3SS mutant were internalised (78.2 ± 7.8%) in contrast to EPEC where only a minority (29 ± 5.8%) of bacteria were phagocytosed (Figure 25B). No significant difference in the percentage of uptake was identified for cells infected with EPEC and the *map* or *eae* mutant strains (Figure 25B). These results confirmed that the individual Map or Intimin proteins do not play critical roles in inhibiting EPEC phagocytosis by J774.A1 macrophages (Quitard *et al.*, 2006). By contrast, infection of cells with the *tir* mutant resulted in a small but significant ($p < 0.001$) anti-phagocytic defect (42.9 ± 6.8% versus 29 ± 5.8% EPEC internalised) indicating a minor role in EPEC's anti-phagocytosis activity, consistent with a previous study (Goosney *et al.*, 1999).

As the *tir* mutant is missing residues 50-320 (followed by a stop codon) of this 550 residue protein, it was possible that the short N-terminal polypeptide, that carries a T3SS signal sequence and host kinase-recruitment motif (Crawford and Kaper, 2002; Bommarius *et al.*, 2007), is delivered into cells and could interfere with the phagocytic process. Thus, a tir_{full} mutant was generated in which the entire gene (from start to stop codons) was deleted (see Materials and Methods) as supported by PCR analysis (Figure 26A).

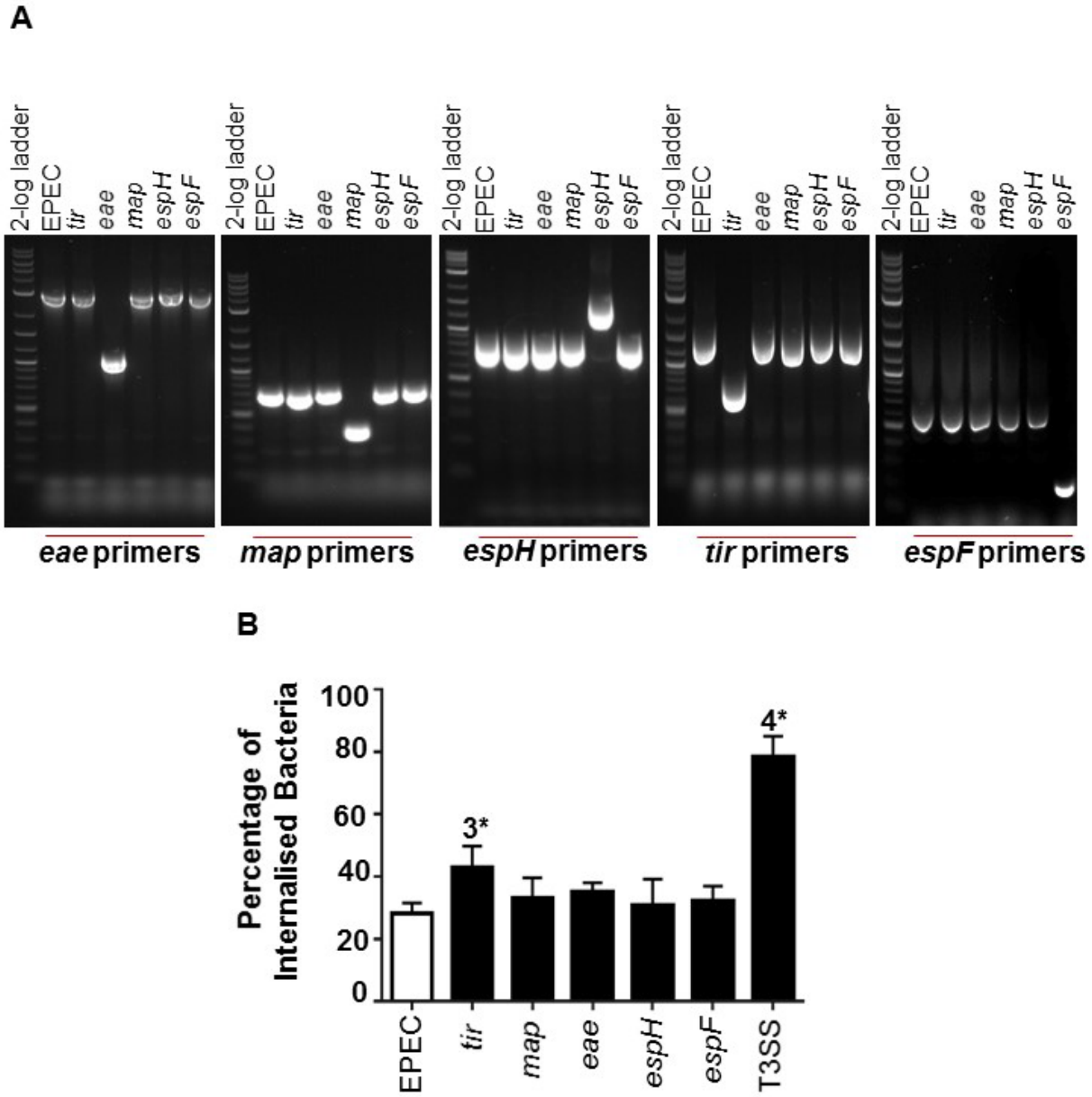


Figure 25. Individual role for Tir but not Map, EspF, EspH or Intimin proteins in EPEC's anti-phagocytic activity in the J774.A1 macrophage model. Indicated strains were used for (A) PCR analysis or to infect (B) J774.A1 macrophages. (A) Oligonucleotides specific to the *eae*, *map*, *espH*, *tir* and *espF* gene were used in PCR analyses to determine the presence of an intact or deleted genes with PCR products visualised, alongside molecular mass markers 2-log (1000Kb ladder), on a 1% agarose gel. (B) J774.A1 macrophages were infected for 1 h (MOI 100:1) with indicated strains (pre-grown in DMEM for 3 h) prior to determining the percentage of internalised bacteria using antibodies to label extracellular bacteria (Red) and total cell-associated bacteria (Green). Fifty macrophages with 20-50 cell-associated bacteria were randomly chosen to count the number of extracellular and total cell-associated bacteria (Green) enabling the percentage internalisation to be calculated. (B) Quantification studies were carried out in a semi-blind manner with the percentage internalised bacteria displayed as the mean value (± standard deviation) from at least three independent experiments. * indicates statistically significant differences (one-way ANOVA with Dunnett's post-hoc test) compared to EPEC data (4* $p < 0.0001$, 3* $p < 0.001$). Strains used were EPEC and strains lacking a functional EspF (*espF*), EspH (*espH*), Map (*map*), Tir (*tir*), Intimin (*eae*) protein or T3SS (T3SS).

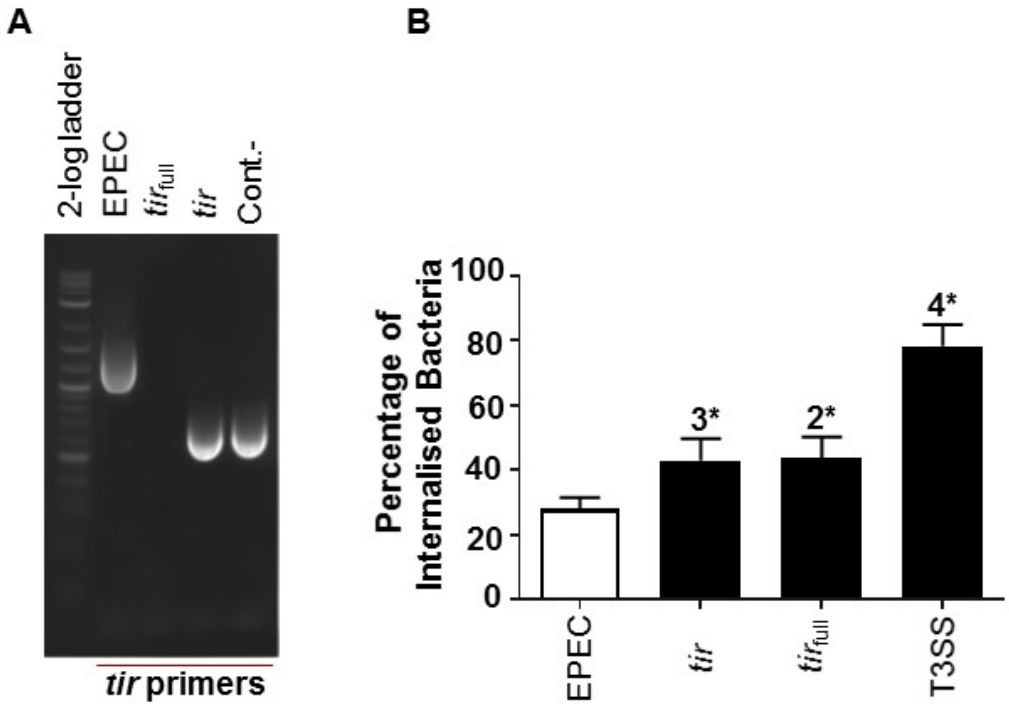


Figure 26. Minor role for the Tir effector in EPEC's anti-phagocytic activity in the J774.A1 macrophage model. Indicated strains were used for (A) PCR analysis or to infect (B) J774.A1 macrophages. (A) Oligonucleotides specific to the *tir* gene were used in PCR reaction to determine absence of intact *tir* gene with PCR products visualised, alongside molecular mass markers 2-log (1000Kb ladder), on a 1% agarose gel. (B) J774.A1 macrophages were infected for 1 h (MOI 100:1) with indicated strains (pre-grown in DMEM for 3 h) prior to determining the percentage of internalised bacteria using antibodies to label extracellular bacteria (Red) and total cell-associated bacteria (Green). Fifty macrophages with 20-50 cell-associated bacteria were randomly chosen to count the number of extracellular and total cell-associated bacteria (Green) enabling the percentage internalisation to be calculated. Quantification studies were carried out in a semi-blind manner with the percentage of internalised bacteria displayed as the mean value (± standard deviation) from at least three independent experiments. * indicates statistically significant differences (one-way ANOVA with Dunnett's post-hoc test) compared to EPEC data (4* $p < 0.0001$, 3* $p < 0.001$, 2* $p < 0.01$). Strains used were EPEC, *tir* (*tir* partially deleted), tir_{full} (*tir* fully deleted) and T3SS (T3SS-deficient).

Similar to the *tir* strain, infecting J774.A1 macrophages with the *tir*$_{full}$ mutant again led to similar, intermediate, anti-phagocytosis activity (43.8 ± 6.5% of bacteria internalised; Figure 26B).

Surprisingly, studies with the *espF* mutant revealed that this strain behaved in a manner indistinguishable to the wildtype, EPEC, strain (Figure 25B) in contrast to its published critical role in EPEC's anti-phagocytic activity (Quitard *et al.*, 2006; Martinez-Argudo *et al.*, 2007; Marchès *et al.*, 2008; Tahoun *et al.*, 2011) and earlier demonstration of an *espF*/pGFP strain failing to inhibits its uptake by J774.A1 macrophages (Figure 14, Chapter 3). The EPEC-like phenotype with this *espF* mutant was predicted to reflect an acquisition of mutations that restored its anti-phagocytic activity. Thus, studies examined the anti-phagocytic activity of *espF* mutant strains (*espF*$_{1-3}$) available in frozen culture stocks of previous laboratory members. PCR analysis confirmed that each strain lacked an intact *espF* gene (Figure 27A) with phagocytic assays revealing EPEC-like phenotypes for each (Figure 27B). Thus, this finding contrasts to earlier studies (Figure 14, Chapter 3) and work with *espF*-deficient mutants (generated in this and/or other laboratories) using cultured macrophages; M-cell model and/or *in vivo* studies (Quitard *et al.*, 2006; Martinez-Argudo *et al.*, 2007; Marchès *et al.*, 2008; Tahoun *et al.*, 2011).

Surprisingly, the examined *espH*::Km mutant displayed no significant defect in the anti-phagocytic assay (Figure 25A) in contrast to its reported inability to inhibit phagocytosis (Dong *et al.*, 2010). Thus, studies examined another available (Nal^{S}) *espH*::Km mutant (Tu *et al.*, 2003) which was found to behave in a near identical manner (Figure 28). To support these findings, the *espH* mutant used in the study illustrating EspH's anti-phagocytic activity (Dong *et al.*, 2010) was requested but unfortunately it displayed binding issues leading to, a kindly provided suicide vector (Dong *et al.*, 2010), being used to generate a new (Nal^{R}) *espH*$_{AA}$ mutant (see Materials and Methods). This *espH*$_{AA}$ mutant lacking a functional *espH* gene was supported by PCR analyses (Figure 29A) and found to behave in a statistically indistinguishable manner to the wild type, EPEC, strain (Figure 29A).

In conclusion, these results unexpectedly failed to support essential roles for EspF and EspH effectors for EPEC to inhibit its uptake by J774A.1 macrophages but revealed a minor role for Tir while confiming no roles for Map or Intimin.

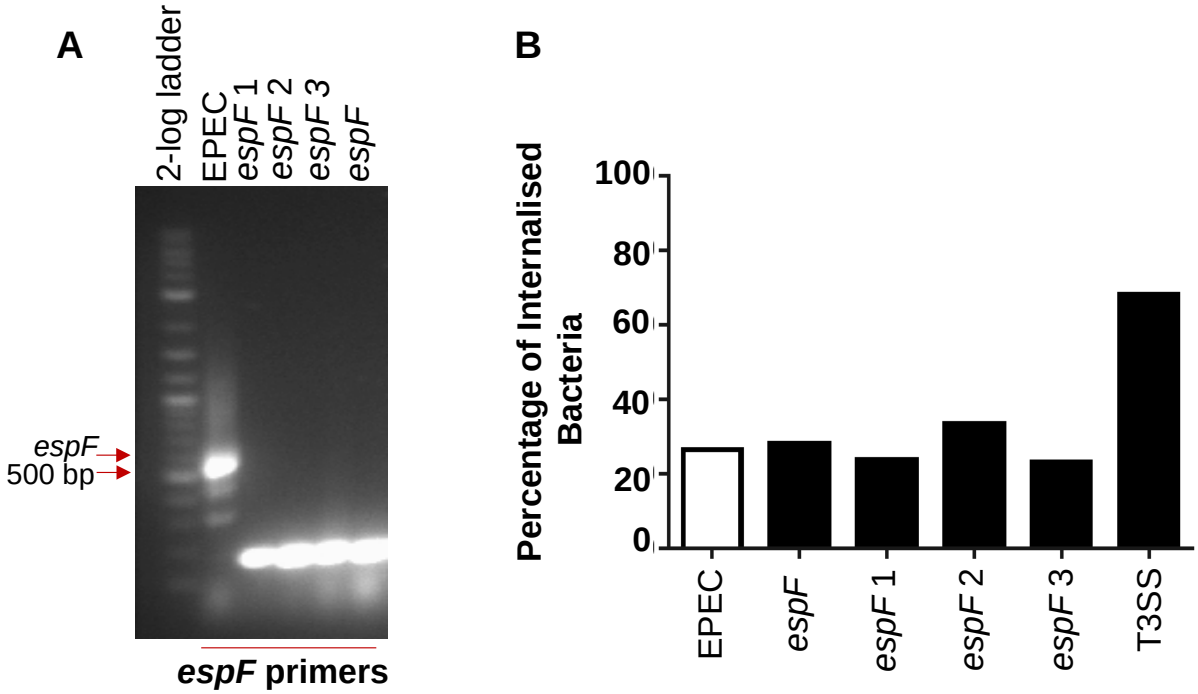


Figure 27. No detectable roles for the EspF effector in EPEC's capacity to inhibit its uptake by J774.A1 macrophages. Indicated strains were used for (A) PCR analysis or to infect (B) J774.A1 macrophages. (A) Oligonucleotides specific to *espF* gene were used in a PCR reaction to determine the presence of an intact (~500bp) or deleted *espF* gene with PCR products visualised, alongside 2-log molecular mass markers on a 1% agarose gel. (B) J774.A1 macrophages were infected for 1 h (MOI 100:1) with indicated strains (pre-grown in DMEM for 3 h) prior to determining the percentage of internalised bacteria using antibodies to label extracellular bacteria (Red) and total cell-associated bacteria (Green). Fifty macrophages with 20-50 cell-associated bacteria were randomly chosen to count the number of extracellular and total cell-associated bacteria (Green) enabling the percentage internalisation to be calculated. (B) Quantification studies were carried out in a semi-blind manner. Strains used were EPEC, T3SS-deficient (T3SS) mutant, previously used EspF-deficient mutant (*espF*) plus *espF* mutants taken from frozen culture stocks of former laboratory workers ($espF_{1\text{-}3}$).

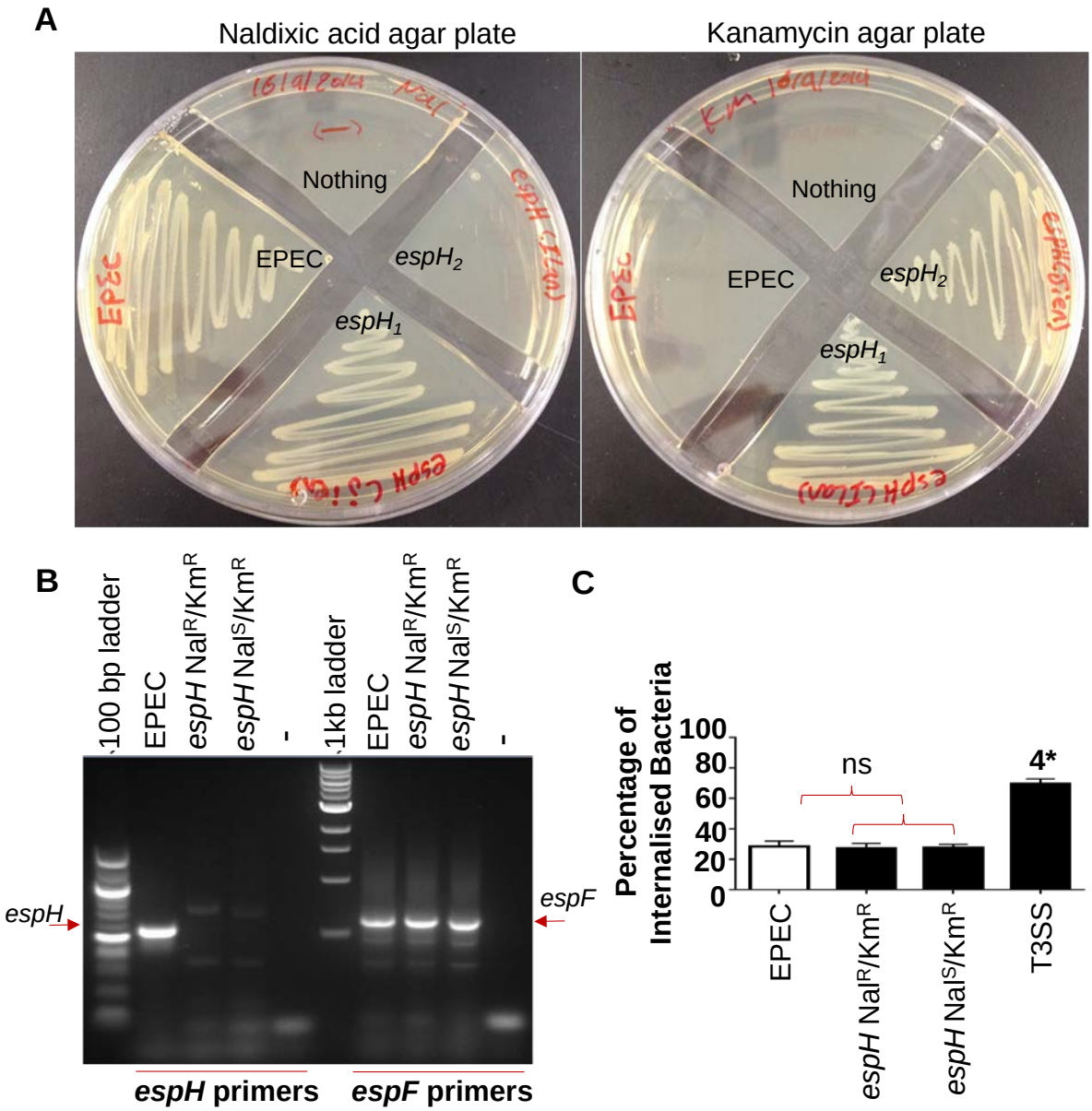


Figure 28. EPEC does not require an intact *espH* gene to inhibit its uptake by J774.A1 macrophages. Indicated strains were used for investigations to support their genotype by (A) confirming expected antibiotic resistance profile and (B) PCR analysis prior to use for infection of (C) J774.A1 macrophages. (A) The genotypes of the two *espH* mutants (*espH* Nal^R/Km^R and *espH* Nal^S/Km^R) were confirmed with antibiotic selective plates. (B) Oligonucleotides specific to *espF* and *espH* genes were used in PCR analyses to determine the presence of an intact or deleted genes with PCR products visualised, alongside 100bp and 1Kb molecular mass markers, on a 1% agarose gel. The oligonucleotides were specific to regions within the *espH* gene and, thus, absent from *espH*::Km variants. (C) J774.A1 macrophages were infected for 1 h (MOI 100:1) with indicated strains (pre-grown in DMEM for 3 h) prior to determining the percentage of internalised bacteria using antibodies to label extracellular bacteria (Red) and total cell-associated bacteria (Green). Fifty macrophages with 20-50 cell-associated bacteria were randomly chosen to count the number of extracellular and total cell-associated bacteria (Green) enabling the percentage internalisation to be calculated. (C) Quantification studies were carried out in a semi-blind manner with the percentage of internalised bacteria displayed as the mean value (± standard deviation) from at least three independent experiments. * indicates statistically significant differences (one-way ANOVA with Dunnett's post-hoc test) compared to EPEC data (4* $p < 0.0001$) or by Tukey's test for multiple comparisons between strains (ns not significant). Strains used were EPEC, T3SS-deficient (T3SS), *espH* Nal^R/Km^R and *espH* Nal^S/Km^R (EspH deficient) mutants.

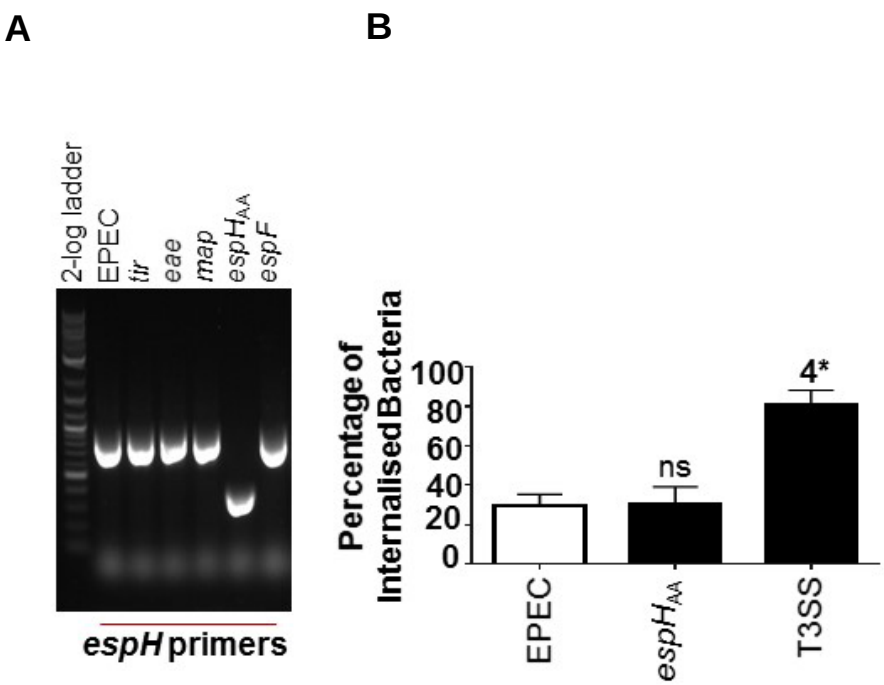


Figure 29. Confirming that EspH dose not play critical role in inhibiting phagocytosis. Indicated strains were used for (A) PCR analysis or to infect (B) J774.A1 macrophages. (A) Oligonucleotides specific to *espH* gene were used in a PCR reaction to determine the presence of an intact (~600bp) or deleted *espH* gene with PCR products visualised, alongside 2-log molecular mass markers on a 1% agarose gel. (B) J774.A1 macrophages were infected for 1 h (MOI 100:1) with indicated strains (pre-grown in DMEM for 3 h) prior to determining the percentage of internalised bacteria using antibodies to label extracellular bacteria (Red) and total cell-associated bacteria (Green). Fifty macrophages with 20-50 cell-associated bacteria were randomly chosen to count the number of extracellular and total cell-associated bacteria (Green) enabling the percentage internalisation to be calculated. Quantification studies were carried out in a semi-blind manner with the percentage of internalised bacteria displayed as the mean value (± standard deviation) from at least three independent experiments. * indicates statistically significant differences (one-way ANOVA with Dunnett's post-hoc test) compared to EPEC data (4* $p < 0.0001$, ns not significant). Strains used were EPEC and strains lacking a functional EspF (*espF*), *espH*$_{AA}$ (*espH*), Map (*map*), Tir (*tir*), Intimin (*eae*) protein or T3SS (T3SS).

4.2.2 Double mutant data uncovers cooperative relationships enabling EPEC to inhibit its uptake by J774.A1 macrophages

Examining the double mutant (10 strains) data revealed some strains to be linked to a much higher level of outlier data (i.e. mean of an experiment distinctly different to other repeats which cluster together; Table 8, Figure 30A). Thus, while there was only one outlier per 28 (3.5%) EPEC and 17 (5.9%) T3SS mutant experiments there was one in four (25%) for the *eh*, *tm* mutants and one in six (16.6%) for *th* mutant (Figure 30A) leading to removal of the outlier with the resulting data (Figure 30B) used for statistical analysis. Remarkably, one double (*ft*) mutant behaved very unusually as 3 (of 6) data points clustered to provide an EPEC-like phenotype (37.7 ± 3.7% versus 29 ± 6%) while the remaining three in a T3SS mutant-like clustering (67.9 ± 2.5% versus 78.2 ± 7.9%; Figure 30B). The latter results indicates that deleting both EspF and Tir may cause some sort of instability in the anti-phagocytic assay. It should be noted that the genotype of each double mutant was supported by PCR analyses (Figure 30C).

Interestingly, the data of only three double mutants was statistically indistinguishable (*mh*, *fm*) or very close (*me*; $p<0.05$) to the positive (EPEC) control data (Figure 30B), consistent with the single (*m*, *f*, *h*, *e*) mutant data (Figure 25B). By contrast, the remaining (7) double mutants displayed major, statistically significant ($p<0.0001$; Figure 30B), defects with one (*fh*) as defective as the T3SS mutant while a slightly reduced, but significant, defect was evident for the other double mutants except *et* and *ft*; the latter due to two distinct data clusters (Figure 30B). This data is suggestive of EspF/Tir; Intimin/Tir; Tir/EspH; Intimin/EspH; EspF/Intimin; Tir/Map and EspF/EspH cooperatively promotes EPEC's ability to inhibit its uptake by J774A.1 macrophages.

A concern for the four *espF*-related double mutants stemmed from the fact that they were generated (Kenny Unpublished) from an *espF* mutant which inhibited phagocytosis (Quitard *et al.*, 2006; Martinez-Argudo *et al.*, 2007) but not in this study. Therefore, these double mutants were re-made using the *espF* mutant assayed in this study, producing fm_{AA}, ft_{AA}, fe_{AA} and fh_{AA} (See Materials and Methods). PCR analysis supported the correct genotype of these newly-generated double mutants (Figure 31A). It should be noted that strain name reflects the order genes were disrupted; for example, *fh* reveals *espH* gene inactivated from the *espF* mutant background.

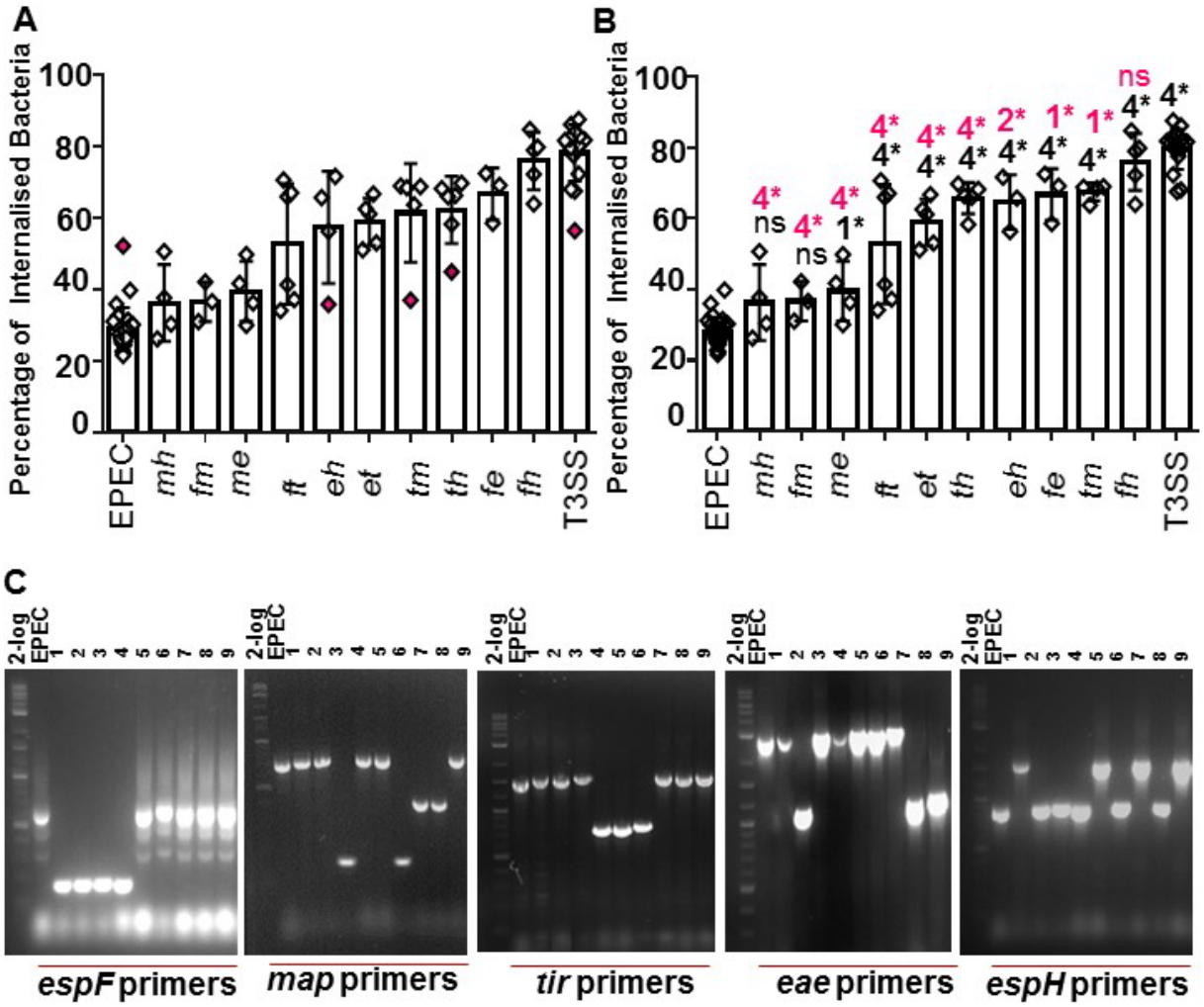


Figure 30. Examining double mutant reveals cooperative relationships in EPEC's ability to inhibit its uptake by J774.A1 macrophages. Indicated strains were used to infect (A&B) J774.A1 macrophages with, post-infection, non-adherent bacteria isolated for (C) PCR analysis. (A&B) J774.A1 macrophages were infected for 1 h (MOI 100:1) with indicated strains (pre-grown in DMEM for 3 h) prior to determining the percentage of internalised bacteria using antibodies to label extracellular bacteria (Red) and total cell-associated bacteria (Green). Fifty macrophages with 20-50 cell-associated bacteria were randomly chosen to count the number of extracellular and total cell-associated bacteria (Green) enabling the percentage internalisation to be calculated. (A&B) Quantification studies were carried out in a semi-blind manner with the percentage of internalised bacteria displayed as the mean value (± standard deviation) from at least three independent experiments. The data from each independent experiment is shown (◊) with outliers (♦) removed providing data (B) used for statistical analysis. * indicating statistically significant differences (one-way ANOVA with Dunnett's post-hoc test) compared to EPEC data (1* $p< 0.05$, 4* $p< 0.0001$, ns not significant; B).* indicates statistically significant differences (one-way ANOVA with Dunnett's post-hoc test) compared to T3SS data (1* $p< 0.05$, 2* $p< 0.01$, 4* $p< 0.0001$, ns not significant). (C) Oligonucleotides specific to *eae*, *map*, *espH*, *tir* and *espF* genes were used in PCR analyses to determine the presence of an intact or deleted genes with PCR products visualised, alongside molecular mass markers 2-log (1000Kb ladder), on a 1% agarose gel. Studies used EPEC and strains lacking a functional T3SS (T3SS) or two LEE proteins: EspF and EspH (*fh*; #1 in C), EspF and Intimin (*fe;* #2 in C), EspF and Map (*fm*; #3 in C), EspF and Tir (*ft*; #4 in C), Tir and EspH (*th*; #5 in C), Tir and Map (*tm*; #6 in C), Map and EspH (*mh*; #7 in C),Map and Intimin (*me*; #8 in C), Intimin and EspH (*eh*; #9 in C). It should be noted that PCR supporting *et* mutant genotype was not demonstrated here but see Figure 33A.

Strain	1	2	3	4	5	6	Av.	SD	Av./without outlier	SD./without outlier	# of n	Outlier
EPEC	31.5	28.7	29.9	22.9	27.3	39.9						
	30.0	52.3	29.1	26.3	27.3	22.3						
	27.0	27.7	31.2	30.3	26.6	32.8						
	36.0	25.0	27.5	21.7	24.5	28.5						
	25.6	25.7	29.1	26.4			29	6.1	28.2	3.9	28	1
map	36.4	25.3	37.2				33	6.6	33	6.6	3	0
tir	36.4	50.1	42.3				43.0	6.9	43.0	6.9	3	0
tir_{full}	48.4	39.1					43.8	6.6	43.8	6.6	2	0
eae	32.7	38.7	32.7	36.3			35.1	2.9	35.1	2.9	4	0
espHNal^R/Km^R	30.4	28.1	27.9	22.9			27.3	13.6	60.4	3.1	4	0
espH Nal^S/Km^R	25.7	28.4	30.4	26.5			27.8	8.3	61.8	2.1	4	0
espH	24.9	40.4	27.1				30.8	8.4	30.8	8.4	3	0
espF	37.5	30.9	28.2				32.2	4.7	32.2	4.7	3	0
espZ	27.1	27.7	25.4				26.7	1.2	26.7	1.2	3	0

Strain	1	2	3	4	5	6	Av.	SD	Av./without outlier	SD./without outlier	# of n	Outlier
me	30.1	49.9	41.8	36.4			39.6	8.4	39.6	8.4	4	0
tm	37.1	69.0	68.5	68.9	63.8		61.5	13.8	67.5	2.5	5	1
et	66.8	53.1	51.2	61.2	62.6		59.0	6.6	59.0	6.6	5	0
et_{AA}	65.4	50.9	52.2				56.2	8.0	56.2	8.0	3	0
fh	72.3	79.0	79.9	84.8	64.0		76.0	8.0	76.0	8.0	5	0
fh_{AA}	38.1	45.4	53.0				45.5	7.5	45.5	7.5	3	0
fe	58.6	72.5	69.2				66.8	7.3	66.8	7.3	3	0
fe_{AA}	44.7	68.2	33.6				48.8	17.7	48.8	17.7	3	0
ft	34.2	37.3	70.7	66.0	41.5	67.1	52.8	16.8	52.2	16.8	6	0
ft_{AA}	58.6	65.3					62.0	4.7	62.0	4.7	2	0
tk	66.4	58.4	69.8	45.0	66.1	68.3	62.3	9.3	65.8	4.4	6	1
mh	26.3	50.7	37.8	30.4			36.3	10.7	36.3	10.7	4	0
fm	31.1	42.2	36.7				36.7	5.5	36.7	5.5	3	0
fm_{AA}	32.7	41.5					37.1	6.2	37.1	6.2	2	0
eh	71.7	56.4	65.8	35.9			57.5	15.7	64.6	7.7	4	1

Strain	1	2	3	4	5	6	Av.	SD	Av./without outlier	SD./without outlier	# of n	Outlier
feh	28.1	72.7	35.5	35.7	39.4		42.3	17.5	34.7	4.8	5	1
fth	73.5	36.5	55.4	55.1	64.2		56.9	13.7	62.1	8.7	5	1
tmf	67.4	74.3	63.7				68.4	5.4	68.4	5.4	3	0
fet	66.3	70.9	61.8				66.3	4.5	66.3	4.5	3	0
fme	75.4	41.1	21.1	34.3	36.7	46.8	42.6	18.2	39.7	5.5	6	2
meh	34.2	48.4	55.0	50.9			47.1	9.0	51.4	3.3	4	1
fmh	34.2	41.2	47.1	46.9			42.4	6.1	42.4	6.1	4	0
tmh	47.1	58.8	58.3				54.7	6.6	54.7	6.6	3	0
eth	33.5	64.5	68.6	52.8	55.8		55.0	13.6	60.4	7.4	5	1
etm	73.0	59.5	65.0	49.3	66.7	57.3	61.8	8.3	61.8	8.3	6	0

Strain	1	2	3	4	5	6	Av.	SD	Av./without outlier	SD./without outlier	# of n	Outlier
etmh	30.2	43.7	54.4	53.0	42.5		44.7	9.7	48.4	6.1	5	1
tmfh	34.8	43.9	73.8	64.4	35.7		50.5	17.6	50.5	17.6	5	0
fmeh	36.3	68.9	28.3	53.0	45.4	32.8	44.1	15.1	39.1	10	6	1
feth	66.0	54.4	49.3	62.2	58.6	30.0	53.4	12.9	58.1	6.5	6	1
etmf	67.5	68.0	73.2	64.0	79.8	76.4						
	72.2	63.2	69.6	65.6	66.4	70.2						
	64.7	50.7	67.8	66.3	64.7	66.8	67.6	6.1	68.6	4.5	18	1
etmfh (quin)	74.2	58.1	59.4	33.1			56.2	17.0	63.9	8.9	4	1

Table 8. Percentage internalised data for indicated double, triple, quadruple and quintuplet mutant bacteria with data in red considered to be an outlier. The mean (Av) and standard deviation (SD) data is given for data including or excluding the indicated outliers with columns indicating the number of independent experiments (# of n) and outliers in the data sets.

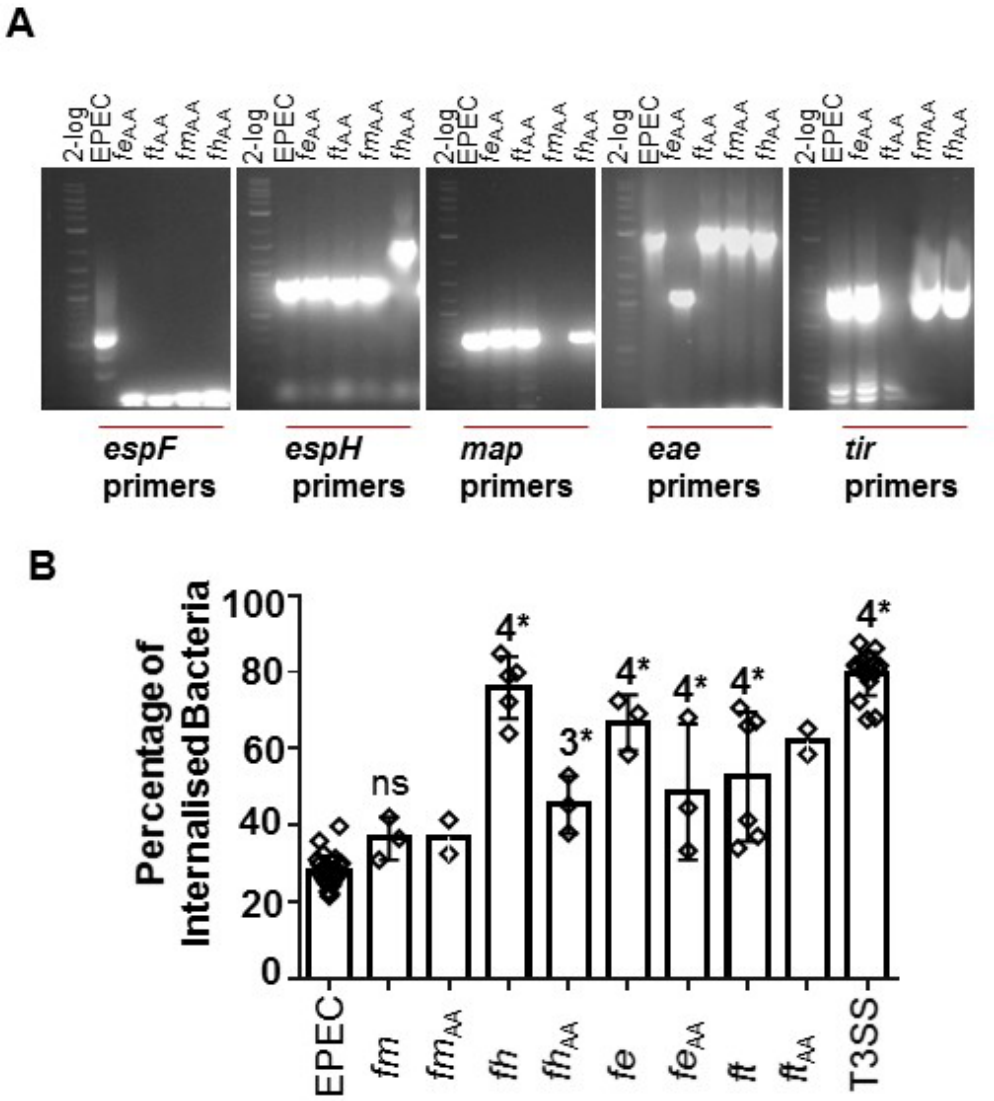


Figure 31. Examining newly generate *espF*-related double mutants. Indicated strains were used for (A) PCR analysis or to infect (B) J774.A1 macrophages. (A) Oligonucleotides specific to *eae*, *map*, *espH*, *tir* and *espF* genes were used in PCR analyses to determine the presence of an intact or deleted genes with PCR products visualised, alongside molecular mass markers 2-log (1000Kb ladder), on a 1% agarose gel. (B) J774.A1 macrophages were infected for 1 h (MOI 100:1) with indicated strains (pre-grown in DMEM for 3 h) prior to determining the percentage of internalised bacteria using antibodies to label extracellular bacteria (Red) and total cell-associated bacteria (Green). Fifty macrophages with 20-50 cell-associated bacteria were randomly chosen to count the number of extracellular and total cell-associated bacteria (Green) enabling the percentage internalisation to be calculated. (B) Quantification studies were carried out in a semi-blind manner with the percentage internalised bacteria displayed as the mean value (± standard deviation) from at least three independent experiments (except fm_{AA} and ft_{AA} where only from two independent experiments) with data from each independent experiment shown (◊).* indicates statistically significant differences (one-way ANOVA with Dunnett's post-hoc test) compared to EPEC data (4* $p < 0.0001$, ns not significant; B). Studies used EPEC and strains lacking functional proteins EspF and Intimin (*fe*, fe_{AA}), EspF and EspH (*fh*, fh_{AA}), EspF and Map (*fm*, fm_{AA}), EspF and Tir (*ft*, ft_{AA}) plus T3SS-deficient (T3SS) mutants. Strains labelled '$_{AA}$' are newly generated with data for other strains taken from Figure 31B.

Assessing these new double mutants revealed (Figure 31B) similar findings for the new *fm*$_{AA}$ mutant (37.1 [n=2] versus 37.6 ± 5.5% [n=3]), while the new *ft*$_{AA}$ mutant displayed an T3SS mutant like phenotype (62 [n=2]) that contrasts to the 'dual' phenotype of the original mutant (37.7 ± 3.7% [n=3 of 6] and 67.9 ± 2.5% [n=3 of 6]; Figure 31B). By contrast, the new *fh*$_{AA}$ mutant displayed an intermediate defect (45.5 ± 7.5% [n=3]) contrasting with 76 ± 8% [n=5] for the *fh* mutant (Table 8) whereas the newly generated *fe*$_{AA}$ mutant produced variable data (48.8 ± 17.7% [n=3; ~45, 68, 34%]) in contrast to original *fe* mutant (66.8 ± 7.3% [n=3]). Despite this variability, the data supports the idea that EspF functions together with either Intimin, Tir or EspH (but not Map) proteins to promote EPEC's capacity to inhibit its uptake by J774.A1 macrophages.

Moreover, a potentially important role for Tir and Intimin cooperativity functions in EPEC's anti-phagocytic activity as supported by finding that the newly generated *et* double mutant (*et*$_{AA}$)- genotype supported by PCR analysis (Figure 32A)- had a similar defect to the original double mutant (56.2 ± 8 [n=3] versus 59 ± 6.6% [n=5]; Figure 32B). Support for the phagocytic-defective of the remaining double mutants (*fe, th, eh, tm, et*) was obtained, due to time constraints, using a different strategy i.e. assessing if plasmid re-introducing a missing gene led to an EPEC-like phenotype by visual observations of the 'Red' (extracellular) and 'Green' (total cell-associated) bacterial signals with findings only used if the identify of control (EPEC and T3SS mutant) strains were, in semi-blind studies, correctly defined. This work confirmed the expectation that introducing Intimin or Tir expressing plasmids into the *et* mutant led to an EPEC-like phenotype. Similarly, introducing a plasmid-expressing Intimin in the *fe* double mutant and Tir or Map into the *tm* double mutants resulted in EPEC-like phenotype in contrast to the T3SS mutant-like signal for the negative controls (T3SS and double mutants). While plasmid-expressing Tir in the *th* double mutant resulted in EPEC-like phenotype, plasmid-expressing EspH in the same double mutant resulted in an intermediate phenotype in contrast to the T3SS mutant. It should be re-iterated that strain identify was only revealed after the assessment was completed. Due to the time constraint, no data was generated with the *fe* mutant carrying an EspF expressing plasmid, thus, further work is needed to complement the *fe* double mutant with plasmid-expressing EspF and other possible complementation of the combinations.

Collectively, this work argues for important cooperative, but redundant, roles for Intimin and EspH (*eh* mutant), EspF and Tir (*ft* mutant), Tir and Map (*tm* mutant), Intimin and Tir (*et* mutant), EspF and EspH (*fh* mutant), EspF and Intimin (*fe* mutant), Tir and EspH (*th* mutant) but not Map and Intimin (*me* mutant), Map and EspH (*mh* mutant) or EspF and Map

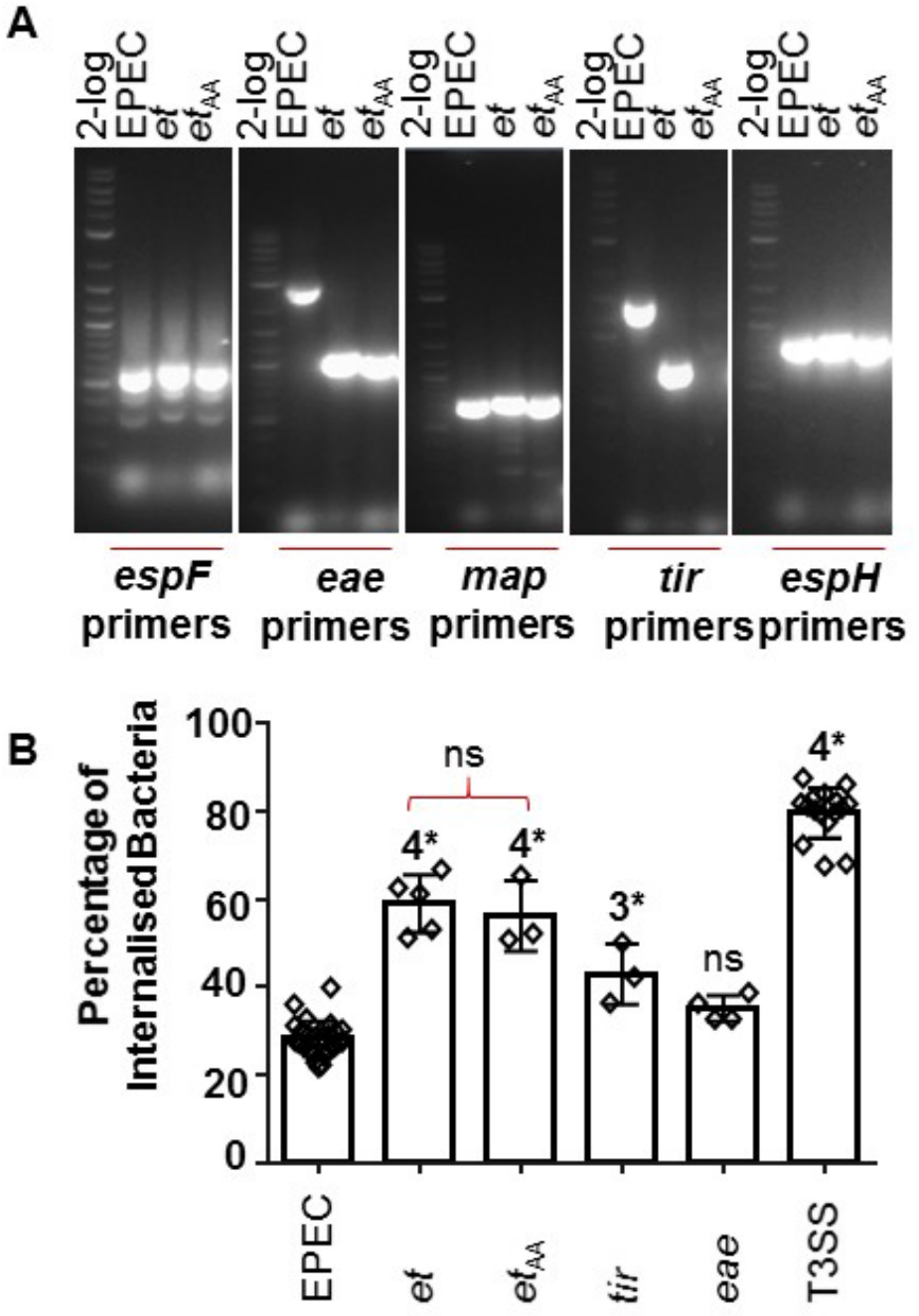


Figure 32. Confirming critical cooperative roles for Intimin and Tir proteins in promoting EPEC's capacity to inhibit its uptake in the J774.A1 model. Indicated strains were used for (A) PCR analysis or to infect (B) J774.A1 macrophages. (A) Oligonucleotides specific to *eae*, *map*, *espH*, *tir* and *espF* genes were used in PCR analyses to determine the presence of an intact or deleted genes with PCR products visualised, alongside molecular mass markers 2-log (1000Kb ladder), on a 1% agarose gel. (B) J774.A1 macrophages were infected for 1 h (MOI 100:1) with indicated strains (pre-grown in DMEM for 3 h) prior to determining the percentage of internalised bacteria using antibodies to label extracellular bacteria (Red) and total cell-associated bacteria (Green). Fifty macrophages with 20-50 cell-associated bacteria were randomly chosen to count the number of extracellular and total cell-associated bacteria (Green) enabling the percentage internalisation to be calculated. (B) Quantification studies were carried out in a semi-blind manner with the percentage of internalised bacteria displayed as the mean value (± standard deviation) with data from each independent experiment shown (◊).* indicates statistically significant differences (one-way ANOVA with Dunnett's post-hoc test) compared to EPEC data (4* $p < 0.0001$, 3* $p < 0.001$ and ns not significant) or by Tukey's test for multiple comparisons between strains (ns not significant; B). Studies used EPEC and strains lacking functional proteins Intimin and Tir (*et* and et_{AA}), Tir (*tir*), Intimin (*eae*) and T3SS (T3SS). Strains labelled '$_{AA}$' are newly generated with data for other strains taken from Figure 30.

(*fm* mutant) for EPEC to inhibits its uptake in this J774A.1 model. However, the finding that the phagocytosis defect associated with most double mutants was not as great as the T3SS mutant (1* p< 0.05, 2* p< 0.01, 4* p< 0.0001, ns not significant; Figure 30B) is suggestive of contributions of other factors.

4.2.3 Additional complexity in EPEC's anti-phagocytic activity

Examination of the ten triple mutant data, from three to six independent experiments, revealed outlier data with four (20% rate for *feh*, *fth* and *eth* mutants with 30% for the *fme* triple mutant; Table 8, Figure 33A) with the data also presented lacking the outliers (Figure 33B). As before, strain genotype was supported by PCR analysis of bacteria obtained from post macrophage infection (Figure 33C). Examining the triple mutant (include outlier) data revealed only one triple (*fmh*) mutant was statistically indistinguishable or very close (*feh* and *fme*; p<0.05) to the positive (EPEC) control data (Figure 33A). By contrast, the remaining (5) triple mutants displayed major, statistically significant (p<0.0001; Figure 33B), defects with a slightly reduced, but significant, defect was evident for two triple mutants (*meh* and *tmh*) (Figure 33B). Statistical analysis (after outlier data deletion) revealed only one triple (*feh*) mutant to behave like EPEC which is in contrast to prominent defects for the related double (*fe*, *fh*, *eh*) mutant strains (Figure 30B). One triple mutant (*fme*) had small, but statistically significant (Figure 33B), defects similar to two, of three, of the corresponding double (*fm*/*me*; Figure 30B, Table 8) but contrasts to prominent defects of the other double (*fe*; Figure 30B, Table 8) mutant. The remaining triple mutants had large defects, similar to the quintuplet (*etmfh*) mutant (Table 8), but significantly less than the T3SS mutant Figure 33B, Table 8). The triple mutant data could be placed into three distinct classes: first class contain *fet*, *fth* and *eth* which share a similar phenotype to the corresponding double mutants (Figure 34). For example, the *fet* triple mutant (66.3 ± 4.5% internalisation) behaves like the *et* (59 ± 6.6%), *fe* (66.8 ± 7.3%) and half of the *ft* (58.2 ± 14.5%) double mutants. It should be noted that we used the T3SS-like *ft* (n=3), not EPEC-like (n=3), data and that this phenotype 'instability' was not evident with the *fet* triple mutant suggesting that it relates to Intimin protein expression in the *ft* genetic background.

The second class contains the *tmf, etm* and *tmh* triple mutants which had similar defects to two of their related double mutants while the remaining double mutant had significant anti-phagocytic activity (Figure 35, Table 8). For instance, the *tmf* mutant (68.4 ± 5.4% internalisation) behaved like the *tm* (67.5 ± 2.5%) and *ft* (58.2 ± 14.5%) mutants but not *fm* (36.7 ± 5.5%) mutant. Again, specific to this example, we used the T3SS-like *ft* (n=3), not EPEC-like (n=3), data with phenotype 'instability' for the *tmf* triple mutant suggesting that it may also relate to Map protein expression in the *ft* genetic background. Moreover, the data

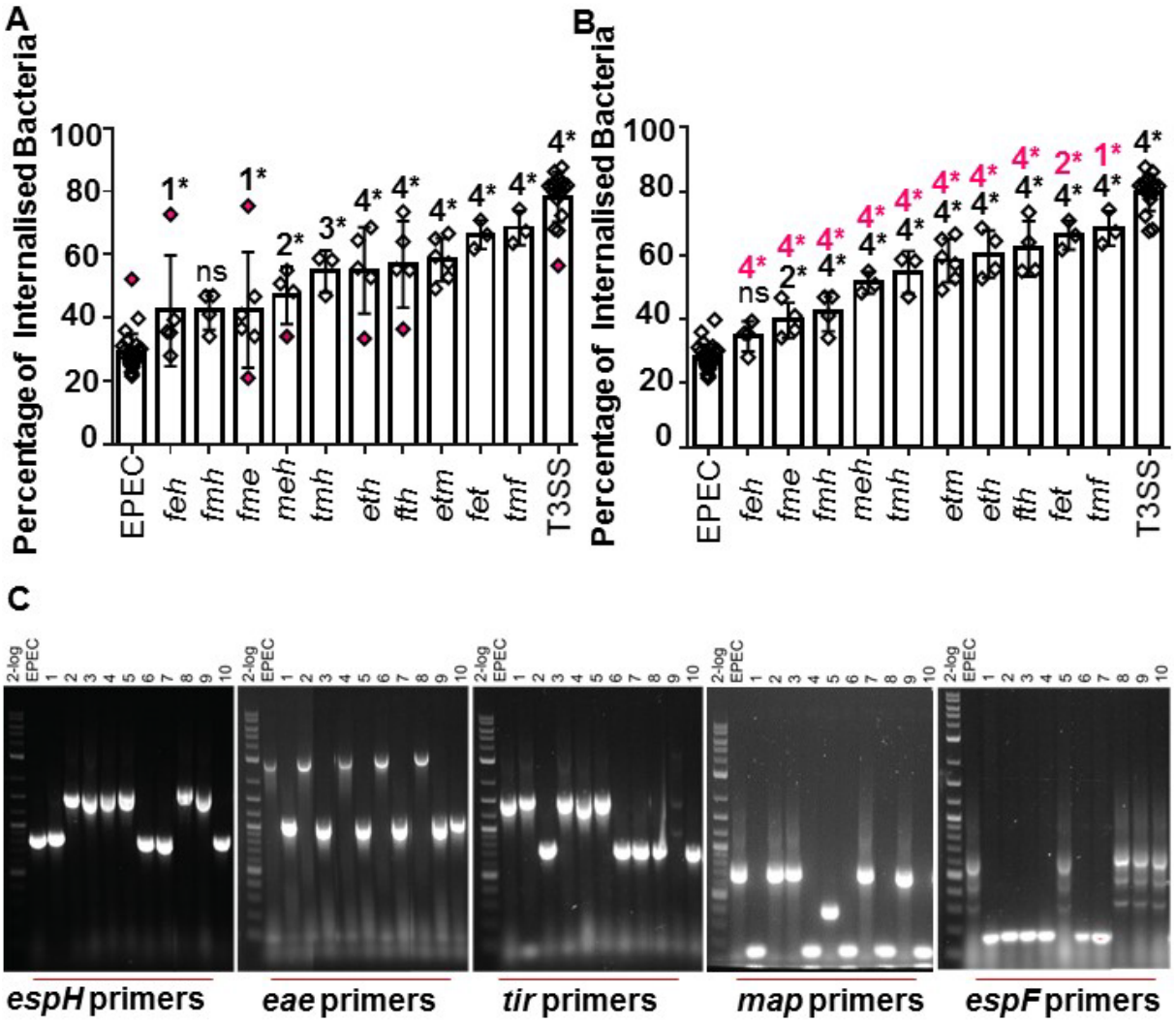


Figure 33. Additional complexity behind EPEC's ability to inhibit phagocytosis demonstrated by triple data. Indicated strains were used to infect (A&B) J774.A1 macrophages with, post-infection, non-adherent bacteria isolated for (C) PCR analysis. (A&B) J774.A1 macrophages were infected for 1 h (MOI 100:1) with indicated strains (pre-grown in DMEM for 3 h) prior to determining the percentage of internalised bacteria using antibodies to label extracellular bacteria (Red) and total cell-associated bacteria (Green). Fifty macrophages with 20-50 cell-associated bacteria were randomly chosen to count the number of extracellular and total cell-associated bacteria (Green) enabling the percentage internalisation to be calculated. (A&B) Quantification studies were carried out in a semi-blind manner with the percentage of internalised bacteria displayed as the mean value (± standard deviation) from at least three independent experiments. The data is also shown lacking outliers ◆(B) and used for statistical analysis with * indicating statistically significant differences (one-way ANOVA with Dunnett's post-hoc test) compared to EPEC data (1* $p < 0.05$, 2* $p < 0.01$, 3* $p < 0.001$, 4* $p < 0.0001$ and ns not significant; A&B). * indicates statistically significant differences (one-way ANOVA with Dunnett's post-hoc test) compared to T3SS data (1* $p < 0.05$, 2* $p < 0.01$, 4* $p < 0.0001$; B). (C) Oligonucleotides specific to *eae*, *map*, *espH*, *tir* and *espF* genes were used in PCR analyses to determine the presence of an intact or deleted genes with PCR products visualised, alongside molecular mass markers 2-log (1000Kb ladder), on a 1% agarose gel. Studies used EPEC and strains lacking a functional T3SS (T3SS) or three LEE proteins: EspF, Map and Intimin (*fme*; #1 in C), EspF, Tir and EspH (*fth*; #2 in C), EspF, Intimin and EspH (*feh;* #3 in C), EspF, Map and EspH (*fmh*; #4 in C), Map, Intimin and EspH (*meh*; #5 in C), Tir, Map and EspF (*tmf*; #6 in C), EspF, Intimin and Tir (*fet;* #7 in C), Tir, Map and EspH (*tmh*; #8 in C), Intimin, Tir and EspH (*eth;* #9 in C), Intimin, Tir and Map (*etm;* #10 in C).

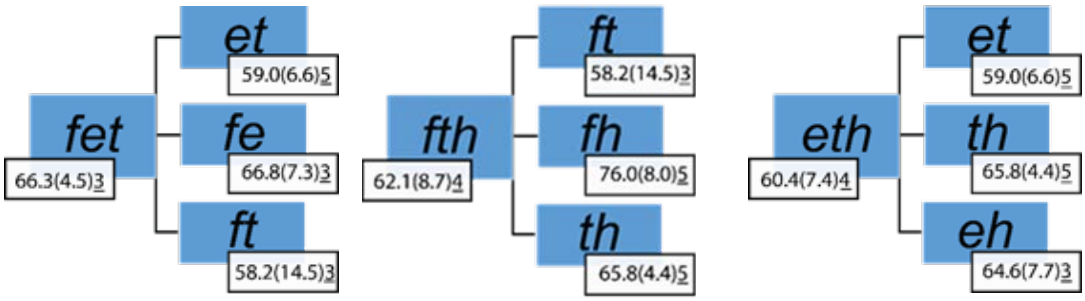


Figure 34. Class 1 mutant strains have T3SS mutant-like phenotypes similar to related double mutants. Shown data is mean data with SD in brackets and number of independent experiments indicated (underlined). It should be noted that we used the T3SS-like *ft* (n=3), not EPEC-like (n=3) for comparison with triple mutant.

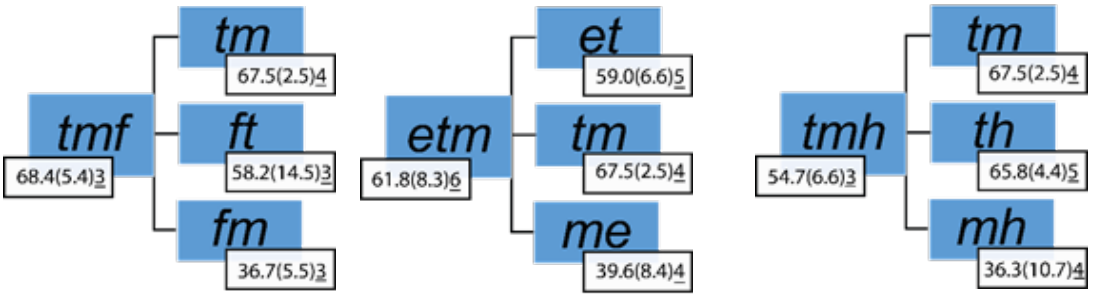


Figure 35. Class 2 mutant strains have T3SS mutant-like phenotypes that contrasts to one of the three corresponding double mutants. Shown data is mean data with SD in brackets and number of independent experiments indicated (underlined).

suggests that the phagocytic activity of the *fm*, *me* and *mh* double mutants depends on Tir activities.

The final class only contains the *meh* triple mutant (51.4 ± 3.3%) which shared a similar defect to only one of the double mutants (*eh* [64.6 ± 7.7%]) with the others (*mh* [36.3 ± 10.7%], *me* [39.6 ± 8.4%]) displaying an EPEC-like phagocytic phenotype (Figure 36, Table 8).

The *eh* mutant defect contrasts to the significant phagocytic activity of the *meh* triple mutant suggesting the Map suppresses the anti-phagocytic activity of other EPEC proteins, presumably Tir or EspF (as *eh* mutant defect similar to that of the *etmfh* mutant; Table 8) present in *meh* triple mutant contrasts to prominent defects of the other double (*fe* and *fh* respectively; Figure 30B, Table 8) mutants.

Interestingly, all but one (*tmfh* [n=5]) of the more complex multi-mutants (quadruple/quintuple) was not associated with outlier data with a 20% rate for the *etmfh* (n=5), ~17% for *etmh* (n=5) and *feth* (n=5) with only 5% for *etmf* (n=19) mutants (Figure 37A, Table 8) with the data also present lacking these outliers and used for statistical analysis (Figure 37B).

As usual, strain genotype was supported by PCR analysis of bacteria (Figure 37C) obtained, at the end of an experiment, from infected wells. While each multi-mutants displayed a significant defect, relative to EPEC, two strains (*fmeh*, *etmh*) appeared to retain significantly anti-phagocytic activity compared to the *feth* and *etmf* mutants which behaved like the quintuplet, *etmfh* (63.9 ± 8.9% internalised), strain (Figure 37B). The latter findings suggest that the *fmeh* and *tmfh* mutant's anti-phagocytic activity depends on the remaining proteins (Tir and Intimin respectively). However, interpretation of the final quadruple mutant (*tmfh*) is hindered by phenotype instability; 3 data points cluster around ~38% and two ~70% (Figure 37B). Interestingly, each multi-mutant was significantly more phagocytic than the T3SS mutant indicating the presence of additional, T3SS-dependent, factors that can inhibit EPEC uptake (Figure 37B).

The multi-mutants also fell into three distinct classes with the first (Figure 38) containing only one member (*fmeh*; 39.1± 10% internalisation) which retains significant levels of anti-phagocytic activity similar to each of the four related triple mutants - *fmh* (42.4 ± 6.1%), *fme* (39.7 ± 5.5%), *feh* (34.7 ± 4.8 %), *meh* (47.1 ± 9.0 %) – and thus does not identify any extra inter-relationships. This work suggests, as mentioned above, that the *fmeh* mutant's phagocytic activity is Tir dependent.

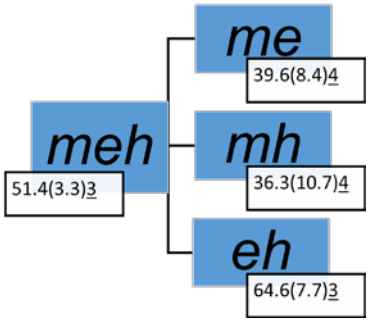


Figure 36. Class 3 mutant strains have T3SS mutant-like phenotypes similar to only one of the three corresponding double mutants. Shown data is mean data with SD in brackets and number of independent experiments indicated (underlined).

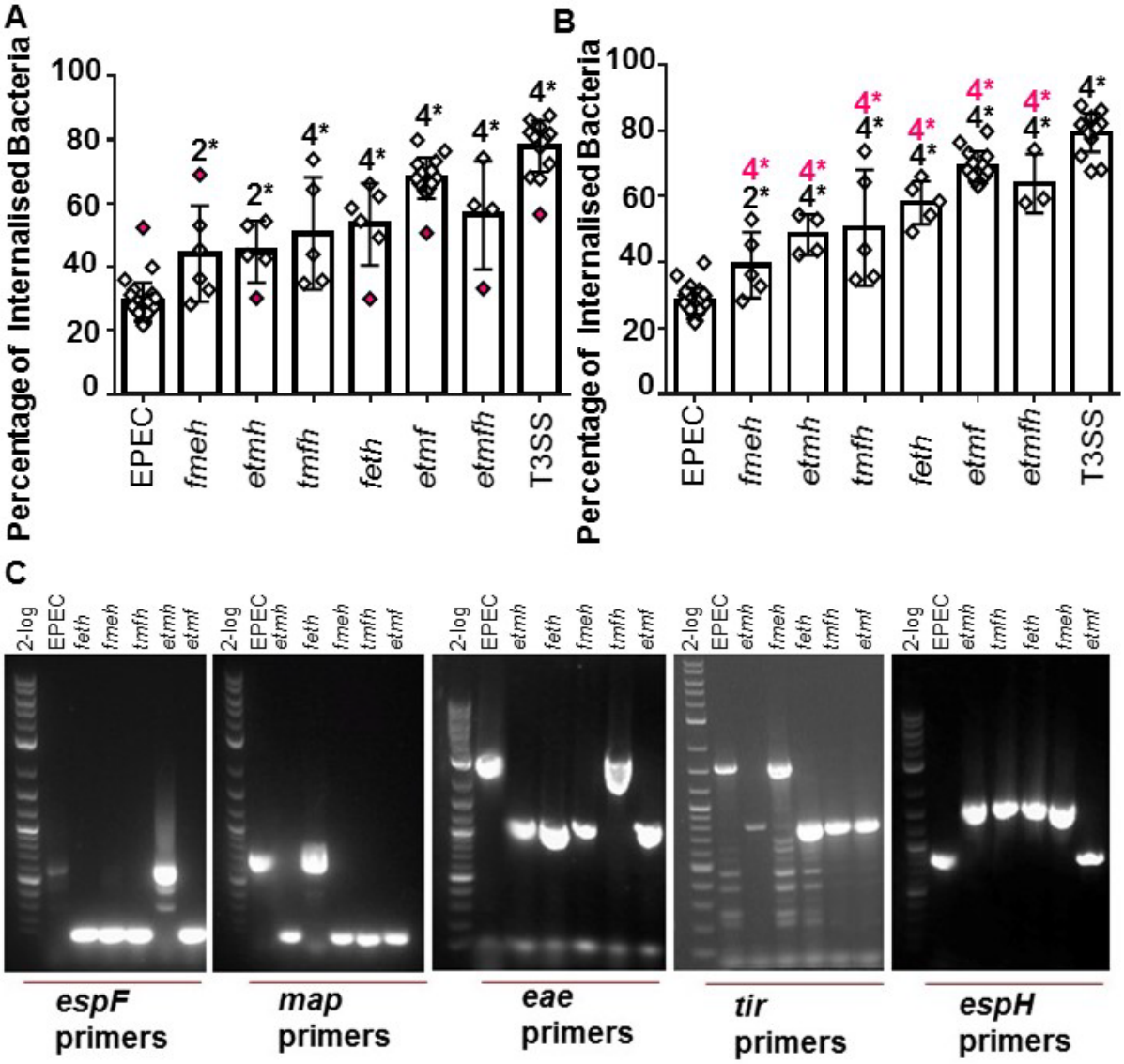


Figure 37. Additional complexity in EPEC's capacity to inhibit its uptake by J774.A1 macrophages. Indicated strains were used to infect (A&B) J774.A1 macrophages with, post-infection, non-adherent bacteria isolated for (C) PCR analysis. (A&B) J774.A1 macrophages were infected for 1 h (MOI 100:1) with the indicated strains (pre-grown in DMEM for 3 h) prior to determining the percentage of internalised bacteria using differential antibody staining to label extracellular bacteria (Red) and total cell-associated bacteria (Green). Fifty macrophages with 20-50 cell-associated bacteria were randomly chosen to count the number of 'Red' and 'Green' bacteria. Quantification studies were carried out in a semi-blind manner with the percentage internalised bacteria displayed as the mean value (± standard deviation) with data from each independent experiment shown (◊) with outliers (◆) removed providing data (B) used for statistical. * indicates statistically significant differences (one-way ANOVA with Dunnett's post-hoc test) compared to EPEC data (2* $p < 0.01$, 4* $p < 0.0001$; B). * indicates statistically significant differences (one-way ANOVA with Dunnett's post-hoc test) compared to T3SS data (4* $p < 0.0001$; B). (C) Oligonucleotides specific to *eae*, *map*, *espH*, *tir* and *espF* genes were used in PCR reactions to determine the presence or absence of intact genes. The PCR products were visualised, alongside 2-log molecular mass markers on a 1% agarose gel. Studies used EPEC and strains lacking a functional T3SS (T3SS) or four or five of the LEE proteins Map (*m*), Intimin (*e*), EspH (*h*), Tir (*t*), and/or EspF (*f*). Note strain name indicates order genes were inactivated, for example, *etmfh* reveals gene encoding Intimin was disrupted first, then Tir, then Map, then EspF and finally EspH.

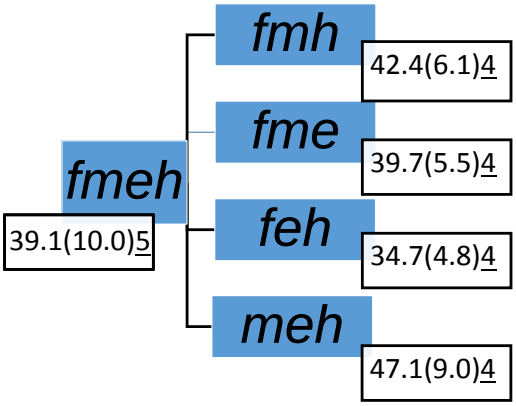


Figure 38. Class 1 mutant strain has significant anti-phagocytic activity similar to related triple mutants. Shown data is mean data with SD in brackets and number of independent experiments indicated (underlined).

The second class (Figure 39) contains the *feth* (58.1 ± 6.5%) and *etmf* (68.5 ± 4.4%) mutants as sharing a similar defect with three triple mutants with the fourth displaying significantly more phagocytic activity i.e. *feh* (34.7 ± 4.8%) and *fme* (39.7 ± 5.5%) respectively. The latter differences suggest that the *feh* and *fme* mutant's anti-phagocytic activity depends on Tir.

The final group (Figure 40) contains the *etmh* (48.4 ± 6.1% internalisation) and *tmfh* (50.5 ± 17.6%) mutants which apparently have significantly more anti-phagocytic activity than several corresponding triple mutants (*etm* [61.8 ± 8.3%]/*eth* [60.4 ± 7.4%] and *tmf* [68.4 ± 5.4%]/*fth* [62.1 ± 8.7%] respectively) and the quintuple (*etmfh* [63.9 ± 8.9%]) mutant. The latter implies that the anti-phagocytic activity of the *etmh* and *tmfh* mutants depends on the remaining proteins (EspF and Intimin respectively) whose contributions are suppressed by Map and/or EspH.

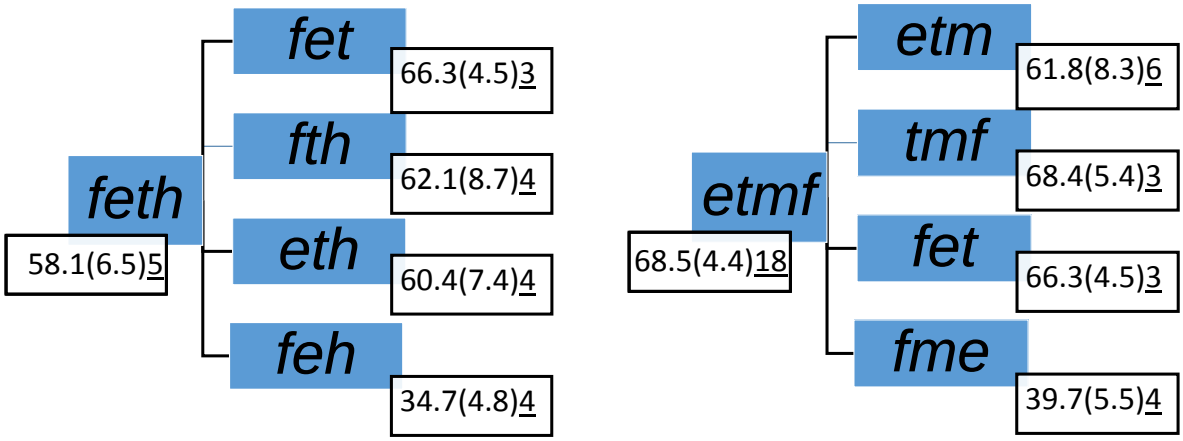


Figure 39. Class 2 mutant strains have T3SS mutant-like phenotypes that contrasts to one of the four corresponding triple mutants. Shown data is mean data with SD in brackets and number of independent experiments indicated (underlined).

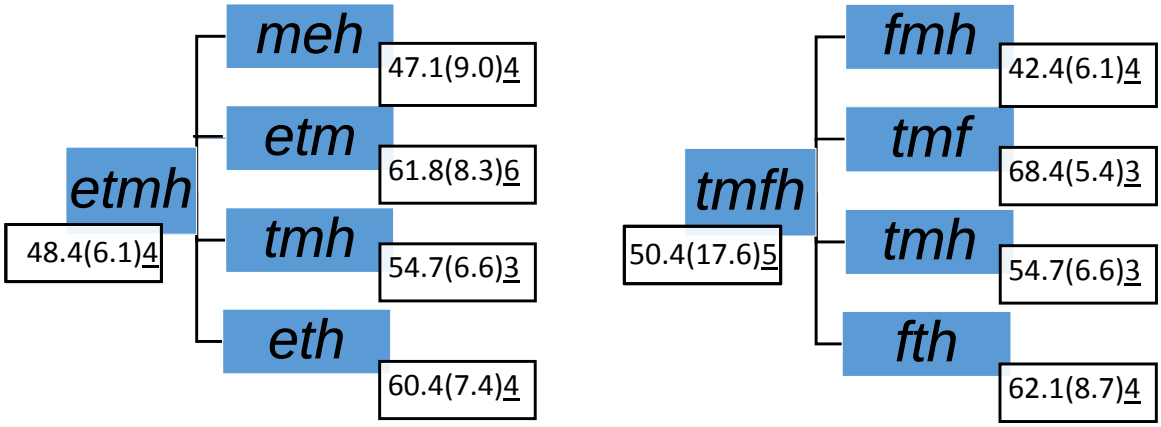


Figure 40. Class 3 mutant strains have significant anti-phagocytic activity in contrast to several related triple mutants which display T3SS mutant-like phenotypes. Shown data is mean data with SD in brackets and number of independent experiments indicated (underlined).

4.3 Discussion

EPEC's ability to inhibit PI 3-kinase dependent phagocytosis has been linked to the subversive activity of the LEE-encoded EspF, EspH EspB and EspG effector proteins (Quitard *et al.*, 2006; Iizumi *et al.*, 2007; Dong *et al.*, 2010; Humphreys *et al.*, 2016). Previous data (Chapter 3) suggests that EspF alone is not sufficient to inhibit EPEC uptake and this may require the LEE *core* region-encoded effectors (Tir, Map, EspH) and/or the Intimin surface protein to aid in the process. Screening of mutants in which genes encoding these five proteins were inactivated in all combinations, linked disruption of some genes with phenotype instability and, surprisingly, failed to support critical roles for EspF or EspH effectors. Indeed, this work suggests that EspF and EspH have indirect roles in EPEC's anti-phagocytic activity and phenotype instability. Crucially, the work in this thesis shows EPEC's anti-phagocytic activity (in the J774A.1 model) is dependent either on the function of Tir or Intimin (redundant roles) and reveals unknown complexity behind EPEC's ability to inhibit its uptake by macrophages.

The critical role of EspF in inhibiting EPEC uptake by macrophages or M-cells has been demonstrated in many studies (Quitard *et al.*, 2006; Martinez-Argudo *et al.*, 2007; Marchès *et al.*, 2008; Tahoun *et al.*, 2011). Indeed, while this idea was supported by my initial studies using an *espF* mutant (carrying a GFP expressing plasmid; chapter 3 Figure15), the screening studies conducted with the *espF* mutant (no GFP plasmid) revealed the mutant to behave like EPEC. A possible explanation for the these findings include the deleterious effects of GFP expression as previously reported in *in vivo* (Krestel *et al.*, 2004; Agbulut *et al.*, 2007) and *in vitro* (Hanazono *et al.*, 1997; Liu *et al.*, 1999), that may affect *espF* mutant's ability to inhibit phagocytosis. This unexpected finding was supported by studies with *espF* clones obtained from frozen stock collections of former lab members thereby arguing against the difference stemming from acquisition of genetic alterations. Indeed, a high level of stability has been found in the EPEC genome (Donnenberg *et al.*, 2015) and clones came from the same 'founder' stock. Crucially, EspF's anti-phagocytic activity has been demonstrated in other laboratories (Quitard *et al.*, 2006; Martinez-Argudo *et al.*, 2007; Marchès *et al.*, 2008; Tahoun *et al.*, 2011), using the *espF* mutant obtained from our group (Quitard *et al.*, 2006; Martinez-Argudo *et al.*, 2007), while others initially failed to define a phagocytic defect (Celli *et al.*, 2001).

This phenotype instability is possibly due to alterations in the infection model, for example, macrophage passaging-induced alterations (genetic or epigenetic incorporated into new frozen stocks) and/or reagent variability (especially cytokine-rich Foetal Calf Serum as known batch-to-batch variability) (Bhatt *et al.*, 2011; Baker, 2016). However, we predict that the phenotype instability relates to EPEC, as inactivating multiple genes was linked to

increasing number of outlier data. Thus, while outlier data was relatively rare for EPEC, for T3SS and single mutants (0-6% rate) there was a 20-30% rate for some multi-mutants. Whereas one double mutant (*ft*) displayed an 50% outlier rate i.e strain acting like the T3SS mutant in half the experiments and like EPEC in the others. The fact that these strains sometimes displayed wild type phenotype (although it is not consist), implies they do possess inhibitory activity(ies) but cannot always deploy them. This could be due to experimental/environmental differences impacting on the expression and/or activity of key factors (T3SS, LEE effectors, Intimin) and/or the delivery of all, or specific effectors. Indeed, LEE protein expression is known to be regulated not only at the transcriptional level but also post-transcriptional and post-translational levels (Bhatt *et al.*, 2011). Interestingly, phenotypic instability seemed to be under the influence of Map and EspH activities as there was a 50% outlier rate for the *ft* (n=6) double mutant decreasing to 20% for the *fth* (n=5) triple and 0% for *tmfh* (n=5) quadruple mutant. Further studies are needed to investigate this further and to determine where their impact is on EPEC (e.g. influencing protein expression/delivery) or the host cells (e.g. altering cellular process with direct/indirect impact on PI-3 kinase uptake pathway). A role for genetic alterations introduced during allelic exchange mediated gene knockout in some of the unexpected findings is possible as re-generating some multiple mutants, from original single mutants, did not always produce similar results. For example *ft* instability was not evident with the *ft*$_{AA}$ (strain name indicates the order genes were inactivated) which now mimicked the T3SS mutant while a T3SS mutant phenotype of the original *fh* (n=5) double mutant was not reproduced by *fh*$_{AA}$ (n=3; 45.5 ± 7.5% internalisation versus 76 ± 8%). The genomes of the latter two strains have been sequenced but data reassembly and comparison analyses have not been completed.

This study suggests that anti-phagocytosis, at least in the J774A.1 model, is a more complex process than recognised. As EspF, EspH and Map activities are not required for the process, unless under specific infection conditions or when EPEC lacks specific gene products - but is driven by the remaining interrogated factors; Intimin and Tir (via redundant and, possibly, cooperative mechanisms).

While *tir* mutant, consistently displayed a higher level of internalisation than wild type, statistically significant, both *et* and *et*$_{AA}$ mutant strains consistently displaying a more prominent defect. In contrast, the *eae* mutant behaves like EPEC phenotype, suggests a more prominent role for Tir in the inhibitory processes consistent with a previous report (Goosney *et al.*, 1999). The validity of our data is supported by the findings that: (i) a *tm* mutant strain is the only Map-deficient double mutant that failed to inhibit its uptake compared with T3SS, as Map

was excluded from inhibition process, supporting that EPEC dependent on Tir to inhibit its internalisation, (ii) the deletion of Tir from double and triple mutant strains (*fm*, *mh*, *me*, *fme*, *feh*) resulted in loss of the ability to inhibit phagocytosis (iii) the mutant strain which only expresses Tir (*fmeh*) demonstrated a greater level of anti-phagocytosis than the T3SS mutant, which indicates implicates an ability of Tir to inhibit EPEC uptake.

Interestingly, while the quadruple *etmf* mutant consistently displayed the reported major defect in inhibiting phagocytosis (Quitard *et al.*, 2006), this was mirrored by the newly-generated *et* and quintuple *etmfh* mutants. None of these strains were as defective as the T3SS mutant, arguing for the existence of additional factors can also influence the macrophage uptake process.

The complex data set raises many predictions for interrogation. For example, the work predicts that Tir or Intimin can drive anti-phagocytosis in the absence of the other three interrogated proteins and that Map, EspF and EspH activity can influence the Tir and/or Intimin-dependent anti-phagocytic processes. Moreover, the finding that the *etmf* anti-phagocytic defect can be effectively rescued by re-introducing *espF* (Quitard *et al.*, 2006)(and this study), is surprising as the corresponding *etm* mutant behaves like the *etmf* mutant. The latter suggests that the EspF copy number is responsible for this difference with associated higher EspF delivery levels. Possibly, altering macrophage processes in a manner that makes the PI3 kinase-dependent phagocytic pathway more sensitive to inhibition by other delivered effectors i.e. the remaining interrogated gene product, EspH, and/or other effectors. Indeed, other data argues for Map, EspF and EspH being able to suppress EPEC's anti-phagocytic activity. For example, the T3SS mutant phenotype of the *eh* double mutant contrasts with EPEC-like phenotype of the *feh* triple suggesting that the EspF, in this genetic background, suppresses the phagocytic activity of Map or, more likely, Tir. Indeed, Map suppresses the anti-phagocytic activity of other effector molecules, as it presence in *fe*, *fh* and *eth*, allow mutant strains to demonstrate low anti-phagocytosis activity. In contrast, the absence of Map from mutant strains (*fme*, *fmh* and *etmh*) increased the anti-phagocytosis activity to a far greater extent than when expressed. There is also evidence for Intimin and EspH possessing suppressive roles, in specific genetic background.

In summary, our studies argue that EPEC's anti-phagocytic activity depends on the activity (redundant) of Tir or Intimin proteins with Map, EspF, EspH and, possibly, other effectors having activities that can impact on the anti-phagocytic process in manner influenced by environmental factors.

Chapter 5. Preliminary investigations on EspF, Tir and Intimin's roles in enabling EPEC to inhibit their uptake by J774.A1 macrophages

5.1 Introduction

Work in the previous chapter implied that EspF has an indirect role in EPEC's anti-phagocytic activity while Tir or Intimin activities may drive the inhibitory process and thus the focus was on these three proteins.

5.1.1 EspF

EspF is a small (206 amino-acids) protein with the N-terminal 73 amino-acids containing three targeting signals: for T3S secretion (residues 1-20), targeting to mitochondria (MTS; residues 1-101) and the host nucleolus (residues 21-74) (Charpentier and Oswald, 2004; Nagai *et al.*, 2005; Dean *et al.*, 2010b). Substitution of leucine 16 to glutamic acid (L16E) prevents EspF targeting the mitochondria (Nagai *et al.*, 2005). By contrast, the rest of EspF is composed of three highly similar polyproline-rich repeat regions (PPR) (Figure 41A) (McNamara and Donnenberg, 1998). Each PPR has binding sites for sorting nexin 9 (SNX9) and N-WASP leading to a complex that remodels the host plasma membrane (Marchès *et al.*, 2006; Alto *et al.*, 2007). EspF has been reported to interact with at least 12 host proteins in several cellular compartments i.e. host cytoplasm, mitochondria, plasma membrane (apical and lateral surfaces) and nucleus (Dean and Kenny, 2009; Holmes *et al.*, 2010). EspF is multifunctional linked to many subversive activites (Dean and Kenny, 2009). For instance, EspF targeting mitochondria is linked to permeabilise the mitochondrial membrane, which releases cytochrome *c* into the cytoplasm. The latter in turn activates caspases and triggers apoptosis as evidenced by phosphatidylserine on the cell surface, internucleosomal DNA cleavage and caspase cleavage of cytokeratins (Crane *et al.*, 2001; Nagai *et al.*, 2005). *In vivo* studies (mouse model with mouse-specific strain), linked cell death with EspF targeting mitochondria (Nagai *et al.*, 2005). EspF activity is also linked to disease-associated processes such as (i) disrupting enterocyte-enterocyte interactions i.e. tight junctions thereby compromising barrier function, (ii) the elongation-associated loss/effacement of absorptive microvilli, (iii) altering activity of ion/nutrient/fluid transporters and (iv) inhibiting EPEC uptake by phagocytic Microfold (M-cells) (Dean *et al.*, 2006; Guttman *et al.*, 2006; Quitard *et al.*, 2006; Martinez-Argudo *et al.*, 2007; Hodges *et al.*, 2008; Marchès *et al.*, 2008; Peralta-Ramirez *et al.*, 2008; Dean and Kenny, 2009; Tahoun *et al.*, 2011). Importantly, EspF can act with other effectors to subvert cellular processes, for example with Tir, Map and Intimin activities to disrupt intestinal barrier function and efface microvilli (Dean and Kenny, 2004; Dean *et al.*, 2006).

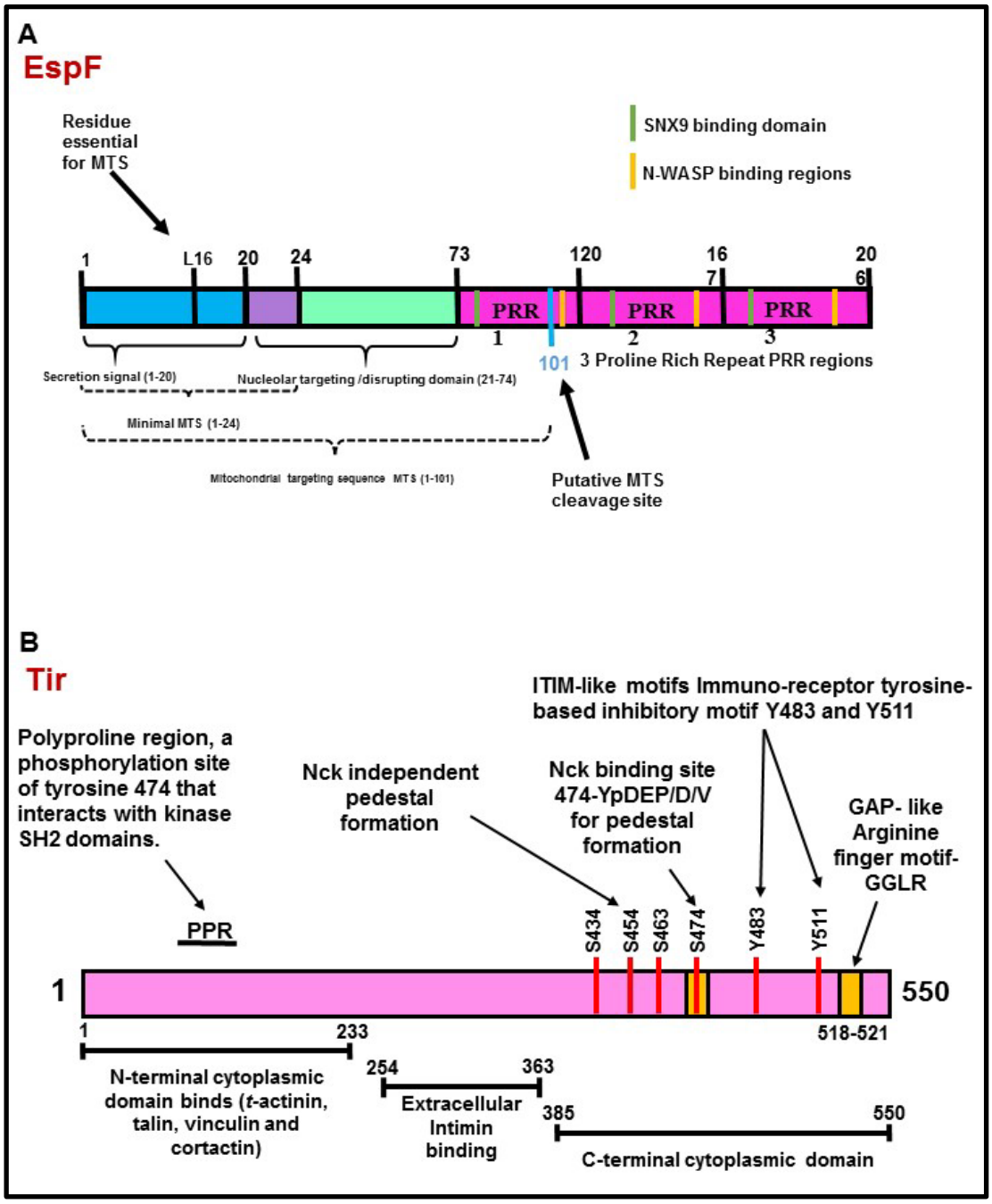


Figure 41. Schematic representation of EspF and Tir effector proteins. (A) EspF is a 206 amino acid protein with its N-terminus (residues 1-20) required for secretion; while the mitochondrial targeting sequence (MTS, residues 1-24) is required for mitochondrial targeting with an essential leucine 16 residue; and a nucleolus targeting signal (residues 21-74). The remainder of the protein (C-terminus) is composed of three polyproline-rich repeat regions (PPR). Each PPR possess a number of eukaryote-like binding domains (sorting nexin 9 (SNX9) and N-WASP, respectively) that are thought to enable the recruitment of various host proteins. (B) Tir is a 550 amino acid protein with an extracellular domain (residues 254-363) that binds the EPEC surface protein intimin. The remaining residues of Tir are at the N-terminus (residues 1-233) and the C-terminus (residues 383-550), the latter located in the cytoplasm of the host and are the target of numerous kinases. Two serine residues, S434 and S463, are phosphorylated by protein kinase A, while Y474 phosphorylation is critical for Nck recruitment and N-WASP activation. This leads to Arp2/3-dependent actin nucleation and consequently, pedestal formation. Moreover, Y454 phosphorylation leads to the recruitment of PI3-kinase (phosphatidylinositide 3-kinase). Recruitment of the latter kinase is associated with an increase of phosphatidylinositol (3, 4, 5)-trisphosphate, which is believed to cause pedestal elongation, aid bacterial attachment and inhibit cell death. Adapted from Dean and Kenny *et al.*,(Dean and Kenny, 2009).

Key to EspF's subversive activity is its efficient translocation into host cells which depends on the chaperone CesF (Elliott *et al.*, 2002). CesF promotes EspF stability within EPEC and its efficient translocation into cells but is not needed for T3SS-dependent secretion of EspF into the extracellular milieu (Elliott *et al.*, 2002). While previous studies have demonstrated a key role for EspF to enable EPEC to inhibit its uptake in cultured macrophages and M-cells (*in vitro* and *in vivo*) models (Quitard *et al.*, 2006; Martinez-Argudo *et al.*, 2007; Marchès *et al.*, 2008; Tahoun *et al.*, 2011), studies argue against a need for EspF to target mitochondria or binding the SNX9 or N-WASP proteins in the anti-phagocytosis process (Quitard *et al.*, 2006; Weflen *et al.*, 2010; Young, 2013). By contrast, one study implicated a critical role for the N-terminal 101 residues: swapping this region for the first 101 residue of Tir led to a fusion protein, TirΔMTSEspF, that did not rescue the anti-phagocytic defect of an EspF-deficient strain (Quitard *et al.*, 2006). The N-terminal 101 residues of Tir encode signals required for direct T3SS and binding to the Tir chaperone, CesT, as well as features linked to recruiting host kinases and cytoskeletal proteins (see below).

5.1.2 Tir

Tir delivery into cells leads to its insertion into the host plasma membrane to act as a receptor for Intimin linked to mediating intimate host-pathogen interactions and inducing subversive signalling leading to alterations including polymerisation of actin beneath adherent EPEC i.e. pedestal formation (Kenny *et al.*, 1997a; Campellone *et al.*, 2004a). Tir is a 550 amino acid protein with N- (residues 1-233) and C- (residues 383-550) terminal regions exposed to the host cytoplasm with an extracellular Intimin binding domain (residues 254-363) linked by two transmembrane domains (De Grado *et al.*, 1999; Hartland *et al.*, 1999; Kenny, 1999).

As mentioned, the N-terminus contains a feature (polyproline-rich region) that recruits tyrosine kinases to phosphorylate a C-terminal-located tyrosine residue, Y474 (Kenny, 2001a; Phillips *et al.*, 2004; Swimm *et al.*, 2004; Bommarius *et al.*, 2007). The N-terminal domain is also linked to the recruitment of many cytoskeletal-related proteins (Bommarius *et al.*, 2007). By contrast, the C-terminus has several known substrates for phosphorylation by host kinases, including protein kinase A (PKA) and tyrosine kinases. Phosphorylation on two serine residues, S434 and S463 (Figure 41B), is linked, as previously discussed, to shifts in apparent molecular mass and the membrane insertion

process as well as efficient pedestal formation (Warawa and Kenny, 2001). Phosphorylation on two tyrosine residues, Y454 and Y474, is linked to actin nucleation by inefficient and efficient mechanisms respectively. Most is known about the latter process where Y474 phosphorylation recruits an adaptor protein, Nck, which recruits N-WASP to sequester the Arp2/3 actin nucleating machinery to induce pedestal formation (Kenny, 1999; Gruenheid *et al.*, 2001; Campellone *et al.*, 2002). Y454-associated actin nucleation occurs independently of Nck (Campellone and Leong, 2005) but, leads to the recruitment of phosphatidylinositide 3-kinase (PI-3 kinase) and SHP2 (Selbach *et al.*, 2009). PI-3 kinase recruitment is linked to an increase of phosphatidylinositol (3, 4, 5)-trisphosphate levels linked to promoting pedestal elongation, phagocytosis and inhibiting cell death (Franke *et al.*, 1997; Selbach *et al.*, 2009; Rosales and Uribe-Querol, 2017). Tir is also phosphorylated on Y484 and Y511 (ITIM-like motifs) linked to recruitment of SHIP-2 (SH2 domain-containing inositol 5-phosphatase) which suppresses signalling that induces anti-microbial responses (Yan *et al.*, 2013).

Importantly, most of Tir's activities depend on the interaction with Intimin inducing intimate pathogen-host cell interaction, pedestal formation, microvilli effacement, disruption of enterocyte-enterocyte interactions and downregulation of Map activity, i.e. filopodia formation (Dean *et al.*, 2006; Dean *et al.*, 2013) and barrier disruption (Dean and Kenny, 2004; Dean *et al.*, 2006; Dean *et al.*, 2013). The latter event depends on an arginine finger-like motif linked to down-regulation of small GTPase activity with Map activating GTPases including Cdc42 and, indirectly, Rho (Kenny *et al.*, 2002; Berger *et al.*, 2009).

Intimin-independent Tir activity is only a recently discovery, and is linked to suppressing EspG subversive activities and inhibiting signalling that induces anti-microbial and inflammatory responses (Dean *et al.*, 2010a; Ruchaud-Sparagano *et al.*, 2011).

5.1.3 Intimin

Intimin is a 939-residue autotransporter protein encoded by the LEE *eae* gene and inserted into the bacterial outer membrane (Frankel *et al.*, 1998; Fairman *et al.*, 2012). The Intimin N-terminal region (residues 1–550) containing a signal peptide region, which is responsible for its secretion to the bacterial surface and mediating its binding to the peptidoglycan (Leo *et al.*, 2015). Whereas its C-terminal region binds to Tir, and this interaction between Intimin and Tir is important for intimate interaction to the infected

host cells and pedestal formation in a Tir tyrosine phosphorylation dependent manner (Kenny and Finlay, 1997). While Intimin's critical virulence role is related to its binding to Tir, it can also interact with host receptor proteins including β-integrin and nucleolin (Frankel *et al.*, 1996; Sinclair and O'Brien, 2002) linked to cellular alterations (Muza-Moons *et al.*, 2003; Dean and Kenny, 2011). However, Intimin has also Tir independent functions in subverting host pathways. For example, both Intimin and Tir deficient mutants display an independent intermediate anti-phagocytosis activity compared with EPEC (Goosney *et al.*, 1999). Indeed, Intimin has been shown to be essential for EspF and Map to disrupt the intestinal barrier function without being interact with Tir (Dean and Kenny, 2004).

The aim of this chapter was to confirm contributions for EspF, Tir and Intimin proteins in EPEC's anti-phagocytic activity and to identify important features and/or domains.

5.2 Results

5.2.1 EspF's anti-phagocytic activity is linked to the N-terminal domain but not to N-WASP recruitment

While the recent evaluation of an *espF* mutant revealed an EPEC-like phenotype, contrasting with the published T3SS-like phenotype (Quitard *et al.*, 2006), an EspF-deficient quadruple (*etmf*) mutant always displayed a major defect which could be rescued by introducing an EspF-expressing plasmid. To confirm this and reported no roles for EspF binding of N-WASP in contrast to N-terminal 101 residues, anti-phagocytosis assays were carried out with the A3 variant which lacks the three putative N-WASP binding motifs and TirΔMTSEspF variants (pΔ101*espF*) as before. Infection studies were also carried out with HeLa cells with Western blots analysis revealing absence of EspF in the non-complemented strain with complementation linked to EspF protein delivery and, as expected, only the TirΔMTSEspF variant being detected by anti-Tir and anti-EspF antibodies (data not shown).

As before (Chapter 3; Figure 17)(Quitard *et al.*, 2006), the *etmf* mutant strain was readily internalised by macrophages with the majority (71.8 ± 6.2%) of bacteria inside the cells in contrast to only 31 ± 2.7% of EPEC bacteria ($p<0.0001$; Figure 42). Importantly, this work confirmed (Young, 2013) that the A3 variant rescued the anti-phagocytic defect of the *etmf* mutant (Figure 42) supporting the inhibitory process to be independent of EspF binding N-WASP. By contrast, the TirΔMTSEspF variant failed to rescue the *etmf* mutant anti-phagocytic defect (Figure 42) as reported (Quitard *et al.*, 2006). The latter data supports the idea that EspF's role in the anti-phagocytosis process requires features in the N-terminal 101 residues or that the Tir domain (or its impact on the C-terminal EspF domain) interferes with the anti-phagocytic process. To further examine the possible role of the N-terminal domain attempts were made to swap the N-terminal MTS regions between EspF and Map but this was not successful. Thus, another strategy was undertaken to identify domains or features responsible for EspF to inhibit phagocytosis.

5.2.2 Possible role for residues 51-73 in EspF is ability to inhibit phagocytosis

To further explore EspF domains or features contributing to the anti-phagocytic process, we took advantage of variants previously constructed by Jon Scott (this lab). Prior to investigating their anti-phagocytic activity, the plasmids were isolated from the *etmf*

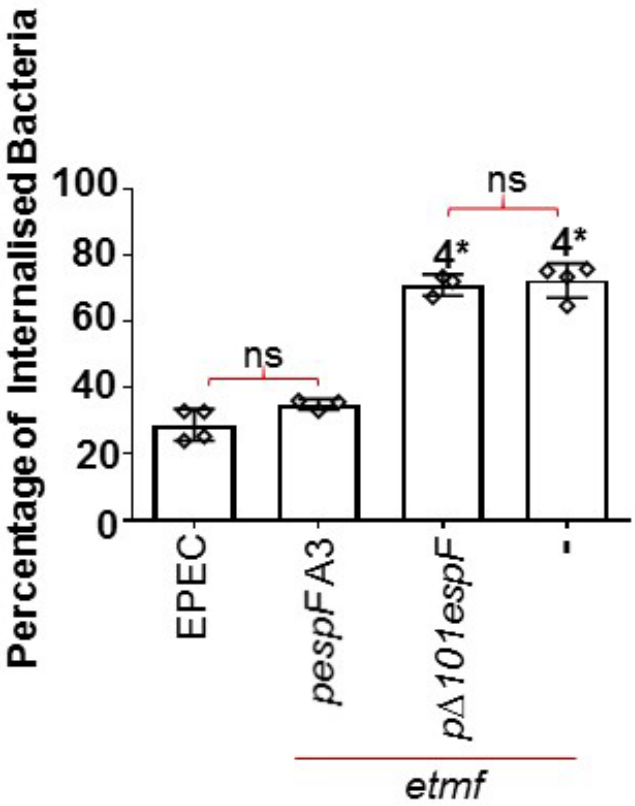


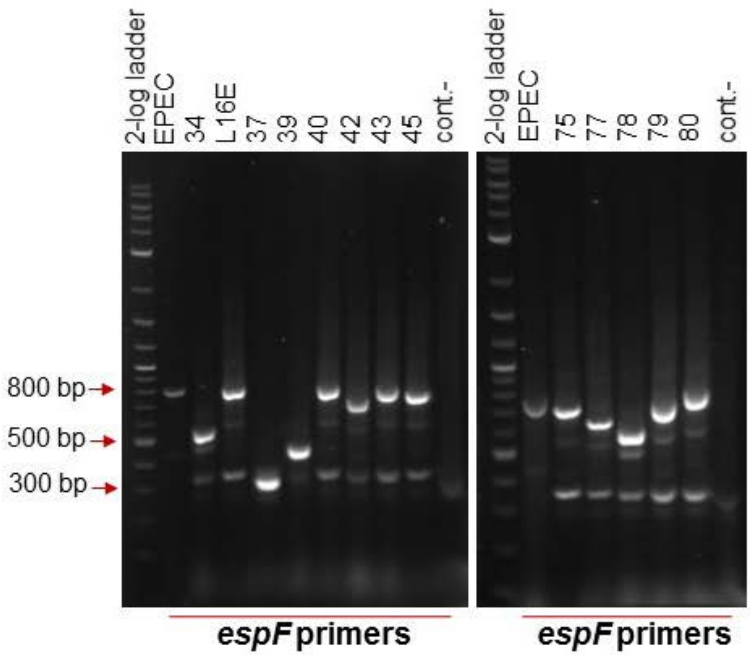
Figure 42. EspF's N-terminal domain but not putative N-WASP binding sites is linked to inhibiting the uptake of the *etmf* quadruple mutant. J774.A1 macrophages were infected for 1 h (MOI 100:1) with indicated strains (pre-grown in DMEM for 3 h) prior to determining the percentage of internalised bacteria using antibodies to label extracellular bacteria (Red) with total cell-associated bacteria detected by plasmid expression of the Green Fluorescent Protein (Green). Fifty macrophages with 20-50 cell-associated bacteria were randomly chosen to count the number of extracellular and total cell-associated bacteria (Green) enabling the percentage internalisation to be calculated. Quantification studies were carried out in a semi-blind manner with the percentage of internalised bacteria displayed as the mean value (± standard deviation) from at least three independent experiments (◊). * indicates statistically significant differences (Dunnett's post-test) compared to EPEC data (ns not significant, 4* $p < 0.0001$; one-way ANOVA*)* or by Tukey's test for multiple comparisons between strains (ns not significant). Strains used were EPEC, *etmf* (lacks LEE-encoded Intimin [*eae* gene product] plus Tir, Map and EspF effectors) and *etmf* carrying no plasmid (-) or plasmid expressing variants that do not recruit N-WASP (p*espF* A3) or the first 101 residues replaced with those from Tir (pΔ101*espF*).

mutant for PCR analysis to confirm the presence and expected size of the *espF* genes. This work revealed that only eleven of fourteen samples displayed *espF* bands of the expected size (Figure 43) i.e. reflecting the expect size of expected deletion, leading to the PCR DNA fragments being sequenced to reveal the true identity of each variant (Figure 43). The confirmed strains were then used to infect HeLa cells to estimate the level of EspF variant expression (Triton X100 insoluble fraction), delivery (Triton X100 soluble fraction) and secretion (obtained from extracellular media; noting DMEM does not contain FCS) with data given in Figure 44 and summarised in Table 9.

Assuming there are no growth or binding differences between the strains, EspF levels in the insoluble fraction act as an indicator of EspF expression levels (i.e. within the cytoplasm of adherent bacteria) while EspF in the soluble fractions indicates delivery levels. Similarly, EspF levels in the extracellular media reveal how much protein is secreted, and not directed into the host cells. Strain genotype was supported by only detecting Tir expression in EPEC but not in *etmf* mutant strains with similar strain growth behaviour suggested by comparable EspB secretion levels (Figure 44C). This analysis detected EspF proteins of the expected molecular mass in all but two strains, #37 and #39, which have the largest deletion lacking 162 and 117 of 206, residues, respectively. The resulting peptides may be too small to be detectable (~5 and 10kDa) or become rapidly degraded. Interestingly, the L16E variant was linked to increased expression (~3 fold) and delivery (~3 fold) but not secretion levels. The observed differences probably reflect EspF expression from plasmids (comparing data to that from EPEC) which is known to increase delivery level (Young, 2013). Features of particular EspF mutants that affect expression, secretion or the translocation process are compiled in Table 9 and provide insight into domains impacting on EspF expression, stability, secretion and/or translocation levels.

Of most interest to this study was the ability, or not, of these different strains to inhibit phagocytosis; comparing data from L16E-carrying variants to the EspF L16E control while variants lacking this substitution (#37 and 77) were compared to wild type EPEC data. Surprisingly, the L16E variant displayed an apparent, though statistically insignificant (due to data variability), defect in fully rescuing the anti-phagocytic defect (Figure 45) in contrast to previous studies (Quitard *et al.*, 2006; Young, 2013). However, this phenotype was mirrored by other L16E variants (for example #80 [serine residue 47 substituted to alanine] and #45 [missing 11 residues]) suggestive of a role for EspF in

A



B

Strains name	Missing residues (amino acid deletion)	PCR (expected size)	PCR (obtained size)	Actual missing residues (amino acid missing)
EPEC	-	700	700	-
L16E	-	700	700	*espF*-L16E
34	Δ101-184	451	450	Δ100-183 (83)
37	Δ21-184	215	300	Δ21-183 (162)
39	Δ51-168	346	400	Δ51-168 (117)
40	Δ101-168	499	700	Δ166-168 (2)
42	Δ50-74	628	610	Δ51-73 (22), #
43	p78-81A	700	700	4 Alanine (79-82) substitute with 4 proline
45	Δ74-101	619	700	Δ62-73 (11), *
75	Δ21-50	613	600	Δ23-52 (29), #
77	Δ21-120	403	650	Δ21-73 (52)
78	Δ50-120	490	580	Δ51-73, Δ117-162 (67),#
79	Δ50-74	628	650	Δ51-73 (22), #
80	S74A	700	700	Serine #47 substitute with Alanine

Figure 43. Correct identity of *espF* variant gene on plasmid in *etmf* quadruple mutant strains. The indicated strains were assessed for the presence of an *espF* gene by PCR with resulting products visualised, alongside molecular mass markers 2-log (1000Kb ladder), on a 1% agarose gel to determine band size relative to the positive control, *espF* (700 bp), gene. Strains used were EPEC, *etmf* (lacks LEE-encoded Intimin [*eae* gene product] plus Tir, Map and EspF effectors) carrying no plasmid (cont.-) or plasmid from strains number # 34, 35, 37, 39, 40, 42, 43, 45, 75, 77, 79 , 80) encoding EspF variants with various deletions or substitution. The table also shows expected and obtained *espF* gene size with the true nature of changes obtained by DNA sequencing leading to renaming the strains. * indicate presence of mutations (number indicated) that do not alter amino acid sequence. # indicate presence of a mutation that alter the amino acid sequence.

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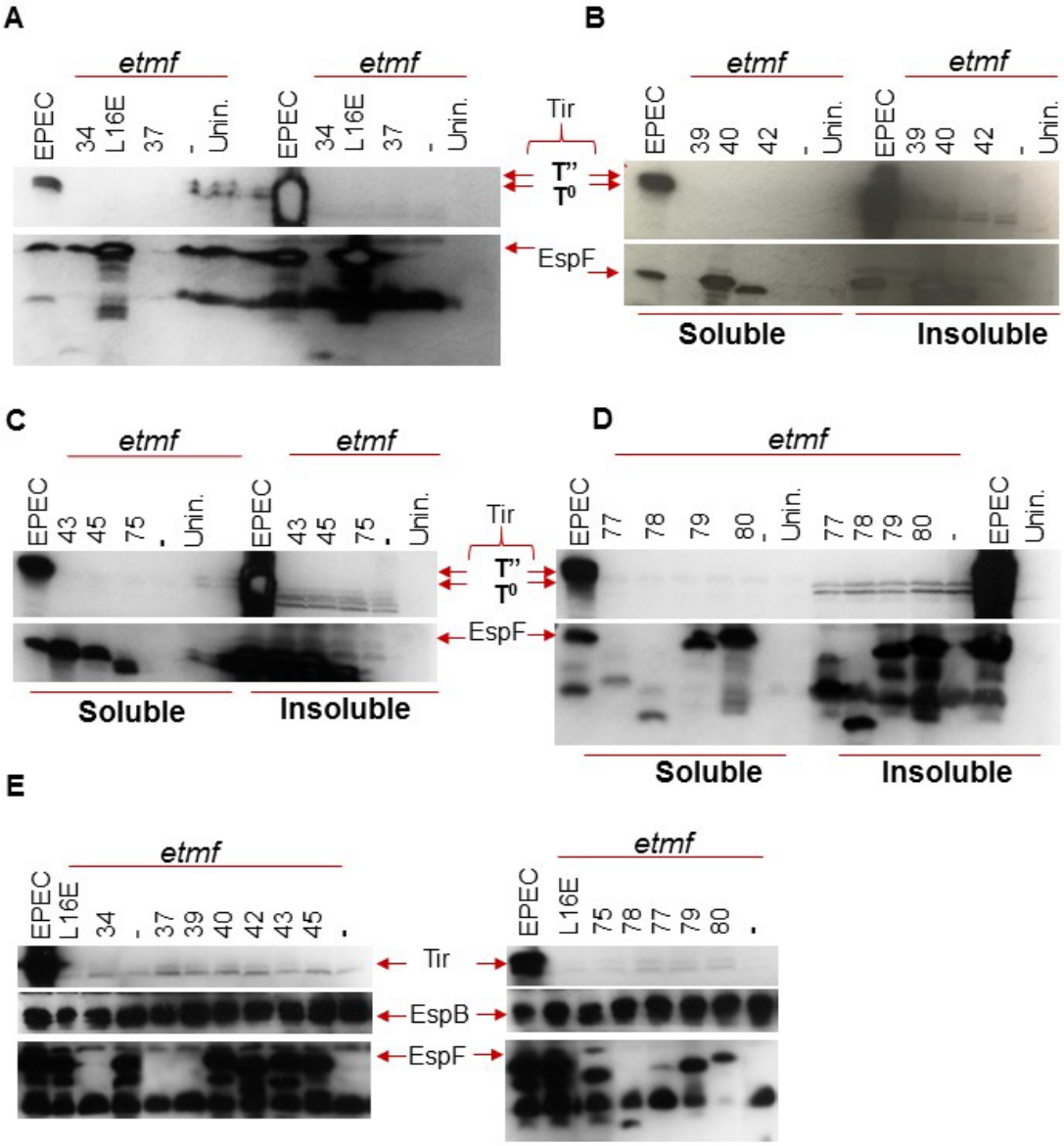
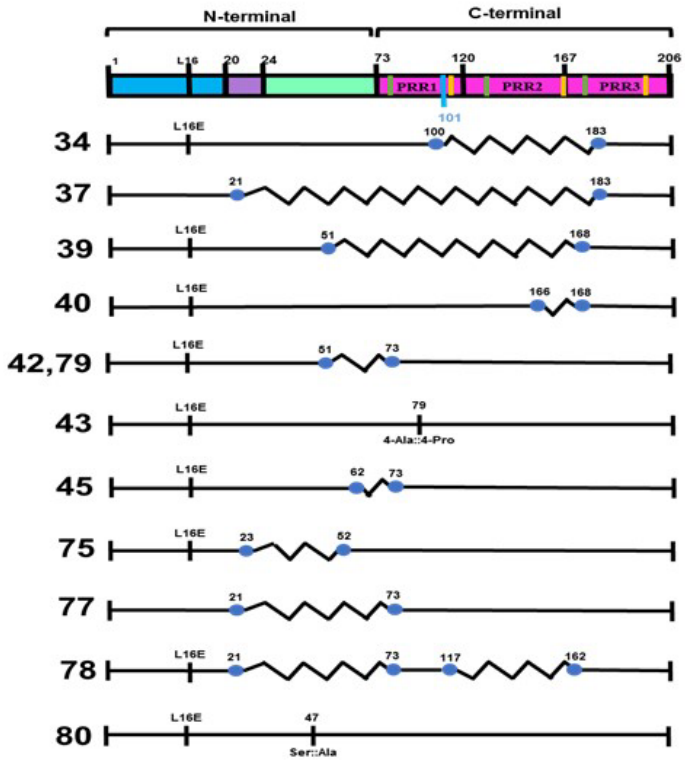


Figure 44. Assessing the expression, delivery and secretion levels of EspF variants by *etmf* quadruple mutant strains during infection of HeLa cells. HeLa cells were infected (MOI of 100:1) with the indicated strains – identity as per Figure 43 - for 3 h before isolating A-D) Triton X-100 soluble fractions (contain host cytoplasmic and membrane proteins plus T3SS-delivered effectors) and insoluble fractions (contain host nuclei and cytoskeleton plus adherent bacteria) or E) proteins secreted into the extracellular milieu. The samples were analysed by western blot (15% SDS-PAGE) probing for EspF and Tir. Arrows indicate the position of Tir (unmodified T^0 and host kinase modified T'' form) and EspF proteins.



Strains name	Missing residues (amino acid)	L16E	Insoluble (EspF expression) size of EspF Kda (estimated level of EspF protein)	Soluble (EspF delivery) size of EspF Kda (estimated level of EspF protein)	EspF secretion during HeLa cells infection, size of EspF Kda (estimated level of EspF protein)	Anti-phagocytosis activity compared with L16E
EPEC	-	-	25 (100%)	25 (100%)	25 (100%)	no
L16E	*espF*-L16E	√	25 (300%)	25 (300%)	25 (100%)	no
34	Δ100-183 (83)	√	13 (<5%)	13 (<5%)	13 (>5%)	no
37	Δ21-183 (162)	X	0	0	0	4*(p< 0.0001) compared to EPEC
39	Δ51-168 (117)	√	0	0	0	*(p< 0.05)
40	Δ166-168 (2)	√	25 (100%)	25 (200%)	25 (100%)	no
42	Δ51-73 (22), #	√	21 (100%)	21 (100%)	21 (100%)	no
43	4 Alanine (79-82) substitute with 4 proline	√	25 (100%)	25 (200%)	25 (100%)	no
45	Δ62-73 (11), *	√	25 (100%)	25 (150%)	25 (100%)	no
75	Δ23-52 (29), #	√	23 (30%)	23 (100%)	23 (20%)	no
77	Δ21-73 (52)	X	19 (50%)	19 (10%)	19 (<10%)	4*(p< 0.0001) compared to EPEC
78	Δ51-73, Δ117-162 (67),#	√	17 (50%)	17 (<10%)	17 (<5%)	no
79	Δ51-73 (22), #	√	23 (50%)	23 (100%)	23 (<20%)	no
80	Serine #47 substitute with Alanine	√	24 (100%)	24 (200%)	24 (<10%)	no

Table 9. Summary of data from *etmf* quadruple mutants carrying plasmids expressing different EspF variants. Schematic of EspF variants DNA sequencing data (see Figure 43) revealing substitutions and amino acid deletions ⋀⋀). Also given is the estimated size of the western-detected EspF protein in indicated fractions from HeLa infected cells (Triton X-100 insoluble/soluble fractions and extracellular supernatant) and the relative level of each band compared to the positive control (EPEC). The last column summarises the phagocytosis data. The western data is only from a single experiment, while the phagocytosis data is from at least three independent experiments

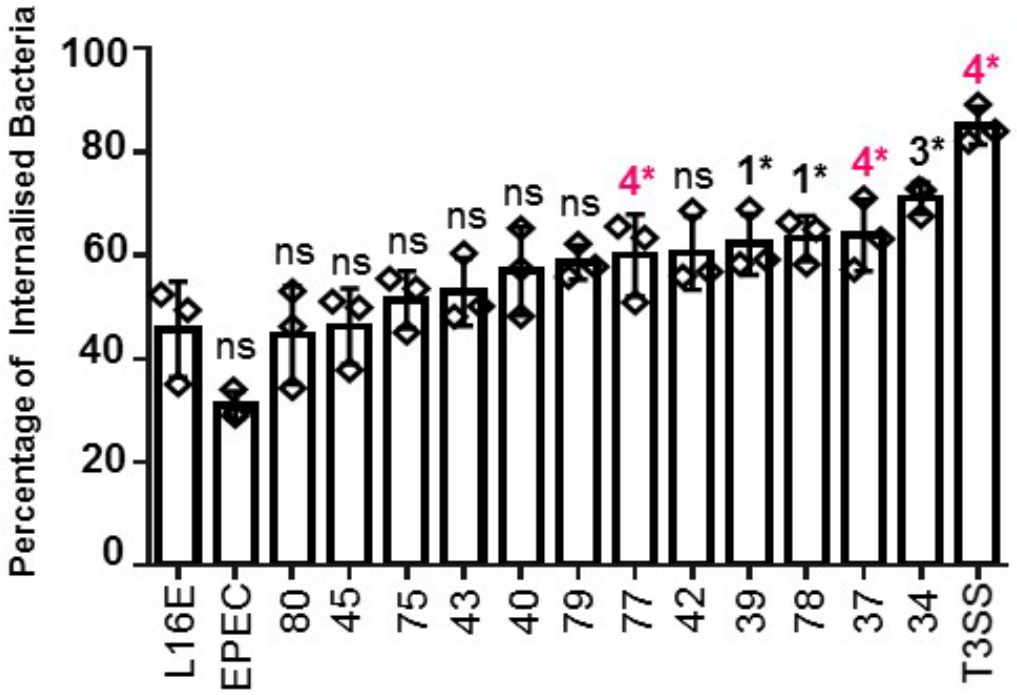


Figure 45. The anti-phagocytosis activity of EspF variants. J774.A1 macrophages were infected (MOI 100:1) with indicated strains (pre-grown in DMEM for 3 h) for 1 h prior to determining the percentage of bacterial internalisation following differential antibody strains labelling extracellular (Red) and total cell-associated (Green) bacteria. Fifty macrophages with 20-50 cell-associated bacteria were randomly chosen to count the number of extracellular and total cell-associated bacteria enabling the percentage internalisation to be calculated. Quantification studies were carried out in a semi-blind manner with the percentage of internalised bacteria displayed as the mean value (± standard deviation) from at least three independent experiments (◊). * indicating statistically significant differences (one-way ANOVA with Dunnett's post-hoc test) compared to L16E data (1* $p < 0.05$, 3* $p < 0.001$ and ns not significant; one-way ANOVA).* indicates statistically significant differences (one-way ANOVA with Dunnett's post-hoc test) compared to EPEC data (4* $p < 0.0001$). Strains used were EPEC, *etmf* (lacks LEE-encoded Intimin [*eae* gene product] plus Tir, Map and EspF effectors) carrying plasmids encoding EspF variants (# 34, 35, 37, 39, 40, 42, 43, 45, 75, 77, 79, 80) carrying alterations given in Table 9.

targeting mitochondria to inhibit phagocytosis in this infection model. Several variants (Figure 45) displayed a large, *etmf* mutant-like defect (Figure 45), linked to not expressing (#37 and #39) or delivering much (0-<10%) of the EspF variant (#34; #78; and #77) with significant defects compared to their controls. Of potential interest for future studies were strains that delivered an EspF variant to similar or greater levels than EPEC (#42, #79, #40, #43 and #75) but appear to display defects, however, the findings are difficult to interpret due to data variability (Figure 45).

5.2.3. Intimin-dependent anti-phagocytosis activity

To test the prediction (Chapter 4) that Intimin when expressed by adherent bacteria with a functional T3SS would provide an anti-phagocytosis response, an Intimin encoding plasmid (p*eae*; See Material & Methods; Table 3) was introduced into the *espGorf3core* mutant as supported by PCR analysis (Figure 46A). The *espGorf3core* mutant strain lacks genes for Intimin, 4 LEE effectors (EspG, EspH, Tir, Map), a Nle effector (EspG2/Orf3) and the two effector-specific chaperones, CesF/CesT. While CesF is essential for EspF intra-bacterial stability and T3SS delivery (Elliott *et al.*, 2002), CesT is required for the delivery of most LEE effectors (Tir, Map, EspH, EspZ) and many of the examined Nle effectors (NleB2; NleG; EspJ; NleH1; NleH2) (Thomas *et al.*, 2005; Mills *et al.*, 2013). However, CesT only promoted the delivery of other Nle effectors (NleB1; NleC) with no roles for CesT or CesF function in the delivery of other effectors (EspG; NleD; NleF) (Thomas *et al.*, 2005; Mills *et al.*, 2013). Thus, this strain is theoretically unable to express and/or deliver most LEE (including EspG, EspF, EspH, Map, EspZ and Tir except EspB) and many Nle effectors.

Phagocytosis assays with the *espGorf3core* mutant confirmed that it had little, if any, ability to inhibit its uptake by J774A.1 macrophages in contrast to the p*eae*-carrying variant which behaved in a statistically indistinguishable manner to EPEC (Figure 46B). This work demonstrates, for the first time, that Intimin has activities that (independent of functions of many effectors including Tir, Map, EspG, EspF and EspH) can directly or indirectly inhibit uptake of EPEC that express a functional effector-delivery system. It must be noted that the T3SS mutant has a functional Intimin surface protein but does not inhibit phagocytosis (Figure 46B).

5.2.4. Tir-dependent anti-phagocytosis activity

A similar approach was taken to test the prediction (Chapter 4) that Tir has Intimin-independent activities that enable EPEC to inhibit its uptake. As Tir delivery is dependent on CesT, a plasmid encoding both proteins was introduced into the *espGorf3core* mutant.

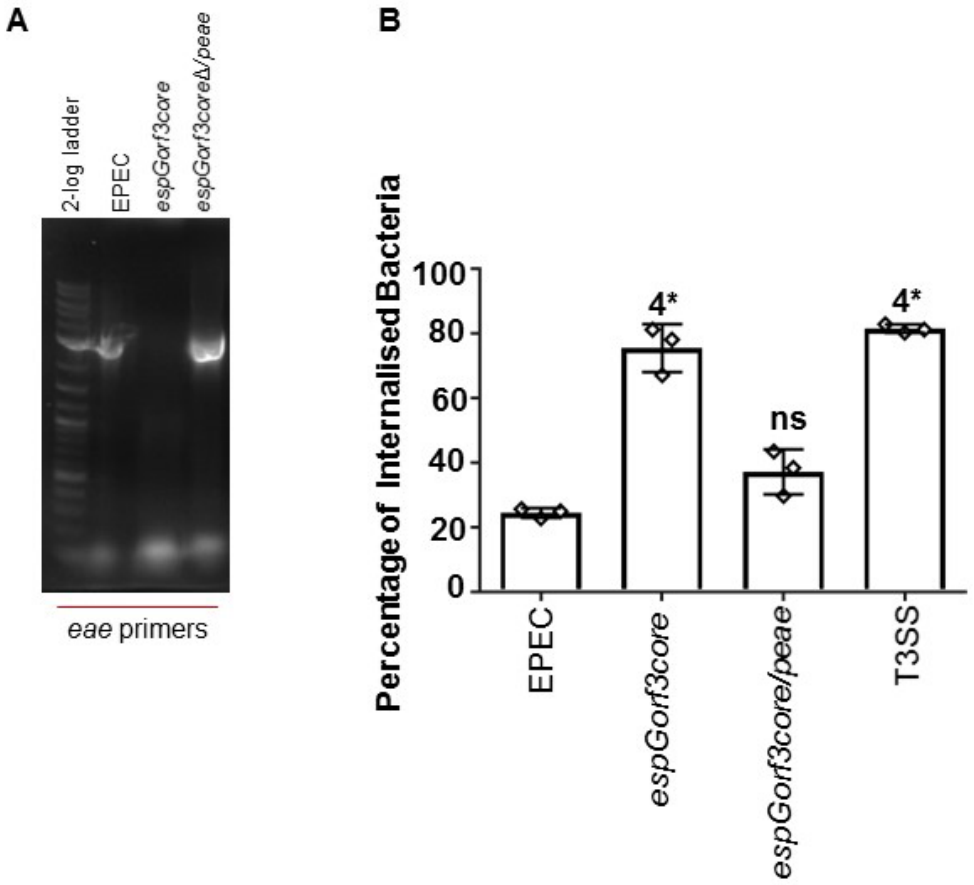


Figure 46. Intimin bestows the *espGorf3core* mutant the capacity to inhibit phagocytosis in the J774.A1 macrophage model. Indicated strains were used for PCR analysis (A) or to infect (B) J774.A1 macrophages. (A) Oligonucleotides specific to *eae* gene (encodes Intimin) were used in PCR analyses to determine the presence of an intact or deleted *eae* gene with PCR products visualised, alongside molecular mass markers 2-log (1000Kb ladder), on a 1% agarose gel. (B) J774.A1 macrophages were infected for 1 h (MOI 100:1) with the indicated strains (pre-grown in DMEM for 3 h) prior to determining the percentage of internalised bacteria using differential antibody staining to label extracellular bacteria (Red) and total cell-associated bacteria (Green). Fifty macrophages with 20-50 cell-associated bacteria were randomly chosen to count the number of 'Red' and 'Green' bacteria. (B) Quantification studies were carried out in a semi-blind manner with the percentage of internalised bacteria displayed as the mean value (± standard deviation) from at least three independent experiments (◊). * indicates statistically significant differences (one-way ANOVA with Dunnett's post-hoc test) compared to EPEC data (4* $p < 0.0001$; ns = not significant). Strains used were EPEC, T3SS mutant (T3SS), or *espGorf3core* mutant (lacks 5 effectors [EspG, Orf3/EspG2, EspH, Tir, Map], two chaperones [CesF, CesT] and Intimin protein) – latter with or without an Intimin-encoding plasmid (p*eae*).

To confirm plasmid introduction, HeLa cells were infected before isolating soluble and insoluble fractions for western blot analysis probing for anti-Tir. This analysis confirmed the features of the *espGorf3core* mutant, i.e. no Tir signal and consistent with the absence of CesF a reduced EspF signal (Figure 47A) (Elliott *et al.*, 2002).

As expected, the introduction of *pcesF*::HA increased EspF levels while introducing the Tir/CesT-encoding plasmid resulted in a Tir signal and, as per EPEC-infected cells detection of host kinase-modified Tir forms thereby illustrating CesT and T3SS functionality (Figure 47A). Crucially, the phagocytic assays carried out with these strains confirmed that the *espGorf3core* mutant strain could not inhibit its uptake whether or not it carried the *pcesF*::HA plasmid to enable EspF delivery while Tir expression/delivery was linked to an EPEC-like phenotype (Figure 47B). These data reveal, for the first time, the existence of a Tir-dependent anti-phagocytic activity that does not require activities of the Intimin, EspG, EspG2, Map, EspF or EspH proteins.

As the re-introduction of CesT would enable the delivery of LEE EspZ and non-LEE encoded effectors (in latter given LEE sufficiency for anti-phagocytosis and no detectable roles for 14 Nles) further studies examined if EspZ may play a role by inactivating the *espZ* gene (Table 1; Materials & Methods). PCR analyses supported *espZ* gene inactivation in EPEC and *espGorf3core* backgrounds with introduction of the Tir/CesT-encoding plasmid (Figure 48A).

Experiments using these newly generated strains to infect J774A.1 macrophages for the anti-phagocytosis assay suggested that EspZ may suppress the activity of, undefined, factors to inhibit EPEC uptake as significantly higher phagocytic activity was observed for *espGorf3coreΔespZ* than with *espGorf3core* ($p<0.05$) and T3SS mutants ($p<0.01$) (Figure 48B). Crucially, introducing the Tir/CesT-expressing plasmid into the *espGorf3coreΔespZ* mutant lead to an EPEC-like phenotype, which was shared by the examined *espZ* single mutant (Figure 48B). This work failed to demonstrate a role for EspZ in EPEC capacity to inhibit its uptake by J774A.1 macrophages and that Tir can inhibit EPEC uptake although the mechanism are not clear.

As the earlier results revealed an important role of Tir in the anti-phagocytosis process, initial studies were undertaken to identify Tir features needed for its anti- phagocytosis process. Thus, plasmids expressing Tir variants (See Material & Methods Table 3) were introduced into the *espGorf3core* mutant strain to examine if any of these plasmids can restore the anti-phagocytosis defect of this mutant. It should be considered that no analysis was provided to confirm the presence of specific mutations. Due to the time constraint, anti-phagocytosis assay for most Tir variants was only done once or twice, thus, no statistical analysis was provided.

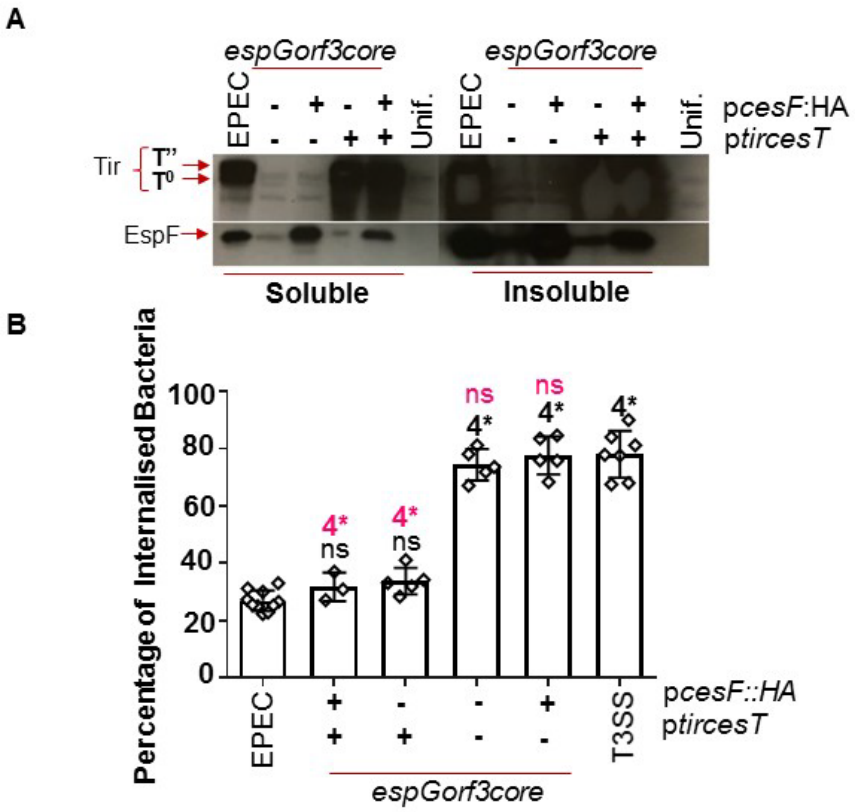


Figure 47. Tir bestows on the *espGorf3core* mutant the capacity to inhibit phagocytosis in the J774.A1 macrophage model. Indicated strains were used to (A) infected (MOI of 100:1) HeLa cells for 3 h prior to isolating Triton X-100 soluble (contains host cytoplasmic and membrane proteins plus T3SS-delivered effectors) and insoluble (contains host nuclei and cytoskeleton plus adherent bacteria) fractions for western blot analysis probing for EspF and Tir. Arrows indicate the position of Tir (unmodified T^0 and host kinase modified T'' form) and EspF proteins. In (B) indicated strains were used to infect J774.A1 macrophages (MOI 100:1; pre-grown in DMEM for 3 h) for 1 h prior to determining the percentage internalised bacteria using differential antibody staining to label extracellular bacteria (Red) and total cell-associated bacteria (Green). Fifty macrophages with 20-50 cell-associated bacteria were randomly chosen to count the number of 'Red' and 'Green' bacteria. (B) Quantification studies were carried out in a semi-blind manner with the percentage of internalised bacteria displayed as the mean value (± standard deviation) from at least three independent experiments (◊). * indicates statistically significant differences (one-way ANOVA with Dunnett's post-hoc test) compared to EPEC data (4* p< 0.0001and ns not significant). * indicates statistically significant differences (one-way ANOVA with Dunnett's post-hoc test) compared to T3SS (4* p<0.0001and ns not significant). Strains used were EPEC, T3SS mutant (T3SS) and *espGorf3*Δ*core* mutant (lacks 5 effectors [EspG, Orf3/EspG2, EspH, Tir, Map], two chaperones [CesF, CesT] and Intimin protein); latter carried (+) or not (-) plasmids encoding Tir and CesT (*ptircesT*) or CesF as a CesF::HA fusion protein (p*cesF*::HA).

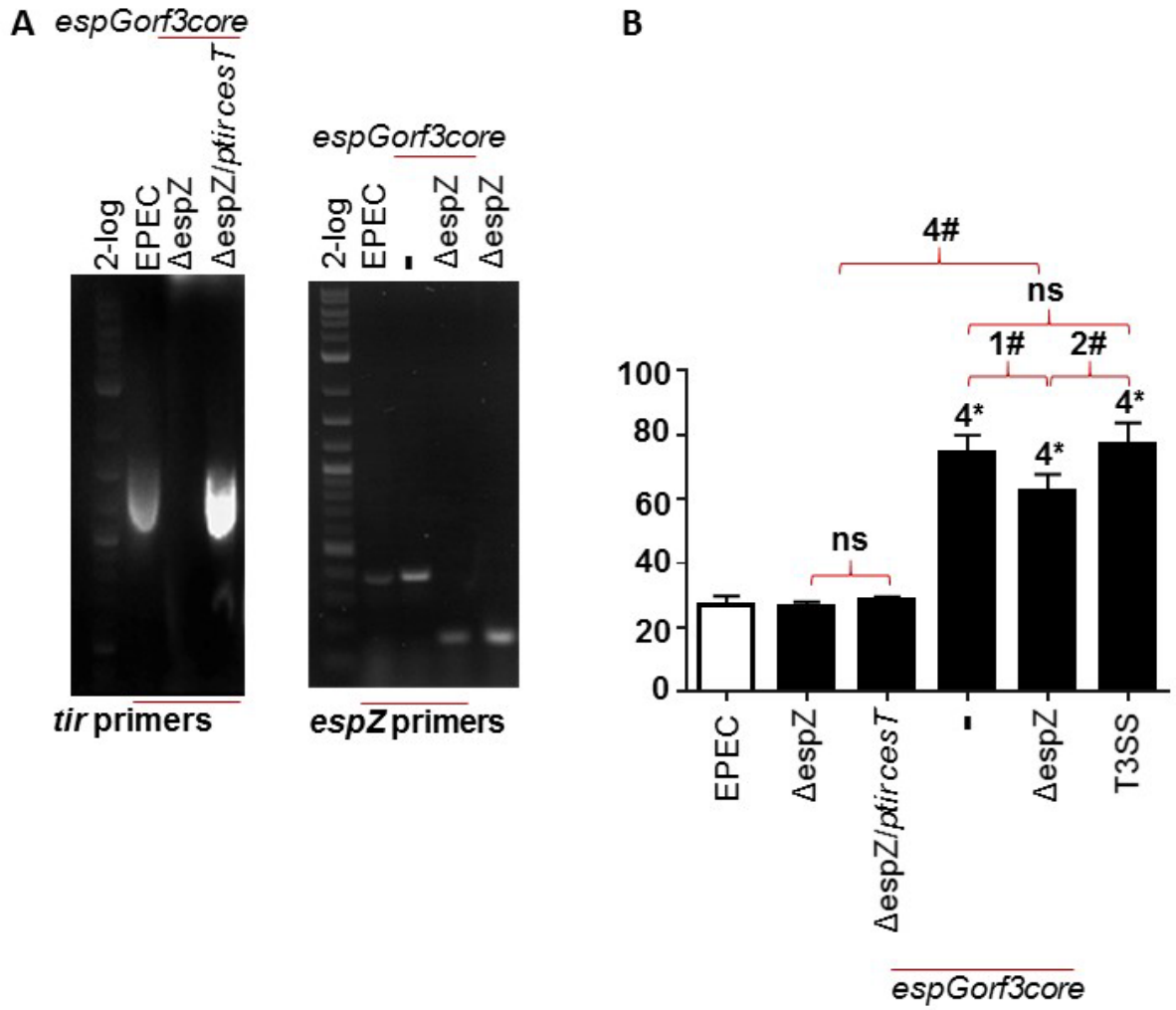


Figure 48. No detectable role for EspZ in anti-phagocytic mechanisms. Indicated strains were used for PCR analysis (A) or to infect (B) J774.A1 macrophages. (A) Oligonucleotides specific to *tir* or *espZ* genes were used in PCR analyses to determine the presence of an intact or deleted genes with PCR products visualised, alongside molecular mass markers 2-log (1000Kb ladder), on a 1% agarose gel. (B) J774.A1 macrophages were infected for 1 h (MOI 100:1) with the indicated strains (pre-grown in DMEM for 3 h) prior to determining the percentage of internalised bacteria using differential antibody staining to label extracellular bacteria (Red) and total cell-associated bacteria (Green). Fifty macrophages with 20-50 cell-associated bacteria were randomly chosen to count the number of 'Red' and 'Green' bacteria. (B) Quantification studies were carried out in a semi-blind manner with the percentage of internalised bacteria displayed as the mean value (± standard deviation) from at least three independent experiments. * indicates statistically significant differences (one-way ANOVA with Dunnett's post-hoc test) compared to EPEC data (4* $p < 0.0001$) or by Tukey's test for multiple comparisons between strains (4# $p<0.0001$, 2# $p<0.01$, 1# $p < 0.05$ and ns not significant). Strains used were EPEC, T3SS mutant (T3SS), *espZ* mutant (lacks EspZ effector) or *espGorf3core* mutant (lacks 5 effectors [EspG, Orf3/EspG2, EspH, Tir, Map], 2 chaperones [CesF, CesT] and Intimin protein). Some strains additionally lacked the EspZ effector (Δ*espZ*) or carried a Tir/CesT-encoding plasmid (p*tircesT*) with the control strain lacking this plasmid (-).

No difference in the percentage of uptake was identified for cells infected with EPEC *espGorf3core*/pACYC*tir* R521. This mutation affects the putative arginine finger motif and is defective for filopodia downregulation and the results suggest no role of this motif in the phagocytosis inhibition (Figure 49). Importantly, all plasmids expressing Tir with different substitutions failed to restore the anti-phagocytosis defect in the *espGorf3core* mutant as all showed high levels of internalisation comparable to EPEC. Importantly, *espGorf3core/p*ACYC*tir S434/463A* displayed a large T3SS mutant-like defect (Figure 49), suggesting that serine phosphorylation may be necessary for phagocytosis inhibition. Future work is therefore required to confirm these results, repeating the anti-phagocytosis analysis for each of these strains and including an analysis to confirm the correct sequence of the strains.

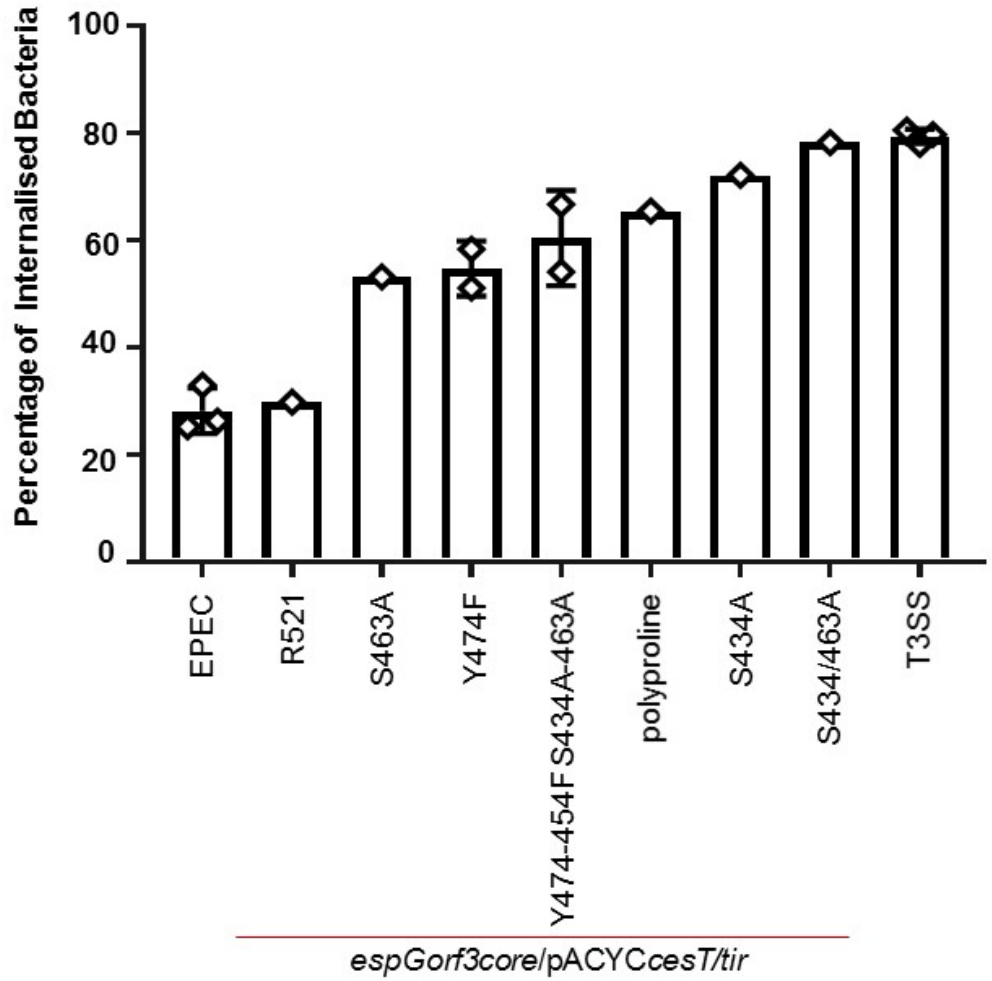


Figure 49. Anti-phagocytosis activity of Tir variants. J774.A1 macrophages were infected for 1 h (MOI 100:1) with indicated strains (pre-grown in DMEM for 3 h) prior to determining the percentage of internalised bacteria using antibodies to label extracellular bacteria (Red) and total cell-associated bacteria (Green). Fifty macrophages with 20-50 cell-associated bacteria were randomly chosen to count the number of extracellular and total cell-associated bacteria (Green) enabling the percentage internalisation to be calculated. Quantification studies were carried out in a semi-blind manner. Strains used were EPEC, T3SS-deficient (T3SS) mutant, *espGorf3core* mutant (lacks 5 effectors [EspG, Orf3/EspG2, EspH, Tir, Map],two chaperones [CesF, CesT] and Intimin protein) carrying no plasmid or a plasmid encoding Tir pACYC *tir*R521, pACYC *tir*S463A, pACYC *tir*Y474F, pACYC*tir* Y474-454F S434A-463A, pACYC *tir* polyproline, pACYC*tir* S434A, pACYC*tir* S434/463A (see Materials & Methods Table 3).

5.3 Discussion

Previous data (Chapter 3) suggests that T3SS delivery of EspF is not sufficient to inhibit phagocytosis of EPEC; however, studies (Chapter 4) with a bank of 32 mutants (lacking genes encoding *map*, *tir*, *espH*, *eae* [encodes Intimin] in all combinations) implicated an indirect role of EspF in the inhibitory process. Thus, to gain insight into the EspF domains and/or features required for this indirect role, complementation studies were undertaken with available EspF variants. The latter variants were introduced into an *etmf* quadruple mutant as its failure to inhibit phagocytosis was always rescued by introducing an EspF-expressing plasmid. Initial complementation of *etmf* generated in this study is consistent with other studies (Young, 2013), confirming no role for EspF features linked to the recruitment of actin nucleating factor, N-WASP. However, N-WASP does have an important role in other EspF disruptive activities, such as disruption of epithelial barrier function and effacement of absorptive microvilli (Dean *et al.*, 2013). Importantly, our results also support an essential role for the EspF N-terminal domain, which is consistent with previous studies (Quitard *et al.*, 2006). The latter study used a Tir- EspF fusion protein where the N-terminal 101 residue of EspF [carries the sequence for chaperone (CesF) binding and targeting to T3SS, mitochondria and nucleolus (Charpentier and Oswald, 2004; Nagai *et al.*, 2005; Dean *et al.*, 2010b)] were exchanged for those of Tir which carries the sequence for chaperone (CesT) binding, T3S signal sequence and kinase recruitment (polyproline-rich) region (Crawford and Kaper, 2002; Bommarius *et al.*, 2007). However, as previously discussed, it is possible that the failure of Tir-EspF fusion protein to rescue the anti-phagocytosis defect of *etmf* mutant could be due to the Tir domain interfering with the anti-phagocytosis activity encoded in the remaining EspF sequence fusion protein. Nevertheless, this fusion protein was able to mimic the ability of the native EspF to cause barrier disruption; an event that requires the C-terminal domain, that carries this N-WASP binding motifs (Dean *et al.*, 2013). Previous research with the L16E variant had discounted a need for EspF mitochondrial targeting sequence (Quitard *et al.*, 2006) and features required to recruit sorting nexin 9 (a protein linked to N-WASP co-recruitment to remodel host membrane) in the phagocytosis inhibition (Weflen *et al.*, 2010; Young, 2013). Unexpectedly, studies with the L16E EspF variant, which was used as a control for variants carrying internal truncations, revealed a partial complementation defect. The latter finding suggested that EspF's targeting to the mitochondria contributed to its, indirect, role in the anti-phagocytosis process. However, the L16E variants data was linked to high level of variability (n=3 only), so the obvious difference between mean data was not statistically significant. Thus, it is likely that a higher number of repeats would decrease the associated error bars, and perhaps reveal a statistical difference between EPEC and *etmf*/*espF* L16E mutant strain. Nevertheless, similar data was

obtained with other data L16E variants such as L16E/S47A double substitution mutant, suggesting that this defect is real, at least in the J774.A1 macrophage model.

Having re-examined the anti-phagocytic activity of variants lacking each of the key features associated with EspF's subversive activities, studies focused on lab-available variants carrying internal truncations or specific substitutions. The internal truncations were made using *espF* encoding the L16E substitutions with oligonucleotides designed to reverse PCR reactions in which specific regions upstream of residue 20 (as residue 1-20 required for EspF translocation) were deleted. However, PCR analysis of available variants, following isolation for *espF* mutant background and introduction into *etmf* quadruple mutant, revealed unexpected fragment sizes leading to each insert being DNA sequenced to reveal the true extent of genetic change. Although how the observed discrepancies arise was unclear, the key finding was identifying these errors to enable data interpretation. As EspF role in the anti-phagocytosis would clearly be dependent on the level of expression and/or delivery into host cells, our study examined the impact of the genetic changes to this process. It is worthwhile noting that, due to the time constraint, this analysis related to a single western blot on samples from a HeLa cell infection experiment probing EspF levels in insoluble, soluble and extracellular protein used as an indication EspF protein expression, T3SS delivery and the T3SS delivery processes respectively. It was assumed that there was no difference in strain binding supported by similar levels of other EPEC proteins (such as EspB or bacterial protein non-specifically detected with antibodies) in the soluble, insoluble and extracellular samples and that EspF antibody detecting many epitopes distributed equally throughout the EspF protein. Plasmid expression of EspF is known to increase delivery levels (Nougayrède and Donnenberg, 2004; Young, 2013). Similarly, this work indicated that while the L16E substitution had no impact on secretion levels, it increased expression and delivery levels by three fold i.e. appeared to promote CesF delivery to the T3SS, chaperone release or delivery kinetics. However, this is not the case with the variant also carrying a S47A substitution, as it was delivered two fold more into HeLa cells with little or no EspF secretion, which may due to interaction with its chaperone, thus increasing the level of delivery and decreasing the level of secretion. Not surprisingly, variants carrying a large internal truncation could not be detected, due to no or few epitopes for detection, rapidly degraded or not captured on percentage of gel used. However, some variants carrying smaller deletion could only be detected at low levels, implicating these regions is chaperone binding or providing antibody-recognised epitopes. Our results are encouraging for future studies to determine features which are implicated in specifically targeting EspF's translocation into cells.

Importantly, the anti-phagocytosis data of EspF variants generated in this chapter related to at least three independent experiments. However, these data must be interpreted with caution due to the unexpected intermediate defect with L16E variants (used as a control) and data variability. Two mutants lacked the L16E substitution and thus could be compared to EPEC data with one #37 behaving like the *etmf* quadruple mutant presumably as it lacks most EspF residues (21-183 from total 206 of EspF protein). Whereas the second mutant #77 (lacks residue 21-73) displayed similar anti-phagocytosis activity as #37. However, the defect of the #77 mutant could be linked to reduced expression (50%), secretion (90%) and translocation (90%) suggesting that the issues related to a translocation defect. Interestingly, mutants which delivered similar levels of EspF into cells as EPEC appear not rescue the anti-phagocytosis defect i.e. #79 (lacks residue 51-73), #75 (lacks residue 23-52) and possibly #43 (lacks the proline rich feature; A79-82P). Further studies are therefore required to re-examine the anti-phagocytosis capacity for each mutants.

In contrast, to the proposed indirect role of EspF, our studies with the single, double, triple, quadruple and quintuplet mutants implicated key roles for Tir and Intimin proteins in promoting EPEC's anti-phagocytosis activity. To test this premise complementation studies were undertaken with a strain, *espGorf3core*, that expressed and/or delivered few T3SS effectors as it lacks genes of 5 effectors (EspG, EspG2, EspH, Tir, Map), Intimin, the chaperones CesF (required for efficient EspF deliver) and CesT (critical for the delivery of EspZ and many non-LEE-effectors, with remaining effectors delivered at reduced or normal levels. Importantly, re-introducing the *cesF*-encoding gene restored EspF delivery without rescuing its anti-phagocytosis defect. Noticeably, EspF delivery levels were supported by western blot analysis (HeLa cells) or immunofluorescent microscopy illustrating T3SS functionally and that EspF delivery is not sufficient to inhibit EPEC uptake (Chapter 3). Crucially, introducing a plasmid encoding Intimin rescued the anti-phagocytic defect of the *espGorf3core*, but there was an apparent small (though not statistically significant) defect. Whereas introducing a plasmid expressing Tir (with CesT to enable delivery into cells) led to data mirroring EPEC- infected macrophages. A concern on the latter data is that co-introduction of the *cesT* gene would restore the delivery of the LEE effector EspZ and many non-LEE effectors. However as we have shown EPEC anti-phagocytosis activity does not require the activity of 14 Nle (and EspG) effectors and is encoded on the LEE region, we only examined a role for EspZ in this event. Interestingly, deleting EspZ from *espGorf3core* mutant was linked to a low level of anti-phagocytic activity suggesting that EspZ supresses the anti-phagocytosis

activity of another factor (probably an effector) expressed by *espGorf3core*. However, this study also discounted a role of EspZ as the single mutant behaved like EPEC.

This work did not provide any data related to Intimin variants, as they were not available at our laboratory and, therefore, studies focused on Tir variants. However, the anti-phagocytosis of Tir variants data is related to a single or two experiments, without support (DNA sequence data and/or western blot) the presence of specific variants. However, this preliminary data suggests no role for the Tir arginine finger-like motif (linked to down regulate GTPase activity) in the phagocytosis inhibition. In contrast, the substitution converting arginine 521 to an alanine demolished Tir's capacity for filopodia downregulation (Kenny *et al.*, 2002). Whereas our data suggests that Tir variants lacking residues or feature linked to recruiting host kinases and/or being substrates for host kinase could have role in Tir anti-phagocytosis activity. Future work therefore is required to re-examine the anti-phagocytosis capacity for each mutants with i) the bioinformatics analysis to confirm presence of required (DNA sequencing PCR-obtained *tir* gene fragment) and ii) increase the number of repeat anti-phagocytosis assay to rule out roles for individual or multiple Tir feature to enable it to bestow onto the *espGorf3core* mutant the capacity to inhibit its uptake by J774.A1 macrophages.

In summary, this study confirmed key, but redundant roles for Tir and Intimin in EPEC's anti-phagocytosis activity and discounted a need for EspZ effector activity. These studies have set the groundwork for future work to define the molecular mechanism by which Tir and Intimin inhibit EPEC uptake by J774.A1 macrophages. However, studies are also needed to examine the reported contribution with other effectors such as EspB.

Chapter 6. Final Discussion

Many bacteria have the capacity to inhibit their uptake by professional phagocytic cells, such as macrophages which phagocytose bacteria in order to kill them and present antigens to promote appropriate immune responses. The anti-phagocytic activity of many bacterial pathogens, including non-invasive EPEC, depend on a T3SS that transfers many effectors into infected cells. EPEC's anti-phagocytic activity is thought to function to inhibit uptake by gut-located M-cells to delay their delivery to associated macrophage and, thus, immune responses.

EPEC's capacity to inhibit phagocytosis has been assessed with a range of cultured macrophage cell lines (e.g. J774.A1), M-cell models and *in vivo* (Goosney *et al.*, 1999; Celli *et al.*, 2001; Quitard *et al.*, 2006; Martinez-Argudo *et al.*, 2007; Marchès *et al.*, 2008; Tahoun *et al.*, 2011). These studies demonstrated that EPEC inhibits PI 3-kinase-dependent phagocytic pathways through the delivery of effectors with inhibitory roles described for four EspB, EspH, EspG and EspF (Quitard *et al.*, 2006; Iizumi *et al.*, 2007; Dong *et al.*, 2010; Humphreys *et al.*, 2016). It should be noted that only EspF's key role has been examined in M-cell and/or *in vivo* models. The EPEC inhibitory mechanism is linked to blocking cytoskeleton rearrangement driving pseudopod membrane extension that engulfs and internalises microorganisms. It has been shown that EspB subverts myosin motor proteins required to drive membrane extension (Iizumi *et al.*, 2007) while EspH block Rho GTPase signalling (by binding RhoGEFs) prevent actin remodelling needed to engulf bacteria (Dong *et al.*, 2010) with EspG preventing WAVE regulatory activity needed for phagocytosis associated actin polymerisation (Humphreys *et al.*, 2016). While a critical role of EspF has been supported in studies with macrophages (Quitard *et al.*, 2006; Marchès *et al.*, 2008) and M-cells (*in vitro* and *in vivo*) the inhibitory mechanism has not been described (Martinez-Argudo *et al.*, 2007; Tahoun *et al.*, 2011). Therefore, the aim of my thesis project was to determine if EspF encodes sufficient information to inhibit EPEC uptake and to investigate the molecular details of the inhibitory mechanism.

The first unexpected finding from my studies came from re-establishing the phagocytic assay, which included a non-pathogenic *E. coli* strain, which displayed higher level of internalisation (~85%) than the T3SS mutant (~65%); noting ~40% of EPEC are internalised via PI-3 kinase-independent pathways. Thus, our studies suggested for the first time, as far as we know, a non-T3SS inhibitory contribution to EPEC's anti-phagocytosis ability. This finding could be interesting for future studies to define the responsible factor(s) and inhibitory mechanism. Crucially, my work also confirmed a critical role for EspF in EPEC anti-phagocytosis activity as both *espF* single and *etmf* multiple mutants (lacking genes expressing Intimin, Map, Tir and EspF) behaved like the T3SS mutant.

An important, but not surprising, finding came from studies assessing the anti-phagocytosis capacity of non-pathogenic *E. coli* to inhibit uptake carrying LEE (on a plasmid),

which revealed LEE sufficiency. However, this LEE region came from another EPEC strain, B171, with the non-pathogenic *E. coli* strain carrying another plasmid encoding other B171 virulence-associated factors (important adhesin [BFP] and regulator [PerA-C] to drive LEE gene expression). Although, the BFP and Per operons did not enable *E. coli* K12 to inhibit phagocytosis studies need to rule out a direct role with LEE, though BFP (EPEC) has been shown not to be required for EPEC to inhibit its uptake (Goosney *et al.*, 1999). Nevertheless, further work is needed to confirm sufficiency with the E2348/69 LEE region. Supporting the idea that all the important factors are LEE encoded was the finding that an E2348/69 strain, TOEA7, that lacks most (14 of 17) known Nle, plus EspG, effectors inhibited its uptake to a similar level as EPEC. This finding was surprising as this strain lacks EspG and EspG2 effectors while a recent study reported that an *espG*/*espG2* double mutant failed to inhibit phagocytosis (Humphreys *et al.*, 2016). This discrepancy presumably reflects differences in the model system, reagents and/or protocols. Ruling out critical roles for the EspG effectors and most known Nle effectors, but apparent LEE sufficiency, suggests that the anti-phagocytosis activity of the TOEA7 mutant is driven by other LEE-encoded effectors linked to this inhibitory process i.e. EspF (Quitard *et al.*, 2006), EspB (Iizumi *et al.*, 2007) and/or EspH (Dong *et al.*, 2010).

Before investigating how EspF inhibited PI-3 kinase-dependent uptake, studies were undertaken to address whether T3SS-dependent delivery of EspF into host cells is sufficient to inhibit phagocytosis. This was achieved through using a previously described approach i.e. the T3SS of an other pathogen (*Yersinia*) as shown to deliver EPEC Tir into cells in a manner greatly promoted by co-expressing the Tir chaperone, CesT (Warawa and Kenny, 2001). Although my work demonstrated that *Yersinia* could deliver EspF into host cells (if co-expressed with its chaperone CesF), the anti-phagocytosis data argued against EspF encoding sufficient information to inhibit phagocytosis. *Yersinia's* ability to deliver a second EPEC effector into host cells may promote studies with other LEE and/or Nle effectors to investigate if they subvert cellular processes when delivered in the absence of other effectors or, indeed, whether EspH, EspB and/or EspG's delivery would inhibit phagocytosis. As we were unable to prove that *Yersinia* delivered EspF into macrophages, a second strategy was undertaken to confirm that EspF does not encode sufficient information to inhibit phagocytosis. This work used a complex mutant, *espGorf3core*, which theoretically is unable to deliver most LEE (including EspF) and many Nle effectors, with EspF delivery restored by plasmid introducing a CesF-expressing plasmid. Crucially, immunofluorescence microscopy studies clearly demonstrated high levels of EspF delivery into macrophages but this failed to inhibit phagocytosis. Thus, we demonstrate that EspF does not encode sufficient information to inhibit phagocytosis implicating roles for other, presumably, LEE-encoded factors. As earlier work

ruled out roles for most non-LEE-encoded effectors, supporting the LEE sufficiency idea, EspF's anti-phagocytosis activity presumably depends on activities of LEE factors missing from the *espGorf3core* mutant i.e. i) EspG; role excluded by TOEA7 strain data, ii) CesT dependent effectors i.e. EspZ (role excluded by examining *espZ* mutant) and, unlikely, Nle effectors, or iii) *core* region proteins: Tir, Map, EspH effectors and/or Intimin.

The key findings from my work came from the decision to test the anti-phagocytosis activity of 32 strains in which the genes of implicated proteins (EspH, Map, Tir, Intimin as well as EspF) were inactivated in all combinations. It should be noted that PCR analysis, on non-adherent bacteria isolated following macrophage infections, supported the genotype of each mutant by illustrating that each gene of interest was either intact or disrupted. Surprisingly, testing the anti-phagocytosis activity of these mutants failed to confirm essential roles for EspF and EspH. Examining *espF* clones from other frozen culture stocks and *espH* clones from two other laboratories, including the one reporting EspH's role in inhibiting phagocytosis (Dong *et al.*, 2010) generated similar findings. It is hard to explain why these mutants displayed an EPEC-like phenotype, especially as my initial data with a *espF*/pGFP strain revealed T3SS mutant-like phenotype with EspF's roles demonstrated in *in vitro* and *in vivo* models (Quitard *et al.*, 2006; Martinez-Argudo *et al.*, 2007; Marchès *et al.*, 2008; Tahoun *et al.*, 2011). An important future experiment would be to compare the phagocytosis activity of the T3SS mutant-like the *espF*/pGFP strain and EPEC-like *espF* mutant to determine the reason for the difference; perhaps relates to genetic (bacterial or host genome), reagent, protocol and/or environmental changes. It should be noted that the variability is unlikely to be related to cells since the MOI used (100:1) consistently resulted in 70-80% of the macrophages having 20-50 cell-associated bacteria, whichever strain was used.

Although, my work revealed that EspF is not always required to inhibit phagocytosis it suggested that it could have indirect role(s). Indeed, the work also suggests that EspH may also have indirect contributions to perhaps help explain why the *espH* mutants behaved like EPEC in my model. It is possible that EspF and EspH can have critical or non-critical contributions dependent on environmental factors, for example, known FCS batch variability in cytokine composition could alter expression of receptors, regulators, signalling pathways or indeed effector expression/delivery levels to impact on the phagocytic processes and/or ability of one or more effectors to contribute to the inhibitory process. Support for this idea came from finding phenotype instability in the phagocytosis assay with a subset of multiple mutants, for example, the *espF*/*tir* (lacks EspF and Tir) displayed a T3SS mutant-like phenotype in three of six experiments with a prominent anti-phagocytic activity in the other three experiments.

Interestingly, this phenotype was not detected with *espF*/*tir*-related triple mutants suggesting that the presence or absence of specific EPEC genes can, directly or indirectly, impact on EPEC's ability to inhibit phagocytosis. While studies were undertaken to define features needed for its indirect role (*etmf* quadruple) the data is too preliminary to discuss but the work shows the potential to define important features and insights on the process. Indeed, preliminary data suggests that EspF's contribution to anti-phagocytosis, in the *etmf* mutant background, depends on EspH activity which could be explore in future projects. Other studies could focus on other potential interrelationships and hypothesis on how cooperative contributes to anti-phagocytosis suggested by the single, double, triple and quadruple mutant data.

Although my work failed to confirm critical roles for EspH, EspG or EspF effectors (didn't examine EspB's reported role as provide mutant had binding issues) it revealed important, but redundant roles for both Tir and Intimin in EPEC's anti-phagocytosis mechanism (at least in this J774A.1 model). Roles for both proteins was suggested by intermediate defects in a previous study (Goosney *et al.*, 1999), while my work revealed a minor defect for the *tir*, not *eae*, mutant. This variability supports the idea that EPEC's anti-phagocytic activity is sensitive to environmental, protocol and/or reagent differences. Importantly, Intimin's role in the anti-phagocytosis was supported by rescuing the anti-phagocytosis defect of the *espGorf3core* mutant by introducing an Intimin-expressing plasmid. As T3SS mutants (express a functional Tir-binding Intimin protein) do not inhibit phagocytosis this argues that Intimin's contribution depends on other *espGorf3core* mutant-encoded factors, perhaps EspB. The latter idea should be tested and complementation studies carried out with Intimin variants with defect in altering host cell physiology independent of Tir to perhaps provide insight to help define the molecular basis of inhibition.

A similar strategy was used to support the idea that Tir has activities that inhibit phagocytosis but data interpretation was hindered by the need to co-introduce the *cesT* gene to enable delivery of Tir and, unfortunately, another LEE effector (EspZ) and many Nle effectors. Given the LEE sufficiency finding and no critical role for 14 (of 17) examined Nle effectors studies examined, and ruled out a key contribution for EspZ in the Tir-associated anti-phagocytic process. However, it was surprising to find that inactivating *espZ* in the *espGorf3core* mutant restored some ($p<0.05$) anti-phagocytic activity as this strain should not deliver EspZ (Ruchaud-Sparagano *et al.*, 2007) and thus deleting *espZ* should have no impact. The latter finding suggests that EspZ may have an additional, indirect or direct, role and, thus, could be the focus of future studies. While this study provided encouraging data on some Tir variants enabling the *espGorf3core* mutant to inhibit phagocytosis, the findings are too

preliminary and further studies are needed to confirm introduction of the correct variant and carry out repeat experiments. It is likely that such data could provide information to help define the molecular details of how Tir activity enables EPEC to inhibit phagocytosis. It would also be interesting to examine whether this Tir activity depends on other EPEC factors (for example, EspB) by introducing Tir into macrophages without other effectors (for example, via the *Yersinia* delivery) or use of the EspB variant that delivers effectors but cannot inhibit phagocytosis (Iizumi *et al.*, 2007).

To conclude, this study has provided valuable insights into the role of the EPEC effector repertoire in the phagocytosis inhibitory mechanism. Furthermore, by screening mutants where genes encoding five proteins were inactivated in all combinations, this study proposes a critical role for Intimin and its translocator Tir in EPEC's anti-phagocytosis activity. Indeed, this work reveals an unknown complexity behind EPEC's ability to inhibit its uptake by macrophages. These experiments have set the groundwork to enable future studies to identify the virulence factors responsible and to uncover the mechanism for inhibiting phagocytosis, thus providing insights into the role of phagocytosis inhibition in EPEC induced disease.

Implications for the future

- Considerably more work will need to be done to enable the comparison of the phagocytosis activity of different mutants such as the T3SS like mutant *espF*/pGFP strain or EPEC-like *espF* mutants. Experiments to determine the reason for the differences could focus on the genetic background of both bacteria and host, alternatively reagents, protocols and/or environmental factors could have an impact.

- This study suggested a T3SS-independent inhibitory contribution, which may implicate roles for other EPEC-specific T3SS-independent factors. Thus, further work is required to define the EPEC factors and mechanisms mediating this T3SS-independent inhibitory mechanism. Possible mediators would be T2SS-dependent substrates required for EPEC virulence, the EspC protein (autotransporter link to cell cytotoxicity) or surface components such as pili, membrane proteins or capsule.

- As mentioned in chapter four, this study suggests that anti-phagocytosis, at least in the J774A.1 model, is a more complex process than recognised. Future research should, therefore, concentrate on confirming the relationships between EPEC effectors in phagocytic inhibition such as cooperative mechanisms between Intimin and Tir or

antagonistic mechanisms as our data argues for Map, EspF and EspH being able to suppress EPEC's anti-phagocytic activity.

- A future study investigating factor(s) and mechanism by which Intimin and Tir inhibit uptake of EPEC by macrophages would be very interesting.
- The T3SS of *Yersinia* can be used as a good model to examine Tir suffecncy and the reported contribution with other effectors such as EspB in the inhibitory mechanism in the absence of all other EPEC effectors.
- Although extensive research has been carried out on a key role for EspF to enable EPEC to inhibit its uptake in cultured macrophages and M-cells (*in vitro* and *in vivo*) models (Quitard *et al.*, 2006; Martinez-Argudo *et al.*, 2007; Marchès *et al.*, 2008; Tahoun *et al.*, 2011), no single study exists which adequately examined the role of other effectors such as EspH, Tir, EspB and the outer membrane Intimin using M-cells models. Future studies using M-cells models (*in vitro* and *in vivo*) are therefore recommended to test the role of Tir and Intimin and other EPEC effectors in the phagocytic inhibition.

Chapter 7. Appendix

7.1 Bioinformatics analysis

7.1.1 espF-L16E strain

EspF native

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BglII=AGATCT

#35= Δ21-74, no BglII, it is L16E but it has no deletion

espF-L16E

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Alignment of Sequence_1: [35] with Sequence_2: [EspF native]

Similarity: 618/621 (99.52 %)

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Seq_1  181   ATTTTTAATGTGAGCAGCCAGGTGACTTCATTTACTCCCTCTCGTCCGGCACCGCCGCCA  240
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Seq_2  601   GAGCAATCGAAGAAAGGGTAA  621
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Alignment of Sequence_1: [Fragment of 35 [1-621]] with Sequence_2: [Fragment of EspF native [1-621]]

Similarity: 205/206 (99.51 %)

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7.1.2 espF-L16E /34 strain

EspF native

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BglII=AGATCT

#34= Δ 298bp-548bp= 100-183 A.A.

espF-L16E / 34

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Similarity: 368/621 (59.26 %)

Seq_1  1     ATGCTTAATGGAATTAGTAACGCTGCTTCTACACTAGGGCGGCAGGAAGTAGGTATCGCA  60
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Seq_2  1     ATGCTTAATGGAATTAGTAACGCTGCTTCTACACTAGGGCGGCAGCTTGTAGGTATCGCA  60


Seq_1  61    AGTCGAGTGAGCTCTGCGGGGGGAACTGGATTTTCTGTAGCCCCTCAGGCTGTTCGTCTT  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  61    AGTCGAGTGAGCTCTGCGGGGGGAACTGGATTTTCTGTAGCCCCTCAGGCTGTTCGTCTT  120


Seq_1  121   ACTCCGGTGAGAGTTCATTCCCCCTTTTCTCCAGGTTCGTCGAATGTTAATGCGAGGACG  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  121   ACTCCGGTGAGAGTTCATTCCCCCTTTTCTCCAGGTTCGTCGAATGTTAATGCGAGGACG  180


Seq_1  181   ATTTTTAATGTGAGCAGCCAGGTGACTTCATTTACTCCCTCTCGTCCGGCACCGCCGCCA  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  181   ATTTTTAATGTGAGCAGCCAGGTGACTTCATTTACTCCCTCTCGTCCGGCACCGCCGCCA  240
```

```
Seq_1  241   CCGACAAGTGGACAGGCATCCGGGGCATCCCGACCTTTACCGCCCATTGCACAGGCA---  297
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  241   CCGACAAGTGGACAGGCATCCGGGGCATCCCGACCTTTACCGCCCATTGCACAGGCATTA  300


Seq_1  298   ------------------------------------------------------------  297

Seq_2  301   AAAGATCATTTAGCTGCCTATGAACTATCGAAAGCGTCTGAGACTGTAAACTTTAAGCCA  360
Seq_1  298   ------------------------------------------------------------  297

Seq_2  361   ACCCGTCCGGCACCGCCGCCACCGACAAGTGGACAGGCATCCGGGGCATCCCGACCTTTA  420


Seq_1  298   ------------------------------------------------------------  297

Seq_2  421   CCGCCCATTGCACAGGCATTAAAAGATCATTTAGCTGCCTATGAACTATCGAAAGCGTCT  480


Seq_1  298   ------------------------------------------------------------  297

Seq_2  481   GAGACTGTAAGCTTTAAGCCAACCCGTCAGGCACCACCGCCACCGACAAGTGGACAGGCA  540


Seq_1  298   --AGAT-CTGGTGGTCTACCGCCCCTTGCACAGGCACTAAAAGATCATTTAGCTGCCTAT  354
                |   ||||||| ||||||||||||||||||||||||||||||||||||||||||||
Seq_2  541   TCCGGGCCTGGTGGACTACCGCCCCTTGCACAGGCACTAAAAGATCATTTAGCTGCCTAT  600


Seq_1  355   GAGCAATCGAAGAAAGGGTAA  375
             ||||||||||||||||||||
Seq_2  601   GAGCAATCGAAGAAAGGGTAA  621
```

Alignment of Sequence_1: [Fragment of 34 [1-375]] with Sequence_2: [Fragment of EspF native [1-621]]

Similarity: 122/124 (98.39 %)

```
Seq_1  1     MLNGISNAASTLGRQEVGIASRVSSAGGTGFSVAPQAVRLTPVRVHSPFSPGSSNVNART  60
             ||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Seq_2  1     MLNGISNAASTLGRQLVGIASRVSSAGGTGFSVAPQAVRLTPVRVHSPFSPGSSNVNART  60


Seq_1  61    IFNVSSQVTSFTPSRPAPPPPTSGQASGASRPLPPIAQA--------------------  99
             ||||||||||||||||||||||||||||||||||||||||
Seq_2  61    IFNVSSQVTSFTPSRPAPPPPTSGQASGASRPLPPIAQALKDHLAAYELSKASETVNFKP  120


Seq_1  100   -RS-----------------------------------------------------------  101
              |
Seq_2  121   TRPAPPPPTSGQASGASRPLPPIAQALKDHLAAYELSKASETVSFKPTRQAPPPPTSGQA  180


Seq_1  102   ---GGLPPLAQALKDHLAAYEQSKKG  124
                 |||||||||||||||||||||||
Seq_2  181   SGPGGLPPLAQALKDHLAAYEQSKKG  206
```

7.1.3 espF-L16E /37 strain

EspF native

ATGCTTAATGGAATTAGTAACGCTGCTTCTACACTAGGGCGGCAGCTTGTAGGTATCGCAAGTCGAGTGAGCTCTGCGGG
GGGAACTGGATTTTCTGTAGCCCCTCAGGCTGTTCGTCTTACTCCGGTGAGAGTTCATTCCCCCTTTTCTCCAGGTTCGTCG
AATGTTAATGCGAGGACGATTTTTAATGTGAGCAGCCAGGTGACTTCATTTACTCCCTCTCGTCCGGCACCGCCGCCACCG
ACAAGTGGACAGGCATCCGGGGCATCCCGACCTTTACCGCCCATTGCACAGGCATTAAAAGATCATTTAGCTGCCTATGAA
CTATCGAAAGCGTCTGAGACTGTAAACTTTAAGCCAACCCGTCCGGCACCGCCGCCACCGACAAGTGGACAGGCATCCGG
GGCATCCCGACCTTTACCGCCCATTGCACAGGCATTAAAAGATCATTTAGCTGCCTATGAACTATCGAAAGCGTCTGAGAC
TGTAAGCTTTAAGCCAACCCGTCAGGCACCACCGCCACCGACAAGTGGACAGGCATCCGGGCCTGGTGGACTACCGCCCT
TGCACAGGCACTAAAAGCATCATTTAGCTGCCTATGAGCAATCGAAGAAAGGGTAA

MLNGISNAASTLGRQLVGIASRVSSAGGTGFSVAPQAVRLTPVRVHSPFSPGSSNVNARTIFNVSSQVTSFTPSR
PAPPPPTSGQASGASRPLPPIAQALKDHLAAYELSKASETVNFKPTRPAPPPPTSGQASGASRPLPPIAQALKDH
LAAYELSKASETVSFKPTRQAPPPPTSGQASGPGGLPPLAQALKDHLAAYEQSKKG

#37 = It is not L16E # 37 missing 485 bp from (63bp-547bp) missing 21 A.A-183A.A

espF-L16E /37

>430181001_37_FW sequence exported from 430181001_37_FW_A01.ab1 BglII=AGATCT

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AATTAGTAACGCTGCTTCTACACTAGGGCGGCAGCTTGTAGGTATCGCAAGATCTGGTGGTCTACCGCCCCTTGCACAGGCACTAAAAGA
TCATTTAGCTGCCTATGAGCAATCGAAGAAAGGGTAACCAACTAATTTAAAACGATTAATCGGTATTGATTATTTGTTTTCCAACTAATAA
AAACCGATATCAAATTGCGTGG

MLNGISNAASTLGRQLVGIARSGGLPPLAQALKDHLAAYEQSKKG

```
Alignment of Sequence_1:  [37] with Sequence_2: [EspF native]

Similarity: 135/621 (21.74 %)

Seq_1  1     ATGCTTAATGGAATTAGTAACGCTGCTTCTACACTAGGGCGGCAGCTTGTAGGTATCGCA  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  1     ATGCTTAATGGAATTAGTAACGCTGCTTCTACACTAGGGCGGCAGCTTGTAGGTATCGCA  60


Seq_1  61    AG-----------------------------------------------------------  62
             ||
Seq_2  61    AGTCGAGTGAGCTCTGCGGGGGGAACTGGATTTTCTGTAGCCCCTCAGGCTGTTCGTCTT  120


Seq_1  63    ------------------------------------------------------------  62

Seq_2  121   ACTCCGGTGAGAGTTCATTCCCCCTTTTCTCCAGGTTCGTCGAATGTTAATGCGAGGACG  180


Seq_1  63    ------------------------------------------------------------  62

Seq_2  181   ATTTTTAATGTGAGCAGCCAGGTGACTTCATTTACTCCCTCTCGTCCGGCACCGCCGCCA  240


Seq_1  63    ------------------------------------------------------------  62
```

```
Seq_2  241   CCGACAAGTGGACAGGCATCCGGGGCATCCCGACCTTTACCGCCCATTGCACAGGCATTA  300

Seq_1  63    ------------------------------------------------------------  62

Seq_2  301   AAAGATCATTTAGCTGCCTATGAACTATCGAAAGCGTCTGAGACTGTAAACTTTAAGCCA  360

Seq_1  63    ------------------------------------------------------------  62

Seq_2  361   ACCCGTCCGGCACCGCCGCCACCGACAAGTGGACAGGCATCCGGGGCATCCCGACCTTTA  420

Seq_1  63    ------------------------------------------------------------  62

Seq_2  421   CCGCCCATTGCACAGGCATTAAAAGATCATTTAGCTGCCTATGAACTATCGAAAGCGTCT  480

Seq_1  63    ------------------------------------------------------------  62

Seq_2  481   GAGACTGTAAGCTTTAAGCCAACCCGTCAGGCACCACCGCCACCGACAAGTGGACAGGCA  540

Seq_1  63    ----AT-CTGGTGGTCTACCGCCCCTTGCACAGGCACTAAAAGATCATTTAGCTGCCTAT  117
                    ||||||| ||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  541   TCCGGGCCTGGTGGACTACCGCCCCTTGCACAGGCACTAAAAGATCATTTAGCTGCCTAT  600

Seq_1  118   GAGCAATCGAAGAAAGGGTAA  138
              ||||||||||||||||||||
Seq_2  601   GAGCAATCGAAGAAAGGGTAA  621
```

Protein

Alignment of Sequence_1: [Fragment of EspF native [1-621]] with Sequence_2: [Fragment of 37 [1-138]]

Similarity: 45/45 (100.00 %)

```
Seq_1  1     MLNGISNAASTLGRQLVGIASRVSSAGGTGFSVAPQAVRLTPVRVHSPFSPGSSNVNART  60
             |||||||||||||||||| |:
Seq_2  1     MLNGISNAASTLGRQLVGIA-RS-----------------------------------  22

Seq_1  61    IFNVSSQVTSFTPSRPAPPPPTSGQASGASRPLPPIAQALKDHLAAYELSKASETVNFKP  120

Seq_2  23    ------------------------------------------------------------  22

Seq_1  121   TRPAPPPPTSGQASGASRPLPPIAQALKDHLAAYELSKASETVSFKPTRQAPPPPTSGQA  180

Seq_2  23    ------------------------------------------------------------  22

Seq_1  181   SGPGGLPPLAQALKDHLAAYEQSKKG  206
                 |||||||||||||||||||||||
Seq_2  23    ---GGLPPLAQALKDHLAAYEQSKKG  45
```

7.1.4 espF-L16E /39 strain

EspF native

ATGCTTAATGGAATTAGTAACGCTGCTTCTACACTAGGGCGGCAGCTTGTAGGTATCGCAAGTCGAGTGAGCTCTGCGGG
GGGAACTGGATTTTCTGTAGCCCCTCAGGCTGTTCGTCTTACTCCGGTGAGAGTTCATTCCCCCTTTTCTCCAGGTTCGTCG
AATGTTAATGCGAGGACGATTTTTAATGTGAGCAGCCAGGTGACTTCATTTACTCCCTCTCGTCCGGCACCGCCGCCACCG
ACAAGTGGACAGGCATCCGGGGCATCCCGACCTTTACCGCCCATTGCACAGGCATTAAAAGATCATTTAGCTGCCTATGAA
CTATCGAAAGCGTCTGAGACTGTAAACTTTAAGCCAACCCGTCCGGCACCGCCGCCACCGACAAGTGGACAGGCATCCGG
GGCATCCCGACCTTTACCGCCCATTGCACAGGCATTAAAAGATCATTTAGCTGCCTATGAACTATCGAAAGCGTCTGAGAC
TGTAAGCTTTAAGCCAACCCGTCAGGCACCACCGCCACCGACAAGTGGACAGGCATCCGGGCCTGGTGGACTACCGCCCC
TTGCACAGGCACTAAAAGATCATTTAGCTGCCTATGAGCAATCGAAGAAAGGGTAA

MLNGISNAASTLGRQLVGIASRVSSAGGTGFSVAPQAVRLTPVRVHSPFSPGSSNVNARTIFNVSSQVTSFTPSR
PAPPPPTSGQASGASRPLPPIAQALKDHLAAYELSKASETVNFKPTRPAPPPPTSGQASGASRPLPPIAQALKDH
LAAYELSKASETVSFKPTRQAPPPPTSGQASGPGGLPPLAQALKDHLAAYEQSKKG

BglII=AGATCT

39= Δ151bp-505BP missing (51AA-168AA) =117AA

espF-L16E /39

>430181001_39_FW sequence exported from
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TCCCCCTTTTCTAGATCTCGTCAGGCACCACCGCCACCGACAAGTGGACAGGCATCCGGGCCTGGTGGACTACCGCCCCTT
GCACAGGCACTAAAAGATCATTTAGCTGCCTATGAGCAATCGAAGAAAGGGTAACCAACTAATTTAAAACGATTAATCGG
TATTGATTATTTGTTTTCCAACTAATAAAAACCGATATCAAATTGCGTGG

MLNGISNAASTLGRQEVGIASRVSSAGGTGFSVAPQAVRLTPVRVHSPFSRSRQAPPPPTSGQASGPGGLPPLAQ
ALKDHLAAYEQSKKG

```
Alignment of Sequence_1:  [39] with Sequence_2: [EspF]

Similarity: 269/621 (43.32 %)

Seq_1  1     ATGCTTAATGGAATTAGTAACGCTGCTTCTACACTAGGGCGGCAGGAAGTAGGTATCGCA  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||   ||||||||||||
Seq_2  1     ATGCTTAATGGAATTAGTAACGCTGCTTCTACACTAGGGCGGCAGCTTGTAGGTATCGCA  60


Seq_1  61    AGTCGAGTGAGCTCTGCGGGGGGAACTGGATTTTCTGTAGCCCCTCAGGCTGTTCGTCTT  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  61    AGTCGAGTGAGCTCTGCGGGGGGAACTGGATTTTCTGTAGCCCCTCAGGCTGTTCGTCTT  120


Seq_1  121   ACTCCGGTGAGAGTTCATTCCCCCTTTTCT------------------------------  150
             |||||||||||||||||||||||||||||||
Seq_2  121   ACTCCGGTGAGAGTTCATTCCCCCTTTTCTCCAGGTTCGTCGAATGTTAATGCGAGGACG  180


Seq_1  151   ------------------------------------------------------------  150

Seq_2  181   ATTTTTAATGTGAGCAGCCAGGTGACTTCATTTACTCCCTCTCGTCCGGCACCGCCGCCA  240


Seq_1  151   ------------------------------------------------------------  150
```

```
Seq_2  241   CCGACAAGTGGACAGGCATCCGGGGCATCCCGACCTTTACCGCCCATTGCACAGGCATTA  300


Seq_1  151   --AGATCT----------------------------------------------------  156
                |||||
Seq_2  301   AAAGATCATTTAGCTGCCTATGAACTATCGAAAGCGTCTGAGACTGTAAACTTTAAGCCA  360


Seq_1  157   ----------------------------------------------------------  156

Seq_2  361   ACCCGTCCGGCACCGCCGCCACCGACAAGTGGACAGGCATCCGGGGCATCCCGACCTTTA  420


Seq_1  157   ----------------------------------------------------------  156

Seq_2  421   CCGCCCATTGCACAGGCATTAAAAGATCATTTAGCTGCCTATGAACTATCGAAAGCGTCT  480


Seq_1  157   ------------------------CGTCAGGCACCACCGCCACCGACAAGTGGACAGGCA  192
                                     ||||||||||||||||||||||||||||||||||||
Seq_2  481   GAGACTGTAAGCTTTAAGCCAACCCGTCAGGCACCACCGCCACCGACAAGTGGACAGGCA  540


Seq_1  193   TCCGGGCCTGGTGGACTACCGCCCCTTGCACAGGCACTAAAAGATCATTTAGCTGCCTAT  252
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  541   TCCGGGCCTGGTGGACTACCGCCCCTTGCACAGGCACTAAAAGATCATTTAGCTGCCTAT  600


Seq_1  253   GAGCAATCGAAGAAAGGGTAA  273
             ||||||||||||||||||||
Seq_2  601   GAGCAATCGAAGAAAGGGTAA  621

Alignment of Sequence_1:  [Fragment of 39 [1-273]] with Sequence_2: [Fragment of
EspF [1-621]]

Similarity: 88/90 (97.78 %)

Seq_1  1     MLNGISNAASTLGRQEVGIASRVSSAGGTGFSVAPQAVRLTPVRVHSPFS--------RS  52
             |||||||||||||| ||||||||||||||||||||||||||||||||||        |
Seq_2  1     MLNGISNAASTLGRQLVGIASRVSSAGGTGFSVAPQAVRLTPVRVHSPFSPGSSNVNART  60


Seq_1  53    ----------------------------------------------------------  52

Seq_2  61    IFNVSSQVTSFTPSRPAPPPPTSGQASGASRPLPPIAQALKDHLAAYELSKASETVNFKP  120


Seq_1  53    ----------------------------------------------RQAPPPPTSGQA  64
                                                            ||||||||||||
Seq_2  121   TRPAPPPPTSGQASGASRPLPPIAQALKDHLAAYELSKASETVSFKPTRQAPPPPTSGQA  180


Seq_1  65    SGPGGLPPLAQALKDHLAAYEQSKKG  90
             ||||||||||||||||||||||||||
Seq_2  181   SGPGGLPPLAQALKDHLAAYEQSKKG  206
```

7.1.5 espF-L16E /40 strain

EspF native

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AATGTTAATGCGAGGACGATTTTTAATGTGAGCAGCCAGGTGACTTCATTTACTCCCTCTCGTCCGGCACCGCCGCCACCG
ACAAGTGGACAGGCATCCGGGGCATCCCGACCTTTACCGCCCATTGCACAGGCATTAAAAGATCATTTAGCTGCCTATGAA
CTATCGAAAGCGTCTGAGACTGTAAACTTTAAGCCAACCCGTCCGGCACCGCCGCCACCGACAAGTGGACAGGCATCCGG
GGCATCCCGACCTTTACCGCCCATTGCACAGGCATTAAAAGATCATTTAGCTGCCTATGAACTATCGAAAGCGTCTGAGAC
TGTAAGCTTTAAGCCAACCCGTCAGGCACCACCGCCACCGACAAGTGGACAGGCATCCGGGCCTGGTGGACTACCGCCCC
TTGCACAGGCACTAAAAGATCATTTAGCTGCCTATGAGCAATCGAAGAAAGGGTAA

MLNGISNAASTLGRQLVGIASRVSSAGGTGFSVAPQAVRLTPVRVHSPFSPGSSNVNARTIFNVSSQVTSFTPSR
PAPPPPTSGQASGASRPLPPIAQALKDHLAAYELSKASETVNFKPTRPAPPPPTSGQASGASRPLPPIAQALKDH
LAAYELSKASETVSFKPTRQAPPPPTSGQASGPGGLPPLAQALKDHLAAYEQSKKG

BglII=AGATCT

#40= ΔCCAACC only

espF-L16E /40

>430181001_40_FW sequence exported from
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GAGAGTTCATTCCCCCTTTTCTCCAGGTTCGTCGAATGTTAATGCGAGGACGATTTTTAATGTGAGCAGCCAGGTGACTTCA
TTTACTCCCTCTCGTCCGGCACCGCCGCCACCGACAAGTGGACAGGCATCCGGGGCATCCCGACCTTTACCGCCCATTGCA
CAGGCATTAAAAGATCATTTAGCTGCCTATGAACTATCGAAAGCGTCTGAGACTGTAAACTTTAAGCCAACCCGTCCGGCA
CCGCCGCCACCGACAAGTGGACAGGCATCCGGGGCATCCCGACCTTTACCGCCCATTGCACAGGCATTAAAAGATCATTTA
GCTGCCTATGAACTATCGAAAGCGTCTGAGACTGTAAGCTTTAAGAGATCTCGTCAGGCACCACCGCCACCGACAAGTGG
ACAGGCATCCGGGCCTGGTGGACTACCGCCCCTTGCACAGGCACTAAAAGATCATTTAGCTGCCTATGAGCAATCGAAGA
AAGGGTAACCAACTAATTTAAAACGATTAATCGGTATTGATTATTTGTTTTCCAACTAATAAAAACCGATATCAAAATTGCG
TGN

```
Alignment of Sequence_1:  [EspF NATIVE] with Sequence_2: [40]

Similarity: 614/621 (98.87 %)

Seq_1  1     ATGCTTAATGGAATTAGTAACGCTGCTTCTACACTAGGGCGGCAGCTTGTAGGTATCGCA  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  1     ATGCTTAATGGAATTAGTAACGCTGCTTCTACACTAGGGCGGCAGGAAGTAGGTATCGCA  60


Seq_1  61    AGTCGAGTGAGCTCTGCGGGGGGAACTGGATTTTCTGTAGCCCCTCAGGCTGTTCGTCTT  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  61    AGTCGAGTGAGCTCTGCGGGGGGAACTGGATTTTCTGTAGCCCCTCAGGCTGTTCGTCTT  120


Seq_1  121   ACTCCGGTGAGAGTTCATTCCCCCTTTTCTCCAGGTTCGTCGAATGTTAATGCGAGGACG  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  121   ACTCCGGTGAGAGTTCATTCCCCCTTTTCTCCAGGTTCGTCGAATGTTAATGCGAGGACG  180
```

```
Seq_1  181   ATTTTTAATGTGAGCAGCCAGGTGACTTCATTTACTCCCTCTCGTCCGGCACCGCCGCCA  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  181   ATTTTTAATGTGAGCAGCCAGGTGACTTCATTTACTCCCTCTCGTCCGGCACCGCCGCCA  240

Seq_1  241   CCGACAAGTGGACAGGCATCCGGGGCATCCCGACCTTTACCGCCCATTGCACAGGCATTA  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  241   CCGACAAGTGGACAGGCATCCGGGGCATCCCGACCTTTACCGCCCATTGCACAGGCATTA  300

Seq_1  301   AAAGATCATTTAGCTGCCTATGAACTATCGAAAGCGTCTGAGACTGTAAACTTTAAGCCA  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  301   AAAGATCATTTAGCTGCCTATGAACTATCGAAAGCGTCTGAGACTGTAAACTTTAAGCCA  360

Seq_1  361   ACCCGTCCGGCACCGCCGCCACCGACAAGTGGACAGGCATCCGGGGCATCCCGACCTTTA  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  361   ACCCGTCCGGCACCGCCGCCACCGACAAGTGGACAGGCATCCGGGGCATCCCGACCTTTA  420

Seq_1  421   CCGCCCATTGCACAGGCATTAAAAGATCATTTAGCTGCCTATGAACTATCGAAAGCGTCT  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  421   CCGCCCATTGCACAGGCATTAAAAGATCATTTAGCTGCCTATGAACTATCGAAAGCGTCT  480

Seq_1  481   GAGACTGTAAGCTTTAAGCCAACCCGTCAGGCACCACCGCCACCGACAAGTGGACAGGCA  540
             ||||||||||||||||||  | | ||||||||||||||||||||||||||||||||||||
Seq_2  481   GAGACTGTAAGCTTTAAGAGATCTCGTCAGGCACCACCGCCACCGACAAGTGGACAGGCA  540

Seq_1  541   TCCGGGCCTGGTGGACTACCGCCCCTTGCACAGGCACTAAAAGATCATTTAGCTGCCTAT  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  541   TCCGGGCCTGGTGGACTACCGCCCCTTGCACAGGCACTAAAAGATCATTTAGCTGCCTAT  600

Seq_1  601   GAGCAATCGAAGAAAGGGTAA  621
             ||||||||||||||||||||
Seq_2  601   GAGCAATCGAAGAAAGGGTAA  621


Alignment of Sequence_1:  [Fragment of 40 [1-621]] with Sequence_2: [Fragment of
EspF NATIVE  [1-621]]

Similarity: 203/206 (98.54 %)

Seq_1  1     MLNGISNAASTLGRQEVGIASRVSSAGGTGFSVAPQAVRLTPVRVHSPFSPGSSNVNART  60
             ||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Seq_2  1     MLNGISNAASTLGRQLVGIASRVSSAGGTGFSVAPQAVRLTPVRVHSPFSPGSSNVNART  60

Seq_1  61    IFNVSSQVTSFTPSRPAPPPPTSGQASGASRPLPPIAQALKDHLAAYELSKASETVNFKP  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  61    IFNVSSQVTSFTPSRPAPPPPTSGQASGASRPLPPIAQALKDHLAAYELSKASETVNFKP  120

Seq_1  121   TRPAPPPPTSGQASGASRPLPPIAQALKDHLAAYELSKASETVSFKRSRQAPPPPTSGQA  180
             ||||||||||||||||||||||||||||||||||||||||||||||  ||||||||||||
Seq_2  121   TRPAPPPPTSGQASGASRPLPPIAQALKDHLAAYELSKASETVSFKPTRQAPPPPTSGQA  180

Seq_1  181   SGPGGLPPLAQALKDHLAAYEQSKKG  206
             |||||||||||||||||||||||||||
Seq_2  181   SGPGGLPPLAQALKDHLAAYEQSKKG  206
```

7.1.6 espF-L16E /42 strain

EspF native

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ACAAGTGGACAGGCATCCGGGGCATCCCGACCTTTACCGCCCATTGCACAGGCATTAAAAGATCATTTAGCTGCCTATGAA
CTATCGAAAGCGTCTGAGACTGTAAACTTTAAGCCAACCCGTCCGGCACCGCCGCCACCGACAAGTGGACAGGCATCCGG
GGCATCCCGACCTTTACCGCCCATTGCACAGGCATTAAAAGATCATTTAGCTGCCTATGAACTATCGAAAGCGTCTGAGAC
TGTAAGCTTTAAGCCAACCCGTCAGGCACCACCGCCACCGACAAGTGGACAGGCATCCGGGCCTGGTGGACTACCGCCCC
TTGCACAGGCACTAAAAGATCATTTAGCTGCCTATGAGCAATCGAAGAAAGGGTAA

MLNGISNAASTLGRQLVGIASRVSSAGGTGFSVAPQAVRLTPVRVHSPFSPGSSNVNARTIFNVSSQVTSFTPSR
PAPPPPTSGQASGASRPLPPIAQALKDHLAAYELSKASETVNFKPTRPAPPPPTSGQASGASRPLPPIAQALKDH
LAAYELSKASETVSFKPTRQAPPPPTSGQASGPGGLPPLAQALKDHLAAYEQSKKG

BglII=AGATCT

42= Δ151-220 bp with two mutation= 23A.A. (51AA.- 73 A.A.)

espF-L16E /42

>430181001_42_FW sequence exported from
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GCAAGTCGAGTGAGCTCTGCGGGGGGAACTGGATTTCCTGTAGCCCCTCAGGCTGTTCGTCTTACTCCGGTGAGAGTTCAT
TCCCCCTTTTCTAGATCTCGTCCGGCACCGCCGCCACCGACAAGTGGACAGGCATCCGGGGCATCCCGACCTTTACCGCCC
ATTGCACAGGCATTAAAAGATCATTTAGCTGCCTATGAACTATCGAAAGCGTCTGAGACTGTAAACTTTAAGCCAACCCGT
CCGGCACCGCCGCCACCGACAAGTGGACAGGCATCCGGGGCATCCCGACCTTTACCGCCCATTGCACAGGCATCAAAAGA
TCATTTAGCTGCCTATGAACTATCGAAAGCGTCTGAGACTGTAAGCTTTAAGCCAACCCGTCAGGCACCACCGCCACCGAC
AAGTGGACAGGCATCCGGGCCTGGTGGACTACCGCCCCTTGCACAGGCACTAAAAGATCATTTAGCTGCCTATGAGCAAT
CGAAGAAAGGGTAACCAACTAATTTAAAACGATTAATCGGTATTGATTATTTGTTTTCCAACTAATAAAAACCGATATCAAA
TTGCGTGG

MLNGISNAASTLGRQEVGIASRVSSAGGTGFPVAPQAVRLTPVRVHSPFSRSRPAPPPPTSGQASGASRPLPPIAQALKD
HLAAYELSKASETVNFKPTRPAPPPPTSGQASGASRPLPPIAQASKDHLAAYELSKASETVSFKPTRQAPPPPTSGQASG
PGGLPPLAQALKDHLAAYEQSKKG

```
Alignment of Sequence_1:  [42] with Sequence_2: [EspF NATIVE]

Similarity: 549/621 (88.41 %)

Seq_1  1     ATGCTTAATGGAATTAGTAACGCTGCTTCTACACTAGGGCGGCAGGAAGTAGGTATCGCA  60
             ||||||||||||||||||||||||||||||||||||||||||||||||   ||||||||||||
Seq_2  1     ATGCTTAATGGAATTAGTAACGCTGCTTCTACACTAGGGCGGCAGCTTGTAGGTATCGCA  60


Seq_1  61    AGTCGAGTGAGCTCTGCGGGGGGAACTGGATTTCCTGTAGCCCCTCAGGCTGTTCGTCTT  120
             |||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
Seq_2  61    AGTCGAGTGAGCTCTGCGGGGGGAACTGGATTTTCTGTAGCCCCTCAGGCTGTTCGTCTT  120


Seq_1  121   ACTCCGGTGAGAGTTCATTCCCCCTTTTCT--AGA------------------------  153
             ||||||||||||||||||||||||||||||  ||
Seq_2  121   ACTCCGGTGAGAGTTCATTCCCCCTTTTCTCCAGGTTCGTCGAATGTTAATGCGAGGACG  180
```

```
Seq_1  154   ------------------------------------TCTCGTCCGGCACCGCCGCCA  174
                                                 ||||||||||||||||||||
Seq_2  181   ATTTTTAATGTGAGCAGCCAGGTGACTTCATTTACTCCCTCTCGTCCGGCACCGCCGCCA  240


Seq_1  175   CCGACAAGTGGACAGGCATCCGGGGCATCCCGACCTTTACCGCCCATTGCACAGGCATTA  234
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  241   CCGACAAGTGGACAGGCATCCGGGGCATCCCGACCTTTACCGCCCATTGCACAGGCATTA  300


Seq_1  235   AAAGATCATTTAGCTGCCTATGAACTATCGAAAGCGTCTGAGACTGTAAACTTTAAGCCA  294
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  301   AAAGATCATTTAGCTGCCTATGAACTATCGAAAGCGTCTGAGACTGTAAACTTTAAGCCA  360


Seq_1  295   ACCCGTCCGGCACCGCCGCCACCGACAAGTGGACAGGCATCCGGGGCATCCCGACCTTTA  354
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  361   ACCCGTCCGGCACCGCCGCCACCGACAAGTGGACAGGCATCCGGGGCATCCCGACCTTTA  420


Seq_1  355   CCGCCCATTGCACAGGCATCAAAAGATCATTTAGCTGCCTATGAACTATCGAAAGCGTCT  414
             |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
Seq_2  421   CCGCCCATTGCACAGGCATTAAAAGATCATTTAGCTGCCTATGAACTATCGAAAGCGTCT  480


Seq_1  415   GAGACTGTAAGCTTTAAGCCAACCCGTCAGGCACCACCGCCACCGACAAGTGGACAGGCA  474
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  481   GAGACTGTAAGCTTTAAGCCAACCCGTCAGGCACCACCGCCACCGACAAGTGGACAGGCA  540


Seq_1  475   TCCGGGCCTGGTGGACTACCGCCCCTTGCACAGGCACTAAAAGATCATTTAGCTGCCTAT  534
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  541   TCCGGGCCTGGTGGACTACCGCCCCTTGCACAGGCACTAAAAGATCATTTAGCTGCCTAT  600


Seq_1  535   GAGCAATCGAAGAAAGGGTAA  555
             ||||||||||||||||||||
Seq_2  601   GAGCAATCGAAGAAAGGGTAA  621

Alignment of Sequence_1:  [Fragment of EspF NATIVE [1-621]] with  Sequence_2:
[Translation of  42]

Similarity: 181/184 (98.37 %)

Seq_1  1     MLNGISNAASTLGRQLVGIASRVSSAGGTGFSVAPQAVRLTPVRVHSPFSPGSSNVNART  60
             |||||||||||||| |||||||||||||| |||||||||||||||||        |
Seq_2  1     MLNGISNAASTLGRQEVGIASRVSSAGGTGFPVAPQAVRLTPVRVHSPFS--------R-  51


Seq_1  61    IFNVSSQVTSFTPSRPAPPPPTSGQASGASRPLPPIAQALKDHLAAYELSKASETVNFKP  120
                          ||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  52    -------------SRPAPPPPTSGQASGASRPLPPIAQALKDHLAAYELSKASETVNFKP  98


Seq_1  121   TRPAPPPPTSGQASGASRPLPPIAQALKDHLAAYELSKASETVSFKPTRQAPPPPTSGQA  180
             |||||||||||||||||||||||| |||||||||||||||||||||||||||||||||||
Seq_2  99    TRPAPPPPTSGQASGASRPLPPIAQASKDHLAAYELSKASETVSFKPTRQAPPPPTSGQA  158


Seq_1  181   SGPGGLPPLAQALKDHLAAYEQSKKG  206
             |||||||||||||||||||||||||
Seq_2  159   SGPGGLPPLAQALKDHLAAYEQSKKG  184
```

7.1.7 espF-L16E /45 strain

EspF native

ATGCTTAATGGAATTAGTAACGCTGCTTCTACACTAGGGCGGCAGCTTGTAGGTATCGCAAGTCGAGTGAGCTCTGC
GGGGGGAACTGGATTTTCTGTAGCCCCTCAGGCTGTTCGTCTTACTCCGGTGAGAGTTCATTCCCCCTTTTCTCCAGG
TTCGTCGAATGTTAATGCGAGGACGATTTTTAATGTGAGCAGCCAGGTGACTTCATTTACTCCCTCTCGTCCGGCACC
GCCGCCACCGACAAGTGGACAGGCATCCGGGGCATCCCGACCTTTACCGCCCATTGCACAGGCATTAAAAGATCATT
TAGCTGCCTATGAACTATCGAAAGCGTCTGAGACTGTAAACTTTAAGCCAACCCGTCCGGCACCGCCGCCACCGACA
AGTGGACAGGCATCCGGGGCATCCCGACCTTTACCGCCCATTGCACAGGCATTAAAAGATCATTTAGCTGCCTATGA
ACTATCGAAAGCGTCTGAGACTGTAAGCTTTAAGCCAACCCGTCAGGCACCACCGCCACCGACAAGTGGACAGGCA
TCCGGGCCTGGTGGACTACCGCCCCTTGCACAGGCACTAAAAGATCATTTAGCTGCCTATGAGCAATCGAAGAAAG
GGTAA

MLNGISNAASTLGRQLVGIASRVSSAGGTGFSVAPQAVRLTPVRVHSPFSPGSSNVNARTIFNVSSQV
TSFTPSRPAPPPPTSGQASGASRPLPPIAQALKDHLAAYELSKASETVNFKPTRPAPPPPTSGQASGA
SRPLPPIAQALKDHLAAYELSKASETVSFKPTRQAPPPPTSGQASGPGGLPPLAQALKDHLAAYEQSK
KG

BglII=AGATCT

#45= Δ182-219= 62-73 A.A (11 A.A) and there is one mutation, this is exactly similar to 36

espF-L16E /45

>430724901_45_espffw sequence exported from 430724901_45_espffw_B01.ab1

NNNNNNNNNNNNNNNTTTTATTATTCGTTTTGATATATATGAGAGTTAGCCAAGATTAGATATAAAGAGGCATA
AATTATGCTTAATGGAATTAGTAACGCTGCTTCTACACTAGGGCGGCAGGAAGTAGGTATCGCAAGTCGAGTGAGC
TCTGCGGGGGGAACTGGATTTTCTGTAGCCCCTCAGGCTGTTCGTCTTACTCCGGTGAGAGTTCATTCCCCCTTTTCT
CCAGGTTCGTCGAATGTTAATGCGAGGACGAGATCTCGTCCGGCGCCGCCGCCACCGACAAGTGGACAGGCATCCG
GGGCATCCCGACCTTTACCGCCCATTGCACAGGCATTAAAAGATCATTTAGCTGCCTATGAACTATCGAAAGCGTCT
GAGACTGTAAACTTTAAGCCAACCCGTCCGGCACCGCCGCCACCGACAAGTGGACAGGCATCCGGGGCATCCCGAC
CTTTACCGCCCATTGCACAGGCATTAAAAGATCATTTAGCTGCCTATGAACTATCGAAAGCGTCTGAGACTGTAAGCT
TTAAGCCAACCCGTCAGGCACCACCGCCACCGACAAGTGGACAGGCATCCGGGCCTGGTGGACTACCGCCCCTTGC
ACAGGCACTAAAAGATCATTTAGCTGCCTATGAGCAATCGAAGAAAGGGTAACCAACTAATTTAAAACGATTAATCG
GTATTGATTATTTGTTTTCCAACTAATAAAAACCGATATCAAATTGCGTG

MLNGISNAASTLGRQEVGIASRVSSAGGTGFSVAPQAVRLTPVRVHSPFSPGSSNVNARTRSRPAPPPPTSGQASGASRP
LPPIAQALKDHLAAYELSKASETVNFKPTRPAPPPPTSGQASGASRPLPPIAQALKDHLAAYELSKASETVSFKPTRQAP
PPPTSGQASGPGGLPPLAQALKDHLAAYEQSKKG

```
Alignment of Sequence_1:  [#45] with Sequence_2: [EspF NATIVE]

Similarity: 579/621 (93.24 %)
Seq_1  1     ATGCTTAATGGAATTAGTAACGCTGCTTCTACACTAGGGCGGCAGGAAGTAGGTATCGCA  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||   ||||||||||||
Seq_2  1     ATGCTTAATGGAATTAGTAACGCTGCTTCTACACTAGGGCGGCAGCTTGTAGGTATCGCA  60

Seq_1  61    AGTCGAGTGAGCTCTGCGGGGGGAACTGGATTTTCTGTAGCCCCTCAGGCTGTTCGTCTT  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  61    AGTCGAGTGAGCTCTGCGGGGGGAACTGGATTTTCTGTAGCCCCTCAGGCTGTTCGTCTT  120
```

```
Seq_1  121   ACTCCGGTGAGAGTTCATTCCCCCTTTTCTCCAGGTTCGTCGAATGTTAATGCGAGGACG  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  121   ACTCCGGTGAGAGTTCATTCCCCCTTTTCTCCAGGTTCGTCGAATGTTAATGCGAGGACG  180

Seq_1  181   A-----------------------------------GATCTCGTCCGGCGCCGCCGCCA  204
             |                                    ||||||||||| |||||||||
Seq_2  181   ATTTTTAATGTGAGCAGCCAGGTGACTTCATTTACTCCCTCTCGTCCGGCACCGCCGCCA  240


Seq_1  205   CCGACAAGTGGACAGGCATCCGGGGCATCCCGACCTTTACCGCCCATTGCACAGGCATTA  264
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  241   CCGACAAGTGGACAGGCATCCGGGGCATCCCGACCTTTACCGCCCATTGCACAGGCATTA  300


Seq_1  265   AAAGATCATTTAGCTGCCTATGAACTATCGAAAGCGTCTGAGACTGTAAACTTTAAGCCA  324
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  301   AAAGATCATTTAGCTGCCTATGAACTATCGAAAGCGTCTGAGACTGTAAACTTTAAGCCA  360


Seq_1  325   ACCCGTCCGGCACCGCCGCCACCGACAAGTGGACAGGCATCCGGGGCATCCCGACCTTTA  384
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  361   ACCCGTCCGGCACCGCCGCCACCGACAAGTGGACAGGCATCCGGGGCATCCCGACCTTTA  420


Seq_1  385   CCGCCCATTGCACAGGCATTAAAAGATCATTTAGCTGCCTATGAACTATCGAAAGCGTCT  444
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  421   CCGCCCATTGCACAGGCATTAAAAGATCATTTAGCTGCCTATGAACTATCGAAAGCGTCT  480


Seq_1  445   GAGACTGTAAGCTTTAAGCCAACCCGTCAGGCACCACCGCCACCGACAAGTGGACAGGCA  504
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  481   GAGACTGTAAGCTTTAAGCCAACCCGTCAGGCACCACCGCCACCGACAAGTGGACAGGCA  540


Seq_1  505   TCCGGGCCTGGTGGACTACCGCCCCTTGCACAGGCACTAAAAGATCATTTAGCTGCCTAT  564
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  541   TCCGGGCCTGGTGGACTACCGCCCCTTGCACAGGCACTAAAAGATCATTTAGCTGCCTAT  600


Seq_1  565   GAGCAATCGAAGAAAGGGTAA  585
             ||||||||||||||||||||||
Seq_2  601   GAGCAATCGAAGAAAGGGTAA  621


Alignment of Sequence_1:  [Fragment of #45 [1-585]] with Sequence_2: [Fragment of
EspF native  [1-621]]

Similarity: 192/194 (98.97 %)

Seq_1  1     MLNGISNAASTLGRQEVGIASRVSSAGGTGFSVAPQAVRLTPVRVHSPFSPGSSNVNART  60
             ||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Seq_2  1     MLNGISNAASTLGRQLVGIASRVSSAGGTGFSVAPQAVRLTPVRVHSPFSPGSSNVNART  60


Seq_1  61    R------------SRPAPPPPTSGQASGASRPLPPIAQALKDHLAAYELSKASETVNFKP  108
                          ||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  61    IFNVSSQVTSFTPSRPAPPPPTSGQASGASRPLPPIAQALKDHLAAYELSKASETVNFKP  120


Seq_1  109   TRPAPPPPTSGQASGASRPLPPIAQALKDHLAAYELSKASETVSFKPTRQAPPPPTSGQA  168
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  121   TRPAPPPPTSGQASGASRPLPPIAQALKDHLAAYELSKASETVSFKPTRQAPPPPTSGQA  180


Seq_1  169   SGPGGLPPLAQALKDHLAAYEQSKKG  194
             |||||||||||||||||||||||||||
Seq_2  181   SGPGGLPPLAQALKDHLAAYEQSKKG  206
```

7.1.8 espF-L16E /75 strain

EspF native

ATGCTTAATGGAATTAGTAACGCTGCTTCTACACTAGGGCGGCAGCTTGTAGGTATCGCAAGTCGAGTGAGCTCTGCGGG
GGGAACTGGATTTTCTGTAGCCCCTCAGGCTGTTCGTCTTACTCCGGTGAGAGTTCATTCCCCCTTTTCTCCAGGTTCGTCG
AATGTTAATGCGAGGACGATTTTTAATGTGAGCAGCCAGGTGACTTCATTTACTCCCTCTCGTCCGGCACCGCCGCCACCG
ACAAGTGGACAGGCATCCGGGGCATCCCGACCTTTACCGCCCATTGCACAGGCATTAAAAGATCATTTAGCTGCCTATGAA
CTATCGAAAGCGTCTGAGACTGTAAACTTTAAGCCAACCCGTCCGGCACCGCCGCCACCGACAAGTGGACAGGCATCCGG
GGCATCCCGACCTTTACCGCCCATTGCACAGGCATTAAAAGATCATTTAGCTGCCTATGAACTATCGAAAGCGTCTGAGAC
TGTAAGCTTTAAGCCAACCCGTCAGGCACCACCGCCACCGACAAGTGGACAGGCATCCGGGCCTGGTGGACTACCGCCCC
TTGCACAGGCACTAAAAGATCATTTAGCTGCCTATGAGCAATCGAAGAAAGGGTAA

MLNGISNAASTLGRQLVGIASRVSSAGGTGFSVAPQAVRLTPVRVHSPFSPGSSNVNARTIFNVSSQVTSFTPSR
PAPPPPTSGQASGASRPLPPIAQALKDHLAAYELSKASETVNFKPTRPAPPPPTSGQASGASRPLPPIAQALKDH
LAAYELSKASETVSFKPTRQAPPPPTSGQASGPGGLPPLAQALKDHLAAYEQSKKG

BglII=AGATCT

#75= Δ63-156 bp (30 AA from 23-52) mutation in two A.A. # (21 and 22 A.A.)

espF-L16E /75

>430181001_75_FW sequence exported from
430181001_75_FW_H01.ab1NNNNNNNNNNNNNNNCNTTTTATTATTCGTTTTGATATATATGAGAGTTAGCCAAGAT
TAGATATAAAGAGGCATAAATTATGCTTAATGGAATTAGTAACGCTGCTTCTACACTAGGGCGGCAGGAAGTAGGTATCG
CAAGATCTTCGTCGAATGTTAATGCGAGGACGATTTTTAATGTGAGCAGCCAGGTGACTTCATTTACTCCCTCTCGTCCGGC
ACCGCCGCCACCGACAAGTGGACAGGCATCCGGGGCATCCCGACCTTTACCGCCCATTGCACAGGCATTAAAAGATCATTT
AGCTGCCTATGAACTATCGAAAGCGTCTGAGACTGTAAACTTTAAGCCAACCCGTCCGGCACCGCCGCCACCGACAAGTG
GACAGGCATCCGGGGCATCCCGACCTTTACCGCCCATTGCACAGGCATTAAAAGATCATTTAGCTGCCTATGAACTATCGA
AAGCGTCTGAGACTGTAAGCTTTAAGCCAACCCGTCAGGCACCACCGCCACCGACAAGTGGACAGGCATCCGGGCCTGGT
GGACTACCGCCCCTTGCACAGGCACTAAAAGATCATTTAGCTGCCTATGAGCAATCGAAGAAAGGGTAACCAACTAATTTA
AAACGATTAATCGGTATTGATTATTTGTTTTCCAACTAATAAAAACCGATATCAAATTGCGTGG

MLNGISNAASTLGRQEVGIARSSSNVNARTIFNVSSQVTSFTPSRPAPPPPTSGQASGASRPLPPIAQALKDHLAAYELS
KASETVNFKPTRPAPPPPTSGQASGASRPLPPIAQALKDHLAAYELSKASETVSFKPTRQAPPPPTSGQASGPGGLPPLA
QALKDHLAAYEQSKKG

```
Alignment of Sequence_1:  [75] with Sequence_2: [EspF NATIVE]

Similarity: 525/621 (84.54 %)

Seq_1  1     ATGCTTAATGGAATTAGTAACGCTGCTTCTACACTAGGGCGGCAGGAAGTAGGTATCGCA  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||    ||||||||||||
Seq_2  1     ATGCTTAATGGAATTAGTAACGCTGCTTCTACACTAGGGCGGCAGCTTGTAGGTATCGCA  60


Seq_1  61    AGATC-------------------------------------------------------  65
             ||
Seq_2  61    AGTCGAGTGAGCTCTGCGGGGGGAACTGGATTTTCTGTAGCCCCTCAGGCTGTTCGTCTT  120
```

```
Seq_1  66    ----------------------------------TTCGTCGAATGTTAATGCGAGGACG  90
                                               |||||||||||||||||||||||||
Seq_2  121   ACTCCGGTGAGAGTTCATTCCCCCTTTTCTCCAGGTTCGTCGAATGTTAATGCGAGGACG  180


Seq_1  91    ATTTTTAATGTGAGCAGCCAGGTGACTTCATTTACTCCCTCTCGTCCGGCACCGCCGCCA  150
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  181   ATTTTTAATGTGAGCAGCCAGGTGACTTCATTTACTCCCTCTCGTCCGGCACCGCCGCCA  240


Seq_1  151   CCGACAAGTGGACAGGCATCCGGGGCATCCCGACCTTTACCGCCCATTGCACAGGCATTA  210
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  241   CCGACAAGTGGACAGGCATCCGGGGCATCCCGACCTTTACCGCCCATTGCACAGGCATTA  300
Seq_1  211   AAAGATCATTTAGCTGCCTATGAACTATCGAAAGCGTCTGAGACTGTAAACTTTAAGCCA  270
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  301   AAAGATCATTTAGCTGCCTATGAACTATCGAAAGCGTCTGAGACTGTAAACTTTAAGCCA  360


Seq_1  271   ACCCGTCCGGCACCGCCGCCACCGACAAGTGGACAGGCATCCGGGGCATCCCGACCTTTA  330
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  361   ACCCGTCCGGCACCGCCGCCACCGACAAGTGGACAGGCATCCGGGGCATCCCGACCTTTA  420


Seq_1  331   CCGCCCATTGCACAGGCATTAAAAGATCATTTAGCTGCCTATGAACTATCGAAAGCGTCT  390
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  421   CCGCCCATTGCACAGGCATTAAAAGATCATTTAGCTGCCTATGAACTATCGAAAGCGTCT  480


Seq_1  391   GAGACTGTAAGCTTTAAGCCAACCCGTCAGGCACCACCGCCACCGACAAGTGGACAGGCA  450
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  481   GAGACTGTAAGCTTTAAGCCAACCCGTCAGGCACCACCGCCACCGACAAGTGGACAGGCA  540


Seq_1  451   TCCGGGCCTGGTGGACTACCGCCCCTTGCACAGGCACTAAAAGATCATTTAGCTGCCTAT  510
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  541   TCCGGGCCTGGTGGACTACCGCCCCTTGCACAGGCACTAAAAGATCATTTAGCTGCCTAT  600


Seq_1  511   GAGCAATCGAAGAAAGGGTAA  531
             |||||||||||||||||||||
Seq_2  601   GAGCAATCGAAGAAAGGGTAA  621


Alignment of Sequence_1:  [Fragment of EspF NATIVE [1-621]] with Sequence_2:
[Fragment of 75 [1-531]]

Similarity: 176/176 (100.00 %)

Seq_1  1     MLNGISNAASTLGRQLVGIASRVSSAGGTGFSVAPQAVRLTPVRVHSPFSPGSSNVNART  60
             ||||||||||||||:||||::                                ||||||||
Seq_2  1     MLNGISNAASTLGRQEVGIARS----------------------------SSNVNART  30


Seq_1  61    IFNVSSQVTSFTPSRPAPPPPTSGQASGASRPLPPIAQALKDHLAAYELSKASETVNFKP  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  31    IFNVSSQVTSFTPSRPAPPPPTSGQASGASRPLPPIAQALKDHLAAYELSKASETVNFKP  90


Seq_1  121   TRPAPPPPTSGQASGASRPLPPIAQALKDHLAAYELSKASETVSFKPTRQAPPPPTSGQA  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  91    TRPAPPPPTSGQASGASRPLPPIAQALKDHLAAYELSKASETVSFKPTRQAPPPPTSGQA  150


Seq_1  181   SGPGGLPPLAQALKDHLAAYEQSKKG  206
             |||||||||||||||||||||||||
Seq_2  151   SGPGGLPPLAQALKDHLAAYEQSKKG  176
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7.1.9 espF-L16E /77 strain

EspF native

ATGCTTAATGGAATTAGTAACGCTGCTTCTACACTAGGGCGGCAGCTTGTAGGTATCGCAAGTCGAGTGAGCTCTGCGGG
GGGAACTGGATTTTCTGTAGCCCCTCAGGCTGTTCGTCTTACTCCGGTGAGAGTTCATTCCCCCTTTTCTCCAGGTTCGTCG
AATGTTAATGCGAGGACGATTTTTAATGTGAGCAGCCAGGTGACTTCATTTACTCCCTCTCGTCCGGCACCGCCGCCACCG
ACAAGTGGACAGGCATCCGGGGCATCCCGACCTTTACCGCCCATTGCACAGGCATTAAAAGATCATTTAGCTGCCTATGAA
CTATCGAAAGCGTCTGAGACTGTAAACTTTAAGCCAACCCGTCCGGCACCGCCGCCACCGACAAGTGGACAGGCATCCGG
GGCATCCCGACCTTTACCGCCCATTGCACAGGCATTAAAAGATCATTTAGCTGCCTATGAACTATCGAAAGCGTCTGAGAC
TGTAAGCTTTAAGCCAACCCGTCAGGCACCACCGCCACCGACAAGTGGACAGGCATCCGGGCCTGGTGGACTACCGCCCC
TTGCACAGGCACTAAAAGATCATTTAGCTGCCTATGAGCAATCGAAGAAAGGGTAA

MLNGISNAASTLGRQLVGIASRVSSAGGTGFSVAPQAVRLTPVRVHSPFSPGSSNVNARTIFNVSSQVTSFTPSR
PAPPPPTSGQASGASRPLPPIAQALKDHLAAYELSKASETVNFKPTRPAPPPPTSGQASGASRPLPPIAQALKDH
LAAYELSKASETVSFKPTRQAPPPPTSGQASGPGGLPPLAQALKDHLAAYEQSKKG

BglII=AGATCT

#77= It is not L16E missing 63bp- 220bp (157bp) = missing 21-73 A.A (53A.A)

espF-L16E 77

>430181001_77_FW sequence exported from
430181001_77_FW_A02.ab1NNNNNNNNNNNCNNNNNNTNNNNNNNNTTTTATTATTCGTTTTGATATATATGAGAG
TTAGCCAAGATTAGATATAAAGAGGCATAAATTATGCTTAATGGAATTAGTAACGCTGCTTCTACACTAGGGCGGCAGCTT
GTAGGTATCGCAAGATCTCGTCCGGCACCGCCGCCACCGACAAGTGGACAGGCATCCGGGGCATCCCGACCTTTACCGCC
CATTGCACAGGCATTAAAAGATCATTTAGCTGCCTATGAACTATCGAAAGCGTCTGAGACTGTAAACTTTAAGCCAACCCG
TCCGGCACCGCCGCCACCGACAAGTGGACAGGCATCCGGGGCATCCCGACCTTTACCGCCCATTGCACAGGCATTAAAAG
ATCATTTAGCTGCCTATGAACTATCGAAAGCGTCTGAGACTGTAAGCTTTAAGCCAACCCGTCAGGCACCACCGCCACCGA
CAAGTGGACAGGCATCCGGGCCTGGTGGACTACCGCCCCTTGCACAGGCACTAAAAGATCATTTAGCTGCCTATGAGCAA
TCGAAGAAAGGGTAACCAACTAATTTAAAACGATTAATCGGTATTGATTATTTGTTTTCCAACTAATAAAAACCGATATCAA
ATTGCGTGG

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ASGASRPLPPIAQALKDHLAAYELSKASETVSFKPTRQAPPPPTSGQASGPGGLPPLAQALKDHLAAYEQSKKG

```
Alignment of Sequence_1:  [77] with Sequence_2: [EspF native]

Similarity: 465/621 (74.88 %)

Seq_1  1     ATGCTTAATGGAATTAGTAACGCTGCTTCTACACTAGGGCGGCAGCTTGTAGGTATCGCA  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  1     ATGCTTAATGGAATTAGTAACGCTGCTTCTACACTAGGGCGGCAGCTTGTAGGTATCGCA  60


Seq_1  61    AG---A-------------------------------------------------------  63
             ||   |
Seq_2  61    AGTCGAGTGAGCTCTGCGGGGGGAACTGGATTTTCTGTAGCCCCTCAGGCTGTTCGTCTT  120


Seq_1  64    ------------------------------------------------------------  63

Seq_2  121   ACTCCGGTGAGAGTTCATTCCCCCTTTTCTCCAGGTTCGTCGAATGTTAATGCGAGGACG  180
```

```
Seq_1  64    ------------------------------------TCTCGTCCGGCACCGCCGCCA  84
                                                 ||||||||||||||||||||
Seq_2  181   ATTTTTAATGTGAGCAGCCAGGTGACTTCATTTACTCCCTCTCGTCCGGCACCGCCGCCA  240


Seq_1  85    CCGACAAGTGGACAGGCATCCGGGGCATCCCGACCTTTACCGCCCATTGCACAGGCATTA  144
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  241   CCGACAAGTGGACAGGCATCCGGGGCATCCCGACCTTTACCGCCCATTGCACAGGCATTA  300


Seq_1  145   AAAGATCATTTAGCTGCCTATGAACTATCGAAAGCGTCTGAGACTGTAAACTTTAAGCCA  204
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  301   AAAGATCATTTAGCTGCCTATGAACTATCGAAAGCGTCTGAGACTGTAAACTTTAAGCCA  360


Seq_1  205   ACCCGTCCGGCACCGCCGCCACCGACAAGTGGACAGGCATCCGGGGCATCCCGACCTTTA  264
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  361   ACCCGTCCGGCACCGCCGCCACCGACAAGTGGACAGGCATCCGGGGCATCCCGACCTTTA  420


Seq_1  265   CCGCCCATTGCACAGGCATTAAAAGATCATTTAGCTGCCTATGAACTATCGAAAGCGTCT  324
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  421   CCGCCCATTGCACAGGCATTAAAAGATCATTTAGCTGCCTATGAACTATCGAAAGCGTCT  480


Seq_1  325   GAGACTGTAAGCTTTAAGCCAACCCGTCAGGCACCACCGCCACCGACAAGTGGACAGGCA  384
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  481   GAGACTGTAAGCTTTAAGCCAACCCGTCAGGCACCACCGCCACCGACAAGTGGACAGGCA  540


Seq_1  385   TCCGGGCCTGGTGGACTACCGCCCCTTGCACAGGCACTAAAAGATCATTTAGCTGCCTAT  444
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  541   TCCGGGCCTGGTGGACTACCGCCCCTTGCACAGGCACTAAAAGATCATTTAGCTGCCTAT  600


Seq_1  445   GAGCAATCGAAGAAAGGGTAA  465
             ||||||||||||||||||||
Seq_2  601   GAGCAATCGAAGAAAGGGTAA  621
```

Alignment of Sequence_1: [Fragment of 77 [1-465]] with Sequence_2: [Fragment of EspF native [1-621]]

Similarity: 154/154 (100.00 %)

```
Seq_1  1     MLNGISNAASTLGRQLVGIA-R-------------------------------------  21
             |||||||||||||||||||| |
Seq_2  1     MLNGISNAASTLGRQLVGIASRVSSAGGTGFSVAPQAVRLTPVRVHSPFSPGSSNVNART  60


Seq_1  22    -------------SRPAPPPPTSGQASGASRPLPPIAQALKDHLAAYELSKASETVNFKP  68
                         ||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  61    IFNVSSQVTSFTPSRPAPPPPTSGQASGASRPLPPIAQALKDHLAAYELSKASETVNFKP  120


Seq_1  69    TRPAPPPPTSGQASGASRPLPPIAQALKDHLAAYELSKASETVSFKPTRQAPPPPTSGQA  128
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  121   TRPAPPPPTSGQASGASRPLPPIAQALKDHLAAYELSKASETVSFKPTRQAPPPPTSGQA  180


Seq_1  129   SGPGGLPPLAQALKDHLAAYEQSKKG  154
             |||||||||||||||||||||||||
Seq_2  181   SGPGGLPPLAQALKDHLAAYEQSKKG  206
```

7.1.10 espF-L16E /78 strain

EspF native

ATGCTTAATGGAATTAGTAACGCTGCTTCTACACTAGGGCGGCAGCTTGTAGGTATCGCAAGTCGAGTGAGCTCTGCGGG
GGGAACTGGATTTTCTGTAGCCCCTCAGGCTGTTCGTCTTACTCCGGTGAGAGTTCATTCCCCCTTTTCTCCAGGTTCGTCG
AATGTTAATGCGAGGACGATTTTTAATGTGAGCAGCCAGGTGACTTCATTTACTCCCTCTCGTCCGGCACCGCCGCCACCG
ACAAGTGGACAGGCATCCGGGGCATCCCGACCTTTACCGCCCATTGCACAGGCATTAAAAGATCATTTAGCTGCCTATGAA
CTATCGAAAGCGTCTGAGACTGTAAACTTTAAGCCAACCCGTCCGGCACCGCCGCCACCGACAAGTGGACAGGCATCCGG
GGCATCCCGACCTTTACCGCCCATTGCACAGGCATTAAAAGATCATTTAGCTGCCTATGAACTATCGAAAGCGTCTGAGAC
TGTAAGCTTTAAGCCAACCCGTCAGGCACCACCGCCACCGACAAGTGGACAGGCATCCGGGCCTGGTGGACTACCGCCCC
TTGCACAGGCACTAAAAGATCATTTAGCTGCCTATGAGCAATCGAAGAAAGGGTAA

MLNGISNAASTLGRQLVGIASRVSSAGGTGFSVAPQAVRLTPVRVHSPFSPGSSNVNARTIFNVSSQVTSFTPSR
PAPPPPTSGQASGASRPLPPIAQALKDHLAAYELSKASETVNFKPTRPAPPPPTSGQASGASRPLPPIAQALKDH
LAAYELSKASETVSFKPTRQAPPPPTSGQASGPGGLPPLAQALKDHLAAYEQSKKG

BglII=AGATCT

#78= Δ 151bp- 219bp and 350bp- 490bp and 3 mutations

Missing 51-73 A.A. and 117A.A-162 A.A and mutation with one amino acid #199 A.A.

espF-L16E /78

>430181001_78_FW sequence exported from
430181001_78_FW_B02.ab1NNNNNNNNNNNNNNNCNNNNNNNNNNNNNNTTATTATTCGTTTTGATATATATGAGAGT
TAGCCAAGATTAGATATAAAGAGGCATAAATTATGCTTAATGGAATTAGTAACGCTGCTTCTACACTAGGGCGGCAGGAA
GTAGGTATCGCAAGTCGAGTGAGCTCTGCGGGGGGAACTGGATTTTCTGTAGCCCCTCAGGCTGTTCGTCTTACTCCGGTG
AGAGTTCATTCCCCCTTTTCTAGATCTCGTCCGGCGCCGCCGCCACCGACAAGTGGACAGGCATCCGGGGCATCCCGACCT
TTACCGCCCATTGCACAGGCATTAAAAGATCACTTAGCTGCCTATGAACTATCGAAAGCGTCTGAGACTGTAAGCTTTAAG
CCAACCCGTCAGGCACCACCGCCACCGACAAGTGGACAGGCATCCGGGCCTGGTGGACTACCGCCCCTTGCACAGGCACT
AAAAGATCATTTAGCTGCCCATGAGCAATCGAAGAAAGGGTAACCAACTAATTTAAAACGATTAATCGGTATTGATTATTT
GTTTTCCAACTAATAAAAACCGATATCAAATTGCGTGG

```
Alignment of Sequence_1:  [78] with Sequence_2: [EspF native]

Similarity: 405/621 (65.22 %)

Seq_1  1     ATGCTTAATGGAATTAGTAACGCTGCTTCTACACTAGGGCGGCAGGAAGTAGGTATCGCA  60
             ||||||||||||||||||||||||||||||||||||||||||||||||   ||||||||||||
Seq_2  1     ATGCTTAATGGAATTAGTAACGCTGCTTCTACACTAGGGCGGCAGCTTGTAGGTATCGCA  60


Seq_1  61    AGTCGAGTGAGCTCTGCGGGGGGAACTGGATTTTCTGTAGCCCCTCAGGCTGTTCGTCTT  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  61    AGTCGAGTGAGCTCTGCGGGGGGAACTGGATTTTCTGTAGCCCCTCAGGCTGTTCGTCTT  120


Seq_1  121   ACTCCGGTGAGAGTTCATTCCCCCTTTTCT------------------------------  150
             ||||||||||||||||||||||||||||||
Seq_2  121   ACTCCGGTGAGAGTTCATTCCCCCTTTTCTCCAGGTTCGTCGAATGTTAATGCGAGGACG  180
```

```
Seq_1  151   ----------------------------------AGATCTCGTCCGGCGCCGCCGCCA  174
                                                ||||||||||| |||||||||
Seq_2  181   ATTTTTAATGTGAGCAGCCAGGTGACTTCATTTACTCCCTCTCGTCCGGCACCGCCGCCA  240

Seq_1  175   CCGACAAGTGGACAGGCATCCGGGGCATCCCGACCTTTACCGCCCATTGCACAGGCATTA  234
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  241   CCGACAAGTGGACAGGCATCCGGGGCATCCCGACCTTTACCGCCCATTGCACAGGCATTA  300

Seq_1  235   AAAGATCACTTAGCTGCCTATGAACTATCGAAAGCGTCTGAGACTGTAA-----------  283
             |||||||| ||||||||||||||||||||||||||||||||||||||||
Seq_2  301   AAAGATCATTTAGCTGCCTATGAACTATCGAAAGCGTCTGAGACTGTAAACTTTAAGCCA  360

Seq_1  284   ------------------------------------------------------------  283

Seq_2  361   ACCCGTCCGGCACCGCCGCCACCGACAAGTGGACAGGCATCCGGGGCATCCCGACCTTTA  420

Seq_1  284   ------------------------------------------------------------  283

Seq_2  421   CCGCCCATTGCACAGGCATTAAAAGATCATTTAGCTGCCTATGAACTATCGAAAGCGTCT  480

Seq_1  284   ----------GCTTTAAGCCAACCCGTCAGGCACCACCGCCACCGACAAGTGGACAGGCA  333
                       ||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  481   GAGACTGTAAGCTTTAAGCCAACCCGTCAGGCACCACCGCCACCGACAAGTGGACAGGCA  540

Seq_1  334   TCCGGGCCTGGTGGACTACCGCCCCTTGCACAGGCACTAAAAGATCATTTAGCTGCCCAT  393
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||
Seq_2  541   TCCGGGCCTGGTGGACTACCGCCCCTTGCACAGGCACTAAAAGATCATTTAGCTGCCTAT  600

Seq_1  394   GAGCAATCGAAGAAAGGGTAA  414
             ||||||||||||||||||||
Seq_2  601   GAGCAATCGAAGAAAGGGTAA  621


Alignment of Sequence_1:  [Translation of 78] with Sequence_2: [Fragment of EspF
native [1-621]]

Similarity: 136/137 (99.27 %)

Seq_1  1     MLNGISNAASTLGRQEVGIASRVSSAGGTGFSVAPQAVRLTPVRVHSPFS--------R-  51
             ||||||||||||||| |||||||||||||||||||||||||||||||||        |
Seq_2  1     MLNGISNAASTLGRQLVGIASRVSSAGGTGFSVAPQAVRLTPVRVHSPFSPGSSNVNART  60

Seq_1  52    -------------SRPAPPPPTSGQASGASRPLPPIAQALKDHLAAYELSKASETV----  94
                          ||||||||||||||||||||||||||||||||||||||||||||
Seq_2  61    IFNVSSQVTSFTPSRPAPPPPTSGQASGASRPLPPIAQALKDHLAAYELSKASETVNFKP  120

Seq_1  95    -------------------------------------------SFKPTRQAPPPPTSGQA  111
                                                         |||||||||||||||||
Seq_2  121   TRPAPPPPTSGQASGASRPLPPIAQALKDHLAAYELSKASETVSFKPTRQAPPPPTSGQA  180

Seq_1  112   SGPGGLPPLAQALKDHLAAHEQSKKG  137
             |||||||||||||||||||:||||||
Seq_2  181   SGPGGLPPLAQALKDHLAAYEQSKKG  206
```

7.1.11 espF-L16E /79 strain

EspF native

ATGCTTAATGGAATTAGTAACGCTGCTTCTACACTAGGGCGGCAGCTTGTAGGTATCGCAAGTCGAGTGAGCTCTGCGGG
GGGAACTGGATTTTCTGTAGCCCCTCAGGCTGTTCGTCTTACTCCGGTGAGAGTTCATTCCCCCTTTTCTCCAGGTTCGTCG
AATGTTAATGCGAGGACGATTTTTAATGTGAGCAGCCAGGTGACTTCATTTACTCCCTCTCGTCCGGCACCGCCGCCACCG
ACAAGTGGACAGGCATCCGGGGCATCCCGACCTTTACCGCCCATTGCACAGGCATTAAAAGATCATTTAGCTGCCTATGAA
CTATCGAAAGCGTCTGAGACTGTAAACTTTAAGCCAACCCGTCCGGCACCGCCGCCACCGACAAGTGGACAGGCATCCGG
GGCATCCCGACCTTTACCGCCCATTGCACAGGCATTAAAAGATCATTTAGCTGCCTATGAACTATCGAAAGCGTCTGAGAC
TGTAAGCTTTAAGCCAACCCGTCAGGCACCACCGCCACCGACAAGTGGACAGGCATCCGGGCCTGGTGGACTACCGCCCC
TTGCACAGGCACTAAAAGATCATTTAGCTGCCTATGAGCAATCGAAGAAAGGGTAA

MLNGISNAASTLGRQLVGIASRVSSAGGTGFSVAPQAVRLTPVRVHSPFSPGSSNVNARTIFNVSSQVTSFTPSR
PAPPPPTSGQASGASRPLPPIAQALKDHLAAYELSKASETVNFKPTRPAPPPPTSGQASGASRPLPPIAQALKDH
LAAYELSKASETVSFKPTRQAPPPPTSGQASGPGGLPPLAQALKDHLAAYEQSKKG

BglII=AGATCT

#79= Δ151bp-219bp = 51A.A- 73A.A (23A.A) with one amino acid mutation #148 A.A.

espF-L16E /79

>430181001_79_FW sequence exported from
430181001_79_FW_C02.ab1NNNNNNNNNNNNNNNNNNNTNNNNNNNNTTTATTATTCGTTTTGNATATATATGAGA
GTTAGCCAAGATTAGATATAAAGAGGCATAAATTATGCTTAATGGAATTAGTAACGCTGCTTCTACACTAGGGCGGCAGG
AAGTAGGTATCGCAAGTCGAGTGAGCTCTGCGGGGGGAACTGGATTTCCTGTAGCCCCTCAGGCTGTTCGTCTTACTCCG
GTGAGAGTTCATTCCCCCTTTTCTAGATCTCGTCCGGCACCGCCGCCACCGACAAGTGGACAGGCATCCGGGGCATCCCGA
CCTTTACCGCCCATTGCACAGGCATTAAAAGATCATTTAGCTGCCTATGAACTATCGAAAGCGTCTGAGACTGTAAACTTTA
AGCCAACCCGTCCGGCACCGCCGCCACCGACAAGTGGACAGGCATCCGGGGCATCCCGACCTTTACCGCCCATTGCACAG
GCATCAAAAGATCATTTAGCTGCCTATGAACTATCGAAAGCGTCTGAGACTGTAAGCTTTAAGCCAACCCGTCAGGCACCA
CCGCCACCGACAAGTGGACAGGCATCCGGGCCTGGTGGACTACCGCCCCTTGCACAGGCACTAAAAGATCATTTAGCTGC
CTATGAGCAATCGAAGAAAGGGTAACCAACTAATTTAAAACGATTAATCGGTATTGATTATTTGTTTTCCAACTAATAAAAA
CCGATATCAAATTGCGTGG

MLNGISNAASTLGRQEVGIASRVSSAGGTGFPVAPQAVRLTPVRVHSPFSRSRPAPPPPTSGQASGASRPLPPIAQALKD
HLAAYELSKASETVNFKPTRPAPPPPTSGQASGASRPLPPIAQASKDHLAAYELSKASETVSFKPTRQAPPPPTSGQASG
PGGLPPLAQALKDHLAAYEQSKKG

```
Alignment of Sequence_1:  [79] with Sequence_2: [EspF native]

Similarity: 549/621 (88.41 %)

Seq_1  1     ATGCTTAATGGAATTAGTAACGCTGCTTCTACACTAGGGCGGCAGGAAGTAGGTATCGCA  60
             ||||||||||||||||||||||||||||||||||||||||||||||   ||||||||||||
Seq_2  1     ATGCTTAATGGAATTAGTAACGCTGCTTCTACACTAGGGCGGCAGCTTGTAGGTATCGCA  60


Seq_1  61    AGTCGAGTGAGCTCTGCGGGGGGAACTGGATTTCCTGTAGCCCCTCAGGCTGTTCGTCTT  120
             |||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
Seq_2  61    AGTCGAGTGAGCTCTGCGGGGGGAACTGGATTTTCTGTAGCCCCTCAGGCTGTTCGTCTT  120
```

```
Seq_1  121   ACTCCGGTGAGAGTTCATTCCCCCTTTTCT--AGA------------------------  153
             ||||||||||||||||||||||||||||||  ||
Seq_2  121   ACTCCGGTGAGAGTTCATTCCCCCTTTTCTCCAGGTTCGTCGAATGTTAATGCGAGGACG  180

Seq_1  154   -------------------------------------TCTCGTCCGGCACCGCCGCCA  174
                                                  ||||||||||||||||||||
Seq_2  181   ATTTTTAATGTGAGCAGCCAGGTGACTTCATTTACTCCCTCTCGTCCGGCACCGCCGCCA  240

Seq_1  175   CCGACAAGTGGACAGGCATCCGGGGCATCCCGACCTTTACCGCCCATTGCACAGGCATTA  234
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  241   CCGACAAGTGGACAGGCATCCGGGGCATCCCGACCTTTACCGCCCATTGCACAGGCATTA  300

Seq_1  235   AAAGATCATTTAGCTGCCTATGAACTATCGAAAGCGTCTGAGACTGTAAACTTTAAGCCA  294
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  301   AAAGATCATTTAGCTGCCTATGAACTATCGAAAGCGTCTGAGACTGTAAACTTTAAGCCA  360

Seq_1  295   ACCCGTCCGGCACCGCCGCCACCGACAAGTGGACAGGCATCCGGGGCATCCCGACCTTTA  354
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  361   ACCCGTCCGGCACCGCCGCCACCGACAAGTGGACAGGCATCCGGGGCATCCCGACCTTTA  420

Seq_1  355   CCGCCCATTGCACAGGCATCAAAAGATCATTTAGCTGCCTATGAACTATCGAAAGCGTCT  414
             |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
Seq_2  421   CCGCCCATTGCACAGGCATTAAAAGATCATTTAGCTGCCTATGAACTATCGAAAGCGTCT  480

Seq_1  415   GAGACTGTAAGCTTTAAGCCAACCCGTCAGGCACCACCGCCACCGACAAGTGGACAGGCA  474
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  481   GAGACTGTAAGCTTTAAGCCAACCCGTCAGGCACCACCGCCACCGACAAGTGGACAGGCA  540

Seq_1  475   TCCGGGCCTGGTGGACTACCGCCCCTTGCACAGGCACTAAAAGATCATTTAGCTGCCTAT  534
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  541   TCCGGGCCTGGTGGACTACCGCCCCTTGCACAGGCACTAAAAGATCATTTAGCTGCCTAT  600

Seq_1  535   GAGCAATCGAAGAAAGGGTAA  555
             ||||||||||||||||||||
Seq_2  601   GAGCAATCGAAGAAAGGGTAA  621
```

Alignment of Sequence_1: [Fragment of 79 [1-555]] with Sequence_2: [Fragment of EspF native [1-621]]

Similarity: 181/184 (98.37 %)

```
Seq_1  1     MLNGISNAASTLGRQEVGIASRVSSAGGTGFPVAPQAVRLTPVRVHSPFS--------R-  51
             |||||||||||||| |||||||||||||| ||||||||||||||||||        |
Seq_2  1     MLNGISNAASTLGRQLVGIASRVSSAGGTGFSVAPQAVRLTPVRVHSPFSPGSSNVNART  60

Seq_1  52    -------------SRPAPPPPTSGQASGASRPLPPIAQALKDHLAAYELSKASETVNFKP  98
                         ||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  61    IFNVSSQVTSFTPSRPAPPPPTSGQASGASRPLPPIAQALKDHLAAYELSKASETVNFKP  120

Seq_1  99    TRPAPPPPTSGQASGASRPLPPIAQASKDHLAAYELSKASETVSFKPTRQAPPPPTSGQA  158
             |||||||||||||||||||||||||| |||||||||||||||||||||||||||||||||
Seq_2  121   TRPAPPPPTSGQASGASRPLPPIAQALKDHLAAYELSKASETVSFKPTRQAPPPPTSGQA  180

Seq_1  159   SGPGGLPPLAQALKDHLAAYEQSKKG  184
             |||||||||||||||||||||||||
Seq_2  181   SGPGGLPPLAQALKDHLAAYEQSKKG  206
```

7.1.12 espF-L16E /80 strain

EspF native

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GGGAACTGGATTTTCTGTAGCCCCTCAGGCTGTTCGTCTTACTCCGGTGAGAGTTCATTCCCCCTTTTCTCCAGGTTCGTCG
AATGTTAATGCGAGGACGATTTTTAATGTGAGCAGCCAGGTGACTTCATTTACTCCCTCTCGTCCGGCACCGCCGCCACCG
ACAAGTGGACAGGCATCCGGGGCATCCCGACCTTTACCGCCCATTGCACAGGCATTAAAAGATCATTTAGCTGCCTATGAA
CTATCGAAAGCGTCTGAGACTGTAAACTTTAAGCCAACCCGTCCGGCACCGCCGCCACCGACAAGTGGACAGGCATCCGG
GGCATCCCGACCTTTACCGCCCATTGCACAGGCATTAAAAGATCATTTAGCTGCCTATGAACTATCGAAAGCGTCTGAGAC
TGTAAGCTTTAAGCCAACCCGTCAGGCACCACCGCCACCGACAAGTGGACAGGCATCCGGGCCTGGTGGACTACCGCCCC
TTGCACAGGCACTAAAAGATCATTTAGCTGCCTATGAGCAATCGAAGAAAGGGTAA

MLNGISNAASTLGRQLVGIASRVSSAGGTGFSVAPQAVRLTPVRVHSPFSPGSSNVNARTIFNVSSQVTSFTPSR
PAPPPPTSGQASGASRPLPPIAQALKDHLAAYELSKASETVNFKPTRPAPPPPTSGQASGASRPLPPIAQALKDH
LAAYELSKASETVSFKPTRQAPPPPTSGQASGPGGLPPLAQALKDHLAAYEQSKKG

#80= S74A It is L16E but there is no deletion only two mutation (in amino acid #47) Serine to Alanine no BglII,

BglII=AGATCT

espF-L16E /80

>430181001_80_FW sequence exported from 430181001_80_FW_D02.ab1NNNNNNNNNNNNNNNNNNTNNNNNNNNTTTTATTATTCGTTTTGATATATATGAGAG
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GAGAGTGCATGCCCCCTTTTCTCCAGGTTCGTCGAATGTTAATGCGAGGACGATTTTTAATGTGAGCAGCCAGGTGACTTC
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ACCGCCGCCACCGACAAGTGGACAGGCATCCGGGGCATCCCGACCTTTACCGCCCATTGCACAGGCATTAAAAGATCATTT
AGCTGCCTATGAACTATCGAAAGCGTCTGAGACTGTAAGCTTTAAGCCAACCCGTCAGGCACCACCGCCACCGACAAGTG
GACAGGCATCCGGGCCTGGTGGACTACCGCCCCTTGCACAGGCACTAAAAGATCATTTAGCTGCCTATGAGCAATCGAAG
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NN

```
Alignment of Sequence_1:  [79] with Sequence_2: [EspF native]

Similarity: 616/621 (99.19 %)

Seq_1  1     ATGCTTAATGGAATTAGTAACGCTGCTTCTACACTAGGGCGGCAGGAAGTAGGTATCGCA  60
             ||||||||||||||||||||||||||||||||||||||||||||||    ||||||||||||
Seq_2  1     ATGCTTAATGGAATTAGTAACGCTGCTTCTACACTAGGGCGGCAGCTTGTAGGTATCGCA  60


Seq_1  61    AGTCGAGTGAGCTCTGCGGGGGGAACTGGATTTTCTGTAGCCCCTCAGGCTGTTCGTCTT  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  61    AGTCGAGTGAGCTCTGCGGGGGGAACTGGATTTTCTGTAGCCCCTCAGGCTGTTCGTCTT  120


Seq_1  121   ACTCCGGTGAGAGTGCATGCCCCCTTTTCTCCAGGTTCGTCGAATGTTAATGCGAGGACG  180
             |||||||||||||| ||| ||||||||||||||||||||||||||||||||||||||||||
Seq_2  121   ACTCCGGTGAGAGTTCATTCCCCCTTTTCTCCAGGTTCGTCGAATGTTAATGCGAGGACG  180
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Seq_1  181   ATTTTTAATGTGAGCAGCCAGGTGACTTCATTTACTCCCTCTCGTCCGGCACCGCCGCCA  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  181   ATTTTTAATGTGAGCAGCCAGGTGACTTCATTTACTCCCTCTCGTCCGGCACCGCCGCCA  240

Seq_1  241   CCGACAAGTGGACAGGCATCCGGGGCATCCCGACCTTTACCGCCCATTGCACAGGCATTA  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  241   CCGACAAGTGGACAGGCATCCGGGGCATCCCGACCTTTACCGCCCATTGCACAGGCATTA  300

Seq_1  301   AAAGATCATTTAGCTGCCTATGAACTATCGAAAGCGTCTGAGACTGTAAACTTTAAGCCA  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  301   AAAGATCATTTAGCTGCCTATGAACTATCGAAAGCGTCTGAGACTGTAAACTTTAAGCCA  360

Seq_1  361   ACCCGTCCGGCACCGCCGCCACCGACAAGTGGACAGGCATCCGGGGCATCCCGACCTTTA  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  361   ACCCGTCCGGCACCGCCGCCACCGACAAGTGGACAGGCATCCGGGGCATCCCGACCTTTA  420

Seq_1  421   CCGCCCATTGCACAGGCATTAAAAGATCATTTAGCTGCCTATGAACTATCGAAAGCGTCT  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  421   CCGCCCATTGCACAGGCATTAAAAGATCATTTAGCTGCCTATGAACTATCGAAAGCGTCT  480

Seq_1  481   GAGACTGTAAGCTTTAAGCCAACCCGTCAGGCACCACCGCCACCGACAAGTGGACAGGCA  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  481   GAGACTGTAAGCTTTAAGCCAACCCGTCAGGCACCACCGCCACCGACAAGTGGACAGGCA  540

Seq_1  541   TCCGGGCCTGGTGGACTACCGCCCCTTGCACAGGCACTAAAAGATCATTTAGCTGCCTAT  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  541   TCCGGGCCTGGTGGACTACCGCCCCTTGCACAGGCACTAAAAGATCATTTAGCTGCCTAT  600

Seq_1  601   GAGCAATCGAAGAAAGGGTAA  621
             ||||||||||||||||||||
Seq_2  601   GAGCAATCGAAGAAAGGGTAA  621
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Alignment of Sequence_1: [Fragment of 79 [1-621]] with Sequence_2: [Fragment of EspF native [1-621]]

Similarity: 204/206 (99.03 %)

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Seq_1  1     MLNGISNAASTLGRQEVGIASRVSSAGGTGFSVAPQAVRLTPVRVHAPFSPGSSNVNART  60
             |||||||||||||| |||||||||||||||||||||||||| ||||||||||||
Seq_2  1     MLNGISNAASTLGRQLVGIASRVSSAGGTGFSVAPQAVRLTPVRVHSPFSPGSSNVNART  60

Seq_1  61    IFNVSSQVTSFTPSRPAPPPPTSGQASGASRPLPPIAQALKDHLAAYELSKASETVNFKP  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  61    IFNVSSQVTSFTPSRPAPPPPTSGQASGASRPLPPIAQALKDHLAAYELSKASETVNFKP  120

Seq_1  121   TRPAPPPPTSGQASGASRPLPPIAQALKDHLAAYELSKASETVSFKPTRQAPPPPTSGQA  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  121   TRPAPPPPTSGQASGASRPLPPIAQALKDHLAAYELSKASETVSFKPTRQAPPPPTSGQA  180

Seq_1  181   SGPGGLPPLAQALKDHLAAYEQSKKG  206
             |||||||||||||||||||||||||
Seq_2  181   SGPGGLPPLAQALKDHLAAYEQSKKG  206
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